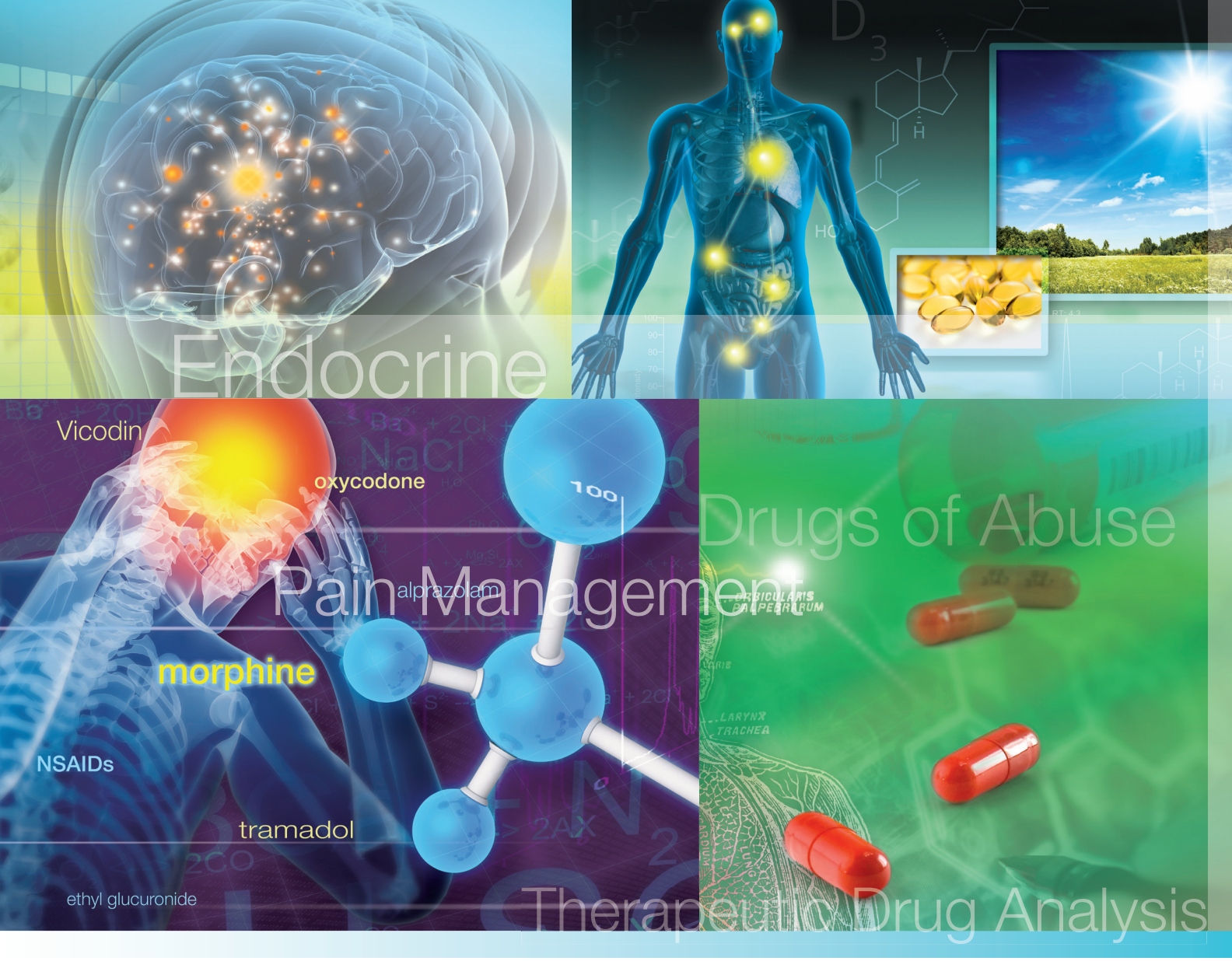

Clinical research and forensic toxicology

Application Compendium

Endocrine Analysis
for Clinical Research*

Therapeutic Drug Analysis
for Clinical Research

Drugs of Abuse Analysis
for Forensic Toxicology**

Pain Management Drug Analysis
for Forensic Toxicology

* For Research Use Only. Not for use in diagnostic procedures.
** For Forensic Use Only.

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Development of a Method for Evaluation of Mass Spectrometer Performance in Real Time

Terry Olney, Oleg Silivra, and Huy Nguyen
Thermo Fisher Scientific, San Jose, CA

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Overview

Purpose: To develop a method which monitors system performance while simultaneously performing quantitation. The research method is used for verification of the internal standard (IS) precursor and product ions. The Internal Standard Verification (ISV) method can be used as any quantitative method would be used. Results of the method are available in real time to immediately determine the confidence that can be placed in the data or to initiate follow-on actions.

Methods: First, a series of testosterone injections are used to demonstrate generation of the confidence data while performing quantitative analysis. Next, conditions are imposed on the mass spectrometer which give rise to fault conditions (mimicking resolution or mass drift) to demonstrate the ability to identify data that is suspicious or incorrect.

Results: The ISV method is shown to have the ability to simultaneously monitor the calibration state or fault state of the system while maintaining the ability to quantitate linearly down the LOD.

Introduction

Manual data review has become a common step in the release of results, this can occupy significant time and effort for a skilled mass spectroscopist. Usually an abnormally low or high result will trigger the need for review and the recourse is usually to manually review the quality of the chromatographic peaks for the various analytes. Here we introduce a method that simultaneously monitors the operation of the mass spectrometer, including resolution, peak width and transmission, during the analytical assay without any sacrifice to analytical performance. This method utilizes the internal standard and does not require any additional steps in sample preparation. The resulting data can speed up manual review and may reduce or eliminate the need for manual review.

While performing routine sample analysis, one can monitor one or two ions to evaluate the performance of the mass spectrometer. However, these monitor ions must be predictable ions, with a relatively intense response, they should not require additional sample handling and should avoid causing negative effects on the measurement of any low concentration anaytes. In most analysis, the internal standard is present as a relatively strong and predicable ion but it is underutilized. A new mode of operating a triple stage quadrupole (TSQ) has been developed that uses the internal standard to evaluate Q1 and Q3 mass position, peak width and intensities, in real time, during routine analysis to alert the user whenever any of these parameters are out of tolerance.

Methods

Sample Preparation

Samples of testosterone and testosterone d3 were made up in 50/50 MeOH/Water. For the purpose of this study the Internal standard concentration in all samples was 0.8 pg/uL.

Liquid Chromatography

Considering the samples were made up in neat solutions, a simple 3 min chromatographic method was used to produce a 3 sec peak, which is representative of anticipated analytical conditions.

Mass Spectrometry

Samples were run using a Thermo Scientific™ TSQ Endura™ triple-stage quadrupole mass spectrometer. The method was developed by starting with a standard Testosterone method that identifies the analyte ions (Testosterone: 289.4→ 97.2, 109.2) and their respective internal standard (Testoserone d_3: 292.4→97.2).

To develop the ISV method from a standard method, the IS is first identified for ISV. The precursor and product ions mass positions are taken from the IS SRM. The resolution value and tolerances for resolution and mass position are automatically populated. The user must add the expected intensity for the parent, product and SRM transitions. Adjustments can be made to all tolerance values as required. The user also has control over the amount of scan time that is taken from the IS transition to perform the ISV scans. Figure 1 shows the method parameters in the TSQ Endura MS method editor. Figure 2 shows the summary of the method, including the ISV parameters.

When the ISV method is generated, in addition to the expected SRM transition specified, the method UI adds two small diagnostic scans: a) Q1 MS full scan of 3 Da about the precursor ion and b) product ion mode over a small 3 Da mass range about the product ion mass. The scan times for the analyte ions are unaffected but time is taken from the internal standard scan to perform the two ISV scans.

FIGURE 1. TSQ Endura MS method. Source parameters and scan parameters for Testosterone analysis using ISV.

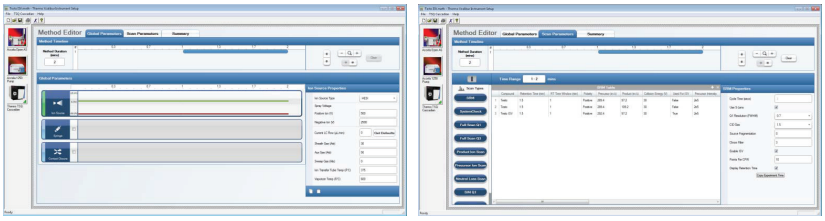
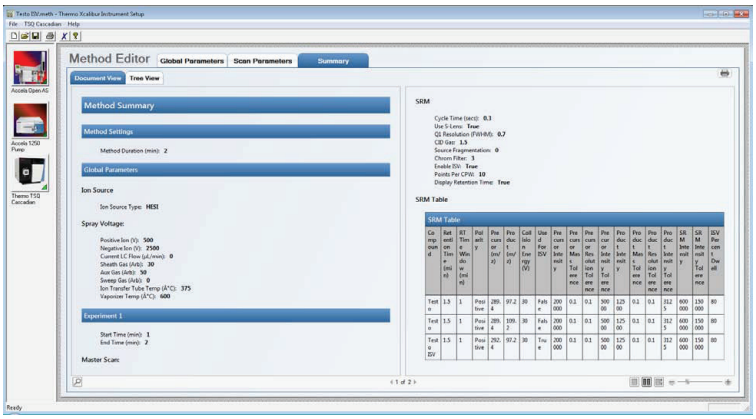


FIGURE 2. TSQ Endura MS method summary.



This method shown here has a chromatographic peakwidth of 3 seconds. Ten scans are specified across the peak. Each cycle has ~300 ms and thus each of the three transitions will have ~100 ms dwell time. The two testosterone transitions are unaffected and have ~100 ms dwell times. The internal standard will provide the dwell time perform the ISV scans. It is assumed that the internal standard is a well behaved, well understood, strong transition. This allows the user to reliably take anywhere from 20 to 80% of the IS dwell time for the ISV measurements.

In the present case 80% of the IS time is given to the ISV. The IS will have a ~20 ms dwell time and both the precursor ion Q1 MS full scan (290.9 – 293.9 Da) and the product ion mode (292.4→ 95.7 – 98.7 Da) scan will each have ~40 ms dwell times.

Data Analysis

Data was collected using Xcalibur 3.0 and processed using Qual Browser. Data was worked up using standard statistical tools in MS Excel.

Results

Quantitation and Monitoring

Routine sample analysis typically consists of monitoring one or two transitions for each analyte ion and monitoring a single transition for the associated internal standard. These analyses can also have multiple analytes and may have multiple internal standards. To reduce complexity and to demonstrate the effectiveness of the new method, a simple testosterone assay with one analyte (two transitions) and one internal standard was used to show the ability to monitor the performance of the mass spectrometer through a series of injections over time. The analyte response was monitored across the useful analytical range while the internal standard transition is used to both normalize the system response (the traditional function) and to evaluate the calibration of the mass spectrometer.

Figure 3 shows the calibration curve using the ISV method. Figure 4 is the curve run using the same method without performing the ISV measurements. This demonstrates that including the ISV portion in the method does not affect the linearity of the assay.

FIGURE 3. Response curve for Testosterone using the ISV method

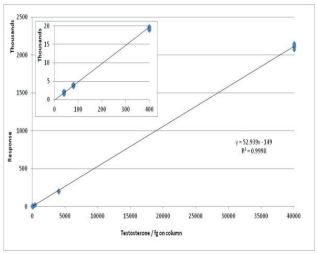
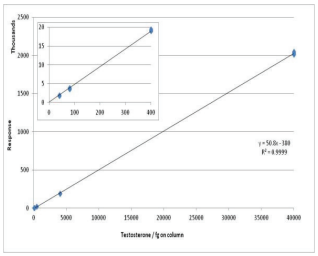


FIGURE 4. Response curve for Testosterone using standard method (No ISV)



In addition, as shown in Table 1, the LLOQ, as defined by <15% RDS, is unchanged at approximately 2 pg/mL for both the ISV and non-ISV methods.

TABLE 1. Testosterone response, corrected for IS. LOD is approximately at the same 2 pg/mL level for both ISV and non-ISV methods.

Testosterone with ISV Measurements			
pg/mL	fg on Col	Response	% St Dev
2	**40**	**2113**	**12.8%**
4	**80**	**3994**	**5.8%**
20	**400**	**19329**	**1.7%**
200	**4000**	**197403**	**1.2%**
2000	**40000**	**2117342**	**1.2%**

Testosterone without ISV Measurements			
pg/mL	fg on Col	Response	% St Dev
2	**40**	**1922**	**9.7%**
4	**80**	**3824**	**5.6%**
20	**400**	**19301**	**1.1%**
200	**4000**	**188281**	**1.3%**
2000	**40000**	**2029272**	**0.8%**

The top panel on Figure 5 shows the SRM chromatographic response for testosterone at 40 pg on column (289.4 → 97.2 + 101.2) and internal standard (292.4 → 97.2) SRMs. The lower panel shows the full scan chromatographic response for the internal standard in the Q1 MS scan (290.9 – 293.9 Da) and the product ion mode (292.4 → 95.7 – 298.7 Da).

FIGURE 5. Testosterone 40 pg on column. Chromatograms shown from top to bottom: Testosterone SRM; Testosterone d3 SRM, Q1 Full Scan, Product Ion Scan.

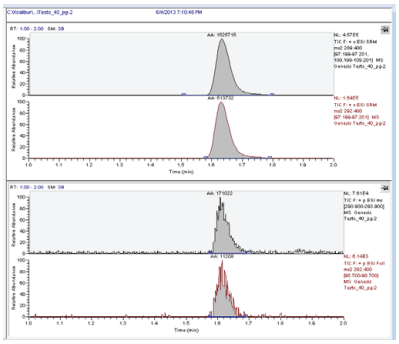


FIGURE 6. Testosterone 40 pg on column. Chromatograms shown from top to bottom: Testosterone d3 Q1 Full Scan, Product Ion Scan; Mass Spectra: Testosterone d3 Q1 Full Scan, Product Ion Scan

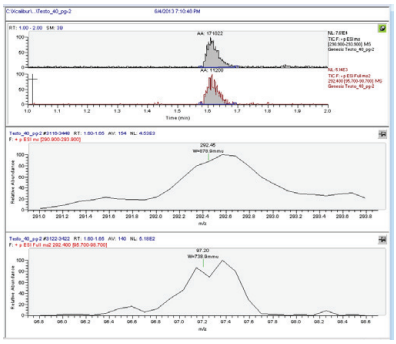


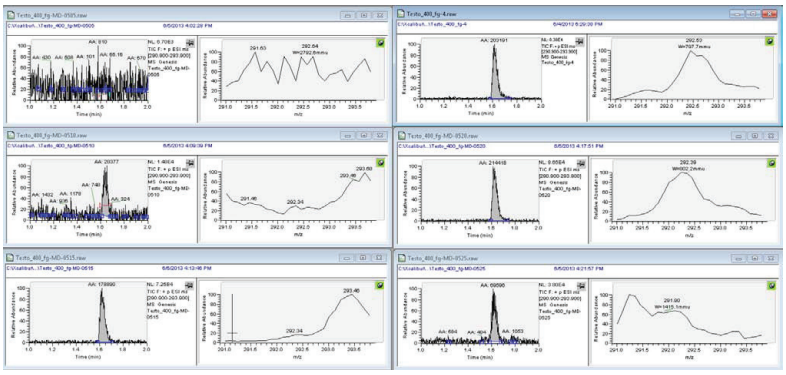
Figure 7 again shown the chromatographic response for the full scan internal standard Q1 MS scan and the product ion scan in the upper panel. In the lower panels the averaged Q1 Full Scan mass spectrum and the Product Ion Mode mass spectrum are shown.

The response for 6 replicate injections over the 2 to 2000 pg/mL range are shown in Table 2. Highlighted in yellow are the values that fall outside of the predetermined tolerances. In most cases, where intensities are low, they track the internal standard and are likely a result of lower injection volume. In only two cases are the values outside the limit for mass or resolution. These deviations were not large enough to indicate a change in calibration or failure in the system.

TABLE 2. Testosterone response corrected for IS. Expected values and tolerance limits are shown at the bottom.

ISV Table for Demonstation									
Conc	Response			Parent			Product		
fg/uL	Analyte	Internal Std	Resp/IS	M/Z	Width	Area	M/Z	Width	Area
40	2948	732910	2396	292.51	0.75	231442	97.23	0.68	13568
40	2492	703486	2050	292.49	0.81	221995	97.22	0.62	14817
40	2775	721359	2270	292.49	0.78	229631	97.22	0.71	15039
40	2432	637542	2247	292.47	0.82	212293	97.21	0.73	13212
40	1731	617986	1517	292.48	0.84	202647	97.23	0.76	13327
40	2425	647537	2196	292.48	0.81	215050	97.20	0.70	11670
80	4480	737877	3871	292.47	0.79	242640	97.21	0.71	14245
80	4513	785476	3637	292.48	0.73	271143	97.21	0.71	16265
80	4246	649547	4207	292.49	0.81	210047	97.22	0.67	13511
80	4036	660396	3900	292.48	0.73	198064	97.23	0.69	13579
80	4896	748874	4207	292.49	0.76	252640	97.21	0.72	17258
80	4401	682479	4143	292.48	0.76	218588	97.22	0.66	13602
400	21176	749495	19843	292.50	0.86	254163	97.24	0.75	16117
400	20982	767396	19186	292.48	0.84	230583	97.20	0.74	14974
400	15863	571678	19479	292.48	0.83	193470	97.21	0.69	9932
400	16293	587573	19465	292.47	0.75	203190	97.23	0.77	12087
400	13233	491762	18875	292.48	0.83	168075	97.20	0.63	9974
400	13344	489467	19129	292.48	0.78	163832	97.25	0.73	9507
4000	197336	702203	201837	292.49	0.79	237080	97.22	0.73	14948
4000	200435	731429	196803	292.49	0.69	239721	97.21	0.65	14157
4000	162271	597776	194950	292.48	0.80	195292	97.22	0.68	12073
4000	159666	579981	197713	292.47	0.75	185614	97.22	0.77	11653
4000	186138	677821	197221	292.49	0.82	227182	97.22	0.72	14333
4000	169513	621454	195893	292.49	0.76	205373	97.20	0.72	12130
40000	1628083	544745	2151369	292.46	0.83	190210	97.20	0.78	11908
40000	1484947	500983	2133628	292.45	0.88	171022	97.20	0.74	11208
40000	1522016	515818	2123993	292.45	0.88	169641	97.22	0.72	11265
40000	1226096	416741	2117816	292.46	0.85	137600	97.20	0.74	7165
40000	1279791	443751	2076001	292.45	0.90	144843	97.19	0.67	9442
40000	1113194	381350	2101243	292.46	0.85	121133	97.19	0.67	8480
Expect		600000		292.4	0.8	200000	97.2	0.7	12500
Error		150000		0.1	0.1	50000	0.1	0.1	3125
Min		450000		292.3	0.7	150000	97.1	0.6	9375
Max		750000		292.5	0.9	250000	97.3	0.8	15625

Figure 7. Simulated Q1 mass drift.



Error Conditions

To demonstrate the utility of the method, a series of failures were induced in the mass spectrometer to show the ability of the method to detect these situations. Figure 7 shows a series of injections representing Q1 mass drift across the peak. The induced drift represents the Q1 mass position moving from 293.7 to 292.5 Da. As the mass

TABLE 3. Effect on Testosterone and Internal Standard for simulated Q1 mass drift.

ISV Table for Demonstation of Q1 Mass Drift								
Conc	Response		Parent			Product		
fg/uL	Analyte	nternal Std	M/Z	Width	Area	M/Z	Width	Area
400	ND	ND	ND	ND	ND	ND	ND	ND
400	3201	101823	293.68	0.78	28377	97.21	0.71	1768
400	15765	568519	292.47	0.75	203190	97.23	0.77	11695
400	18019	568924	293.46	0.78	178890	97.23	0.77	12729
400	21541	669797	292.39	0.81	214418	97.24	0.72	15903
400	7010	221336	291.90	1.44	69596	97.24	0.73	3798
400	502	15619	292.51	1.42	5000	ND	ND	ND

Figure 8. Simulated Q3 resolution / mass drift.

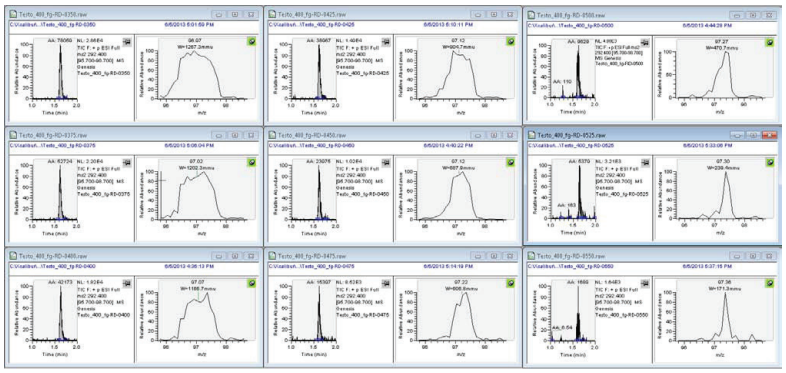


TABLE 4. Effect on Testosterone and Internal Standard for simulated Q3 resolution and mass drift.

ISV Table for Demonstation of Q3 Resolution Drift									
Conc	Response			Parent			Product		
fg/uL	Analyte	nternal Std	Resp/IS	M/Z	Width	Area	M/Z	Width	Area
400	129983	4524398		292.51	0.83	233924	96.97	1.29	78059
400	81433	2660164		292.51	0.79	183366	97.02	1.22	52724
400	62903	2114164		292.51	0.86	199773	97.06	1.16	42173
400	71324	2315379		292.53	0.87	229089	97.12	0.87	38967
400	37355	1268617		292.51	0.80	212764	97.12	0.71	23975
400	25779	813788		292.52	0.85	227637	97.22	0.66	15397
400	15637	550496		292.51	0.88	225702	97.25	0.63	9629
400	7182	259348		292.67	0.73	204002	97.30	0.69	5379
400	2169	77429		292.68	0.90	189833	97.32	0.11	1689
400	572	19136		292.67	0.73	196620	97.27	0.15	400
400	200	6462		292.67	0.76	208027	97.12	0.11	120
400	ND	ND		292.66	0.74	231238	ND	ND	ND
400	ND	ND		292.67	0.85	232639	ND	ND	ND

drifts, the observed response in the analyte and internal standard responses are shown in table 3. This can help to elucidate the cause of a change in internal standard.

Figure 8 shows a series of injections representing Q3 resolution/mass drift across the range. The induced drift represents a Q3 mass position moving from 96 to 97.12 Da and an associated change in peakwidth from 1.29 down to 0.11 Da FWHM. The effect on the analyte and internal standard response are shown in table 4.

The output of these measurements are placed in an instrument database. These results, and evaluation with respect to the given tolerances, are interpreted by the application software. Responses can be limited to: a) simply alerting the user to the failure; b) provide a snapshot of the instrument state at the moment of failure; c) aiding in data evaluation during manual review; d) automatically rejecting data and remove the need for manual review; e) rerun of the sample; f) triggering a system evaluation, an automatic recalibration and / or triggering a series of self-diagnostic routines to fully evaluate and respond to the failure. In the extreme, the response may go to the extent of alerting the operator and service organization that a failure has been detected and needs attention.

Method Complexity

The method is constructed to allow full characterization of the analyte ions without compromise, while taking a portion of the internal standard time to evaluate the precursor and product ions. To this end, limits must be placed on the number of internal standards that can be used at any given time with respect to the analyte ions.

In general two ISV ions with four quantitative ions (eight transitions) is the practical limit of the method as it is currently implemented. This limit can be relaxed for wider chromatographic peaks or must be tightened for faster chromatography.

Conclusion

- A research method has been developed to evaluate the health or status of a mass spectrometer in real time while performing routine quantitative analysis
- This method can be used to flag suspicious data or to reduce or remove the need for manual review. Other possible outcomes are automated initiation of system evaluation and/or system tune and calibration
- The method is constructed to avoid any negative impact on quantification, particularly are the limits of quantitation.

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Selected-Reaction Monitoring–Mass Spectrometric Immunoassay Analysis of Parathyroid Hormone and Related Variants

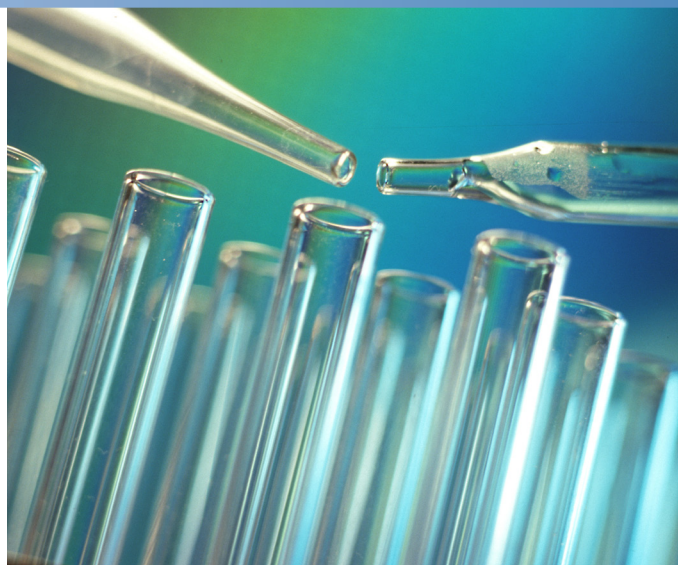
Mary F. Lopez, Taha Rezai, David A. Sarracino, Amol Prakash, Bryan Krastins
Thermo Fisher Scientific BRIMS Center, Cambridge, MA

Application Note MSIA1004

Key Words
LTQ Orbitrap XL, TSQ Vantage, Pinpoint Software, parathyroid hormone, PTH, biomarkers, MSIA

Goal
To develop a highly sensitive and selective selected-reaction monitoring–mass spectrometric immunoassay analysis (SRM-MSIA)-based method for the concurrent detection and quantification of full-length parathyroid hormone (PTH) [amino acid (aa)1–84] and two N-terminal variants [aa7–84 and aa34–84] for clinical research use.

Introduction
Parathyroid hormone is produced in the parathyroid glands through the two-step conversion of prepro-PTH (115 amino acids) to pro-PTH (90 amino acids) to the 84 amino acid peptide (PTH1–84). Conventional PTH measurements typically rely on two-antibody recognition systems coupled to a variety of detection modalities.[1] The most specific modalities are able to differentiate between different truncated forms of PTH and are referred to as second- and third-generation PTH assays.[2] The key to the application of these later-generation assays is the ability to selectively detect and quantify various PTH forms. In particular, two variants are the subject of increased research investigation: full-length PTH1–84 and PTH missing the 6 N-terminal amino acids (PTH7–84). Because of the inability of existing tests to detect microheterogeneity,[3] these variants were historically considered as a single PTH value (by the first-generation assays). The classification of each variant as its own molecular entity, and the analysis of each independently, suggest an antagonistic relationship between the two different forms in regard to calcium homeostasis.[4] In fact, there is mounting research showing that the ratio between PTH1–84 and PTH7–84 could have future clinical relevance for distinguishing between hyper-parathyroid bone turnover and adynamic bone disease.[5-7]



The ratio of PTH1–84 to PTH7–84 is an example of the potential utility of the microheterogeneity within the PTH protein. Another PTH variant, PTH1–34, has been identified as exhibiting biochemical activity comparable to the full-length protein. There are indications that the microheterogeneity of PTH has yet to be fully characterized, challenging researchers' efforts to determine the utility and/or confounding effects on present-day methods. Accurate examination of known PTH variants and the simultaneous evaluation of other possible variants requires a degree of analytical freedom that universally escapes conventional methods. This work describes mass spectrometric immunoassays that, although specifically designed for the detection of PTH1–84 and PTH7–84, also facilitate the simultaneous discovery and evaluation of further microheterogeneity in PTH.

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Experimental

Approach

In addition to the well-characterized truncated PTH variants, PTH1–84 and PTH7–84, four other molecular versions have been reported in the literature as present in human biofluids (primarily plasma or serum). Aligning these fragments to the sequence of PTH1–84 produced a variant map revealing forms stemming predominantly from N-terminal truncations (Figure 1). A conserved region (among several variants) was evident between residues 48 and 84. This region was suitable for immunoaffinity targeting to capture ragged N-terminal variants (for example, PTH1–84 and PTH7–84). Postcapture digestion of retained PTH (and variants) created the basis for SRM-MSIA,[8-11] for which surrogate peptides representative of the different PTH variants were selected for analysis.

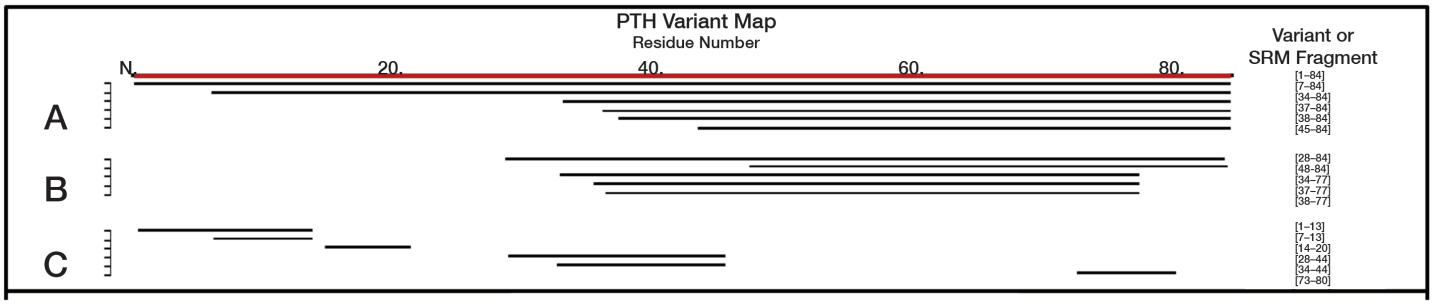


Figure 1. PTH variant map. (A) N-terminally truncated PTH variants identified previously.[7,12] (B) Variants added to map by top-down MS analysis. (C) Conserved and truncated tryptic fragments chosen for SRM-MSIA.

Reagents

Goat polyclonal anti-PTH39–84 antibody was purchased from Immutopics International. Recombinant human PTH (rhPTH) was obtained from Bachem. Premade 0.01 M HEPES-buffered saline with 3 mM EDTA and 0.05% (vol/vol) surfactant P20 (HBS-EP) was purchased from Biacore. Thermo Scientific™ Pierce™ premixed 2-[morpholino]ethanesulfonic acid–buffered saline powder packets and Thermo Scientific synthetic heavy-labeled peptides were used. High purity solvents from Fisher Chemical brand were used.

Samples

A total of 24 plasma samples were used in the research study: 12 from individuals with previously diagnosed severe renal impairment or end-stage renal disease (ten males and two females; mean age 66.7 years) and 12 from healthy individuals (ten males and two females; mean age 65 years). Among the individuals with renal failure, three were Hispanic, two were Asian, two were African American, and six were Caucasian. The ethnicity information for the healthy sample donors was not available.

Calibration Curves Samples

Samples for creation of calibration curves were prepared from pooled human plasma by step-wise, 2-fold serial dilution of an initial sample containing rhPTH at a concentration of 1000 ng/L (eight steps, range 1000–7.8 ng/L). Samples were frozen at -80 °C until use.

Sample Preparation and Immunocapture

Purification and concentration of the PTH was accomplished by immunoaffinity capture. Extraction of PTH from plasma was carried out with proprietary Thermo Scientific™ Mass Spectrometric Immunoassay (MSIA™) pipette tips derivatized with the PTH antibodies via 1,1′-carbonyldiimidazole chemistry.[13-17] After extraction, PTH was digested, separated by liquid chromatography, and analyzed by high-resolution MS/MS on an ion trap-Orbitrap™ hybrid mass spectrometer and by SRM on a triple quadrupole mass spectrometer as described below.

Sample Elution and Trypsin Digestion

Bound proteins were eluted from the tips into a 96 well plate by pipetting 100 µL of 30% acetonitrile/0.5% formic acid up and down for a total of 15 cycles. Samples were lyophilized to dryness and then resuspended in 30 µL of 30% n-propanol/100 mmol/L ammonium bicarbonate, pH 8.0, diluted with 100 µL of 25 M acetic acid containing 100 ng of trypsin. Samples were allowed to digest for 4 hours at 37 °C. After digestion, samples were lyophilized and resuspended in 30 µL of 3% (vol/vol) acetonitrile/0.2% (vol/vol) formic acid/glucagon/PTH heavy peptides.

High-Resolution LC-MS/MS

High-resolution LC-MS/MS analysis was carried out using a Thermo Scientific™ EASY-nLC™ system and Thermo Scientific™ LTQ Orbitrap XL™ hybrid ion trap-Orbitrap mass spectrometer. Samples in 5% (vol/vol) acetonitrile/0.1% (vol/vol) formic acid were injected into a Thermo Scientific™ Hypersil GOLD™ aQ fused-silica capillary column (75 µm x 25 cm, 5 µm particle size) in a 250 µL/min gradient of 5% acetonitrile/0.1% formic acid to 30% acetonitrile/0.1% formic acid over the course of 180 minutes. The total run time was 240 minutes and the flow rate was 285 nL/min. The LTQ Orbitrap XL MS was operated at 60,000 resolution (FWHM at *m/z* 400) for a full scan for data-dependent Top 5 MS/MS experiments (CID or HCD). The top 5 signals were selected with monoisotopic precursor selection enabled, and +1 and unassigned charge states rejected. Analyses were carried out in the ion trap or the Orbitrap analyzer. The experiments were performed using collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) fragmentation modes.

SRM Methods

SRM methods were developed on a Thermo Scientific™ TSQ Vantage™ triple stage quadrupole mass spectrometer with a Thermo Scientific™ Accela™ pump, a CTC PAL® autosampler (Leap Technologies), and a Thermo Scientific™ Ion Max™ source equipped with a high-flow metal needle. A mass window of 0.7 full width at half maximum (FWHM, unit resolution) was used in the SRM assays because the immunoenriched samples had a very high signal-to-noise ratios. Narrower windows were necessary when the matrix background was significant and caused interferences that reduced signal-to-noise in the SRM channels. Reversed-phase separations were carried out on a Hypersil GOLD column (1 mm x 100 mm, 1.9 µm particle size) with a flow rate of 160 µL/min. Solvent A was 0.2% formic acid in LC-MS-grade water, and solvent B was 0.2% formic acid in Fisher Scientific™ Optima™-grade acetonitrile.

Software

Thermo Scientific™ Pinpoint™ software was used for targeted protein quantification, automating the prediction of candidate peptides and the choice of multiple fragment ions for SRM assay design. Pinpoint software was also used for peptide identity confirmation and quantitative data processing. The intact PTH sequence was imported into the software and digested with trypsin *in silico*. Then, transitions for each peptide were predicted and tested with recombinant PTH digest to determine those peptides and transitions delivering optimal signal. After several iterations, a subset of six peptides with multiple transitions was chosen.

Further tests were conducted with this optimized method. After the target peptides were identified, heavy arginine or lysine versions were synthesized to be used as internal quantitative standards. Target peptides were subsequently identified and quantified by coeluting light- and heavy-labeled transitions in the chromatographic separation. Time alignment and relative quantification of the transitions were performed with Pinpoint software. All samples were assayed in triplicate.

Results and Discussion

Top-Down Analysis and Discovery of Novel Variants

The approach described herein coupled targeting a common region of PTH by use of a polyclonal antibody (raised to the C-terminal end of the protein) with subsequent detection by use of SRM MS. Numerous PTH variants were simultaneously extracted with a single, high-affinity polyclonal antibody, and the selection of the epitope was directed by the target of interest (i.e., intact and N-terminal variants). The primary goal was to differentiate between intact PTH1–84 and N-terminal variant PTH7–84 while simultaneously identifying any additional N-terminal heterogeneity throughout the molecule. The results of these top-down experiments allowed the development of an initial standard profile for PTH. Clearly, this profile is not finite, and may be expanded to include additional variants found through literature search and/or complementary full-length studies. However, this standard profile provided an initial determination of target sequences for developing specific SRM assays.

Selection of Transitions for SRM

During LC-MS/MS analysis, multiple charge states and fragmentation ions were generated from each fragment, resulting in upwards of 1000 different precursor/product transitions possible for PTH digested with trypsin. Empirical investigation of each transition was not efficient. Therefore, a workflow incorporating predictive algorithms with iterative optimization was used to predict the optimal transitions for routine monitoring of tryptic fragments (Figure 2). The strategy facilitated the translation of peptide intensity and fragmentation behavior empirically obtained by high-resolution LC-MS/MS analyses to triple quadrupole SRM assays. Inherent to the success of the workflow was the similarity of peptide ion fragmentation behavior in these ion trap and triple quadrupole instruments.[12] Empirical data from such LC-MS/MS experiments were used in conjunction with computational methods (*in silico* tryptic digestions and prediction of SRM transitions) to enhance the design of effective SRM methods for selected PTH peptides.

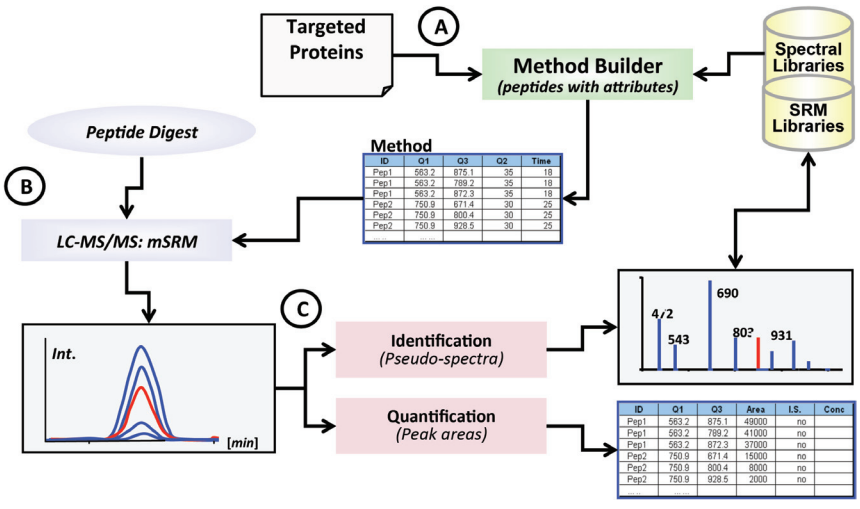


Figure 2. Pinpoint workflow for development of multiplexed SRM assays. [Q = quadrupole; mSRM = multiple SRM; Int. = intensity; I.S. = internal standard; Conc = concentration. Time measurements are in minutes (min).]

The initial list of transitions was queried empirically to produce an LC-MS/MS profile based on four tryptic peptides that collectively spanned >50% (45 of 84 amino acids) of the full PTH sequence. SVSEIQLMHNLGK [amino acid (aa)1–13] was monitored to represent PTH species with an intact N-terminus, such as PTH1–84. Other tryptic peptides, HLNSMER (aa14 –20), DQVHNFVALGAPLAPR (aa28–44), and ADVNVLTK (aa73–80) were included for monitoring across the PTH sequence. In addition, transitions for two truncated tryptic peptides, LMHNLGK (aa7–13) and FVALGAPLAPR (aa34–44), were added to the profile to monitor for truncated variants PTH7–84 and PTH34–84, respectively. In total, 32 SRM transitions tuned to these six peptides were used to monitor intact and variant forms of PTH (Figure 1).

Generation of Standard Curves and Limits of Detection and Quantification

rhPTH was spiked into stock human blood plasma to create calibration curves for all target tryptic peptides through serial dilution. As illustrated in Figure 3 for peptides LQDVHNFVALGAPLAPR (aa28–44) and SVSEIQLMHNLGK (aa1–13), SRM transitions for the four wild-type tryptic fragments exhibited linear responses (R^2 = 0.90–0.99) relative to rhPTH concentration, with limits of detection for intact PTH of 8 ng/L and limits of quantification for these peptides calculated at 31 and 16 ng/L, respectively. Standard error of analysis for all triplicate measurements in the curves ranged from 3% to 12% for all peptides, with <5% chromatographic drift between replicates. In addition, all experimental peptide measurements were calculated relative to heavy-labeled internal standards. CVs of integrated areas under the curve for 54 separate measurements (for each heavy peptide) ranged from 5% to 9%. Monitoring of variant SRM transitions showed no inflections relative to rhPTH concentration, owing to the absence of truncated variants in the stock rhPTH.

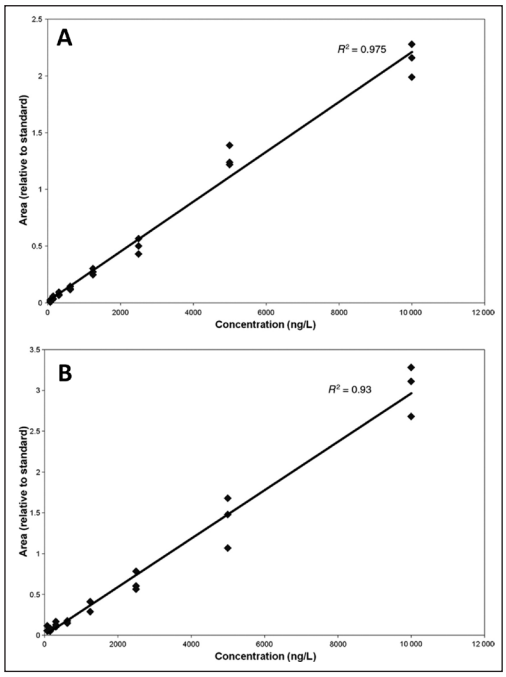


Figure 3. SRM calibration curves for PTH peptides.
(A) Peptide LQDVHNFVALGAPLAPR aa28–44.
(B) Peptide SVSEIQLMHNLGK aa1–13.

Evaluation of Research Study Samples

Initial SRM data were acquired from replicate plasma samples. The light and heavy peptides coeluted precisely in all samples. Further SRM experiments were carried out on the cohort of renal failure (n = 12) and normal (n = 12) samples. The most prominent PTH variant in the renal failure samples was PTH34–84. To quantify this observation with SRM, all samples were interrogated to determine the expression ratios of renal failure to normal for the various target peptides, including FVALGAPLAPR (aa34–84), which should be specific to the 34–84 variant. Chromatographic data from single renal-failure samples for peptides FVALGAPLAPR (aa34–44) and SVSEIQLMHNLGK (aa1–13) are shown in Figure 4. The peak integration area and individual coeluting fragment transitions for each peptide are illustrated. Similar chromatograms were obtained for peptides LQDVHNFVALGAPLAPR (aa28–44), HLNSMER (aa14–20), and ADVNVLTK (aa73–80) (data not shown). The sample variances and expression ratios of renal-failure samples to normal samples for each peptide are shown in Figure 5. The expression ratios for the peptides ranged from 4.4 for FVALGAPLAPR (aa34–44) to 12.3 for SVSEIQLMHNLGK (aa1–13). Notable quantities of peptide LMHNLGK (aa 7–13) were not detected in these samples. Sample variances illustrated in the scatter plots in Figure 5 demonstrate that the renal failure and normal samples groups were clearly segregated by the five target peptides.

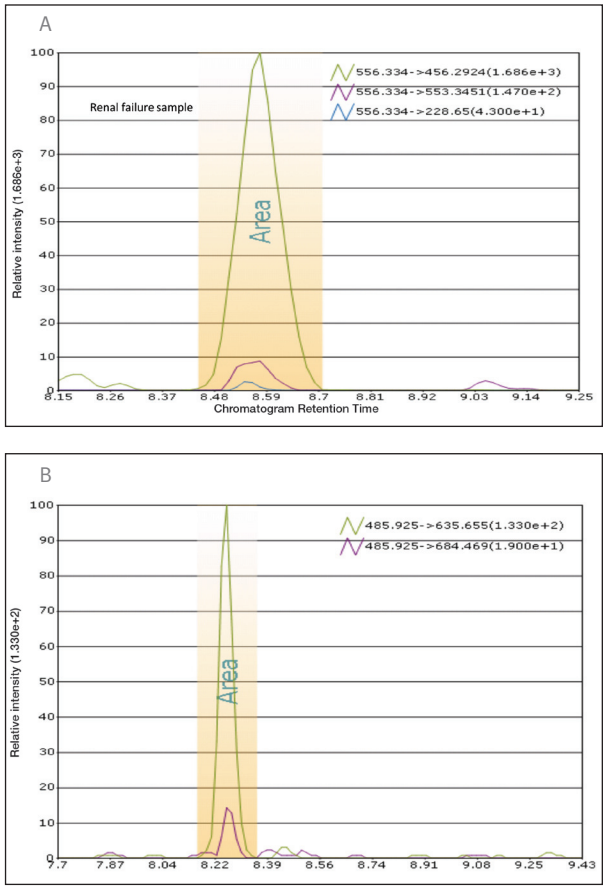
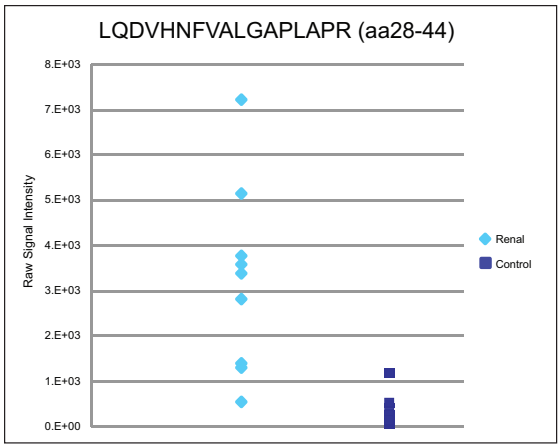
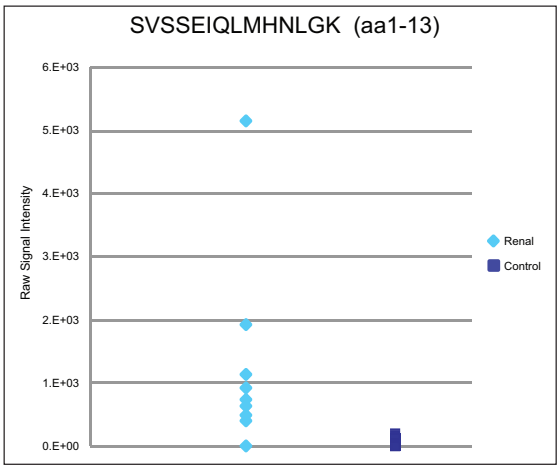
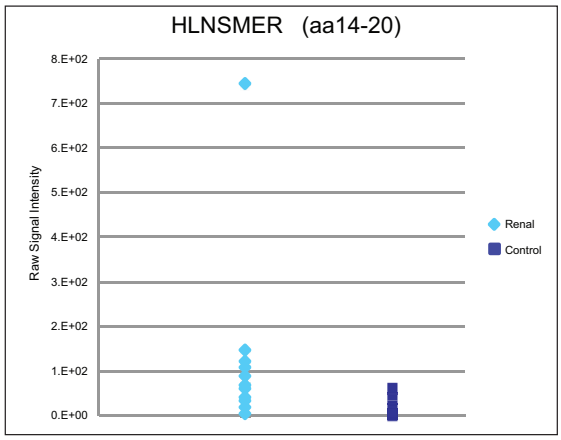
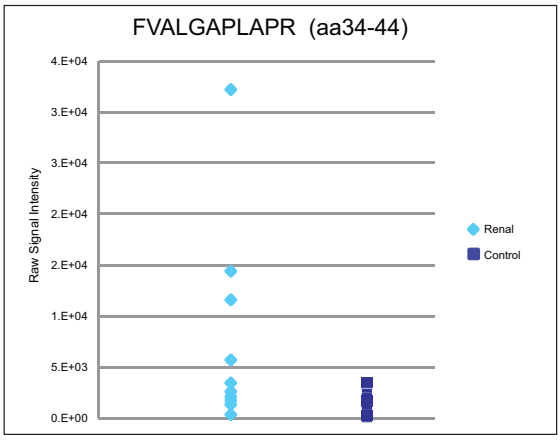


Figure 4. Pinpoint software SRM data from samples of normal and renal failure patients. Chromatographic data illustrate peak integration area and individual fragment transitions for peptides from single renal failure samples. (A) Semitryptic peptide FVALGAPLAPR (aa34–44), specific to the 34–84 variant (see Figure 1). (B) Tryptic peptide SVSEIQLMHNLGK (aa1–13).









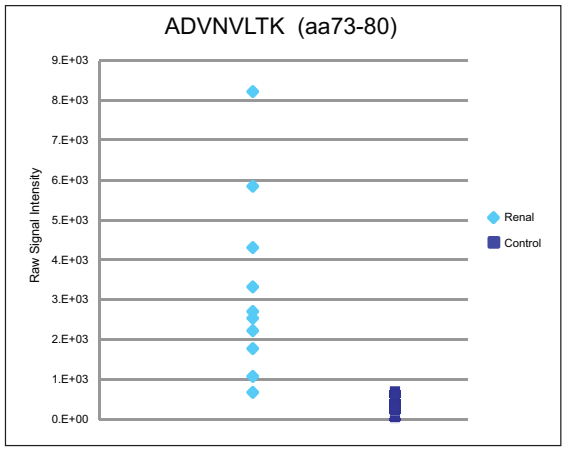


Figure 5. SRM quantitative ratios and sample variances of PTH peptides in samples from renal failure patients (Renal) and healthy controls. Ratios refer to the average value of the renal cohort divided by the average value of the healthy control cohort.

Conclusion

An SRM-MSIA-based analysis method was developed capable of simultaneously monitoring full-length PTH and truncated variants with analytical metrics suitable for clinical research use. Using a workflow incorporating postcapture tryptic digestion, surrogate peptides representative of PTH1–84 and PTH7–84 were generated and then monitored using SRM. In addition, tryptic fragments spanning other regions of PTH were incorporated into the analysis. Relative ion signals for these species confirmed that the clinical research method was functional and created the basis for a standard PTH profile. This standard profile was expanded to include a peptide representative of a novel variant, PTH34–84, clipped at the N-terminus. In total, 32 SRM transitions were analyzed in a multiplexed method to monitor nonvariant PTH sequences with >50% sequence coverage, as well as the two truncated variants. Peptides exhibited linear responses (R^2 = 0.90–0.99) relative to the limit of detection for an intact recombinant human PTH concentration of 8 ng/L. Limits of quantification were 16–31 ng/L, depending on the peptide. Standard error of analysis for all triplicate measurements was 3%–12% for all peptides, with <5% chromatographic drift between replicates. The CVs of integrated areas under the curve for 54 separate measurements of heavy peptides were 5%–9%.

Pinpoint software was used to develop and implement "intelligent SRM" data acquisition strategies, increasing instrument efficiency by avoiding the need to monitor all of the specified transitions at all times. Use of these techniques may be particularly advantageous for clinical research laboratories in methods where a large number of PTH variants are monitored, or where the analyzed sample contains a complex mixture of PTH-derived peptides and components produced by digestion of compounds in the sample matrix.

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Analysis of 25-Hydroxyvitamin D in Serum Using an Automated Online Sample Preparation Technique with a High-Resolution Benchtop Orbitrap Mass Spectrometer

Matthew Berube, Joe DiBussolo, Catherine Lafontaine, Yang Shi
Thermo Fisher Scientific, Franklin, MA

Application Note 567

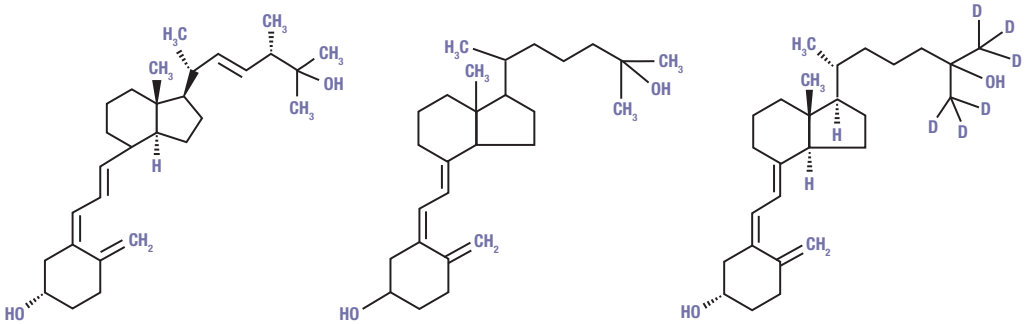
Key Words
Transcend TLX-1 System, TurboFlow Technology, Exactive Plus, Vitamin D

Goal
To demonstrate the effectiveness of a clinical research method for the quantitation of 25-hydroxyvitamin D using online sample preparation and high-resolution, accurate mass (HR/AM) quantitation with a Thermo Scientific Exactive Plus Orbitrap mass spectrometer.

Introduction
Blood levels of 25-hydroxyvitamin D_2 and 25-hydroxy-vitamin D_3 are commonly tested by clinical researchers to assess vitamin D sufficiency. In the last decade, liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS) has become a popular technique for such measurements. Due to their higher resolving power relative to triple-stage quadrupole mass spectrometers, Orbitrap™-based mass spectrometers are better able to resolve analytes from sample matrices. In addition, the ease of initial method set up and daily use provides an advantage over triple-stage quadrupole mass spectrometers for clinical research.

A method has been created that allows precipitated serum to be injected into an HPLC system with minimal sample preparation and analyzed by an Exactive™ Plus benchtop Orbitrap mass spectrometer. Total method time is 7.75 minutes on a Thermo Scientific Transcend TLX-1 system utilizing TurboFlow technology. Throughput can be increased to a sample every 3.7 minutes by using a Transcend™ TLX-2 multiplexed UHPLC system or 1.9 minutes with a Transcend TLX-4 system.

Experimental
Standard solutions of 25-hydroxyvitamin D_2, 25-hydroxy-vitamin D_3, and deuterated 25-hydroxyvitamin D_3 internal standard were obtained from Cerilliant, Inc. (Figure 1). Six calibrators at 2, 5, 10, 25, 50 and 100 ng/mL and three QCs at 5, 40 and 80 ng/mL were prepared by fortifying bovine serum albumin diluent with 200 ng/mL 25-hydryoxyvitamin D_2 and D_3 standard mix. Precipitating reagent was prepared by adding deuterated D_6-25-hydroxy-vitamin D_3 to acetonitrile for a final concentration of 75 ng/mL. In addition, pooled human serum samples were crashed 2 to 1 with acetonitrile and spiked with analytes for a final concentration of 20 ng/mL for 25-hydroxy-vitamin D_2 and 25-hydroxyvitamin D_3, and 50 ng/mL of D_6 25-hydroxyvitamin D_3 internal standard.



25-Hydroxyvitamin D_2
$C_{28}H_{44}O_2$ (MW 412.65)

25-Hydroxyvitamin D_3
$C_{27}H_{44}O_2$ (MW 400.63)

D_6-25-Hydroxyvitamin D_3
$C_{27}H_{38}D_6O_2$ (MW 406.67)

Figure 1. Analytes

Thermo SCIENTIFIC

Samples were prepared by adding 200 µL of precipitating reagent containing internal standard to each centrifuge tube containing 100 µL of calibrants and controls. Tubes were vortexed for 30 seconds and then centrifuged at 5,000 RCF for 10 minutes. Supernatants were then aliquoted into autosampler vials for analysis. Calibration curves and QCs were run in triplicate each day across four days. In addition, 800 pooled serum sample replicates containing 20 ng/mL 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 and 50 ng/mL of D_6-25-hydroxy-vitamin D_3 internal standard were injected to test robustness of the method. Thermo Scientific Xcalibur software was used to collect data and analyze the results. The Exactive Plus mass spectrometer was used with an APCI source in positive ionization mode. Full-scan data was collected from *m/z* 350 to 425.

LC/MS Conditions

TurboFlow Method Parameters (see also Figure 2)	
Plumbing mode:	Focus Mode
Column:	Thermo Scientific TurboFlow XL C-18P 0.5 x 50 mm
Injection volume:	50 µL
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol
Solvent C:	40:40:20 acetonitrile: isopropyl alcohol: acetone (v:v:v)
Analysis time:	7.75 minutes
Cycle time when multiplexed 4x:	1.9 minutes

HPLC Method Parameters	
Analytical column:	Thermo Scientific Accucore C18 3 x 50 mm 2.6 µm
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol

Mass Spectrometer Parameters	
Scan mode:	Full
Scan range:	*m/z* 350 – 425
Fragmentation:	None
Polarity:	Positive
Microscans:	1
Resolution:	70,000
AGC target:	3 x 10^6
Maximum inject time:	200

Ion Source Parameters	
Ion source:	APCI
Discharge current:	3.5 uA
Vaporizer temperature:	500 °C
Sheath gas pressure:	30 units
Ion sweep gas pressure:	1 unit
Aux gas pressure:	5 units
Capillary temperature:	250 °C
S-Lens RF level:	60

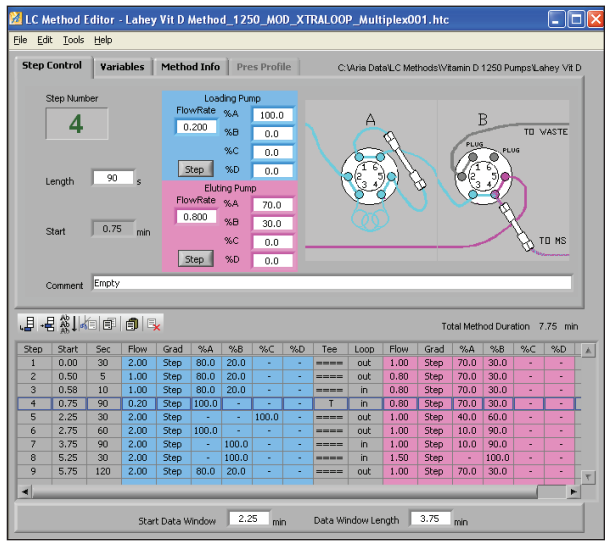


Figure 2. TurboFlow method details

Results and Discussion

The lower limit of quantitation (LLOQ) was determined to be 2 ng/mL for both analytes in BSA as indicated in Figure 3. Limits of quantitation (LOQs) were estimated from the triplicate injections of the standard solutions. The signal-to-noise ratio was greater than 10 and the coefficient of variation (CV) values were less than 10% at the LLOQ of 2 ng/mL for both 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 (Table 1). The correlation coefficients obtained using 1/X weighted linear regression analysis of the standard curves were greater than 0.99 for both analytes (Figures 4 and 5). A relative standard deviation (%RSD) test was performed in pooled human serum fortified with analytes at 20 ng/mL and crashed with internal standard solution for a total internal standard concentration of 50 ng/mL. The RSDs of ten replicate injections were less than 10% for both analytes (Table 2). A recovery study was also performed using a neat standard of 20 ng/mL 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 with 50 ng/mL D_6-25-hydroxy-vitamin D_3. The standard was injected ten times on the TurboFlow™ column and analytical column, and ten times on the analytical column only, and area counts were compared. The relative recoveries were 97% and 99% for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, respectively.

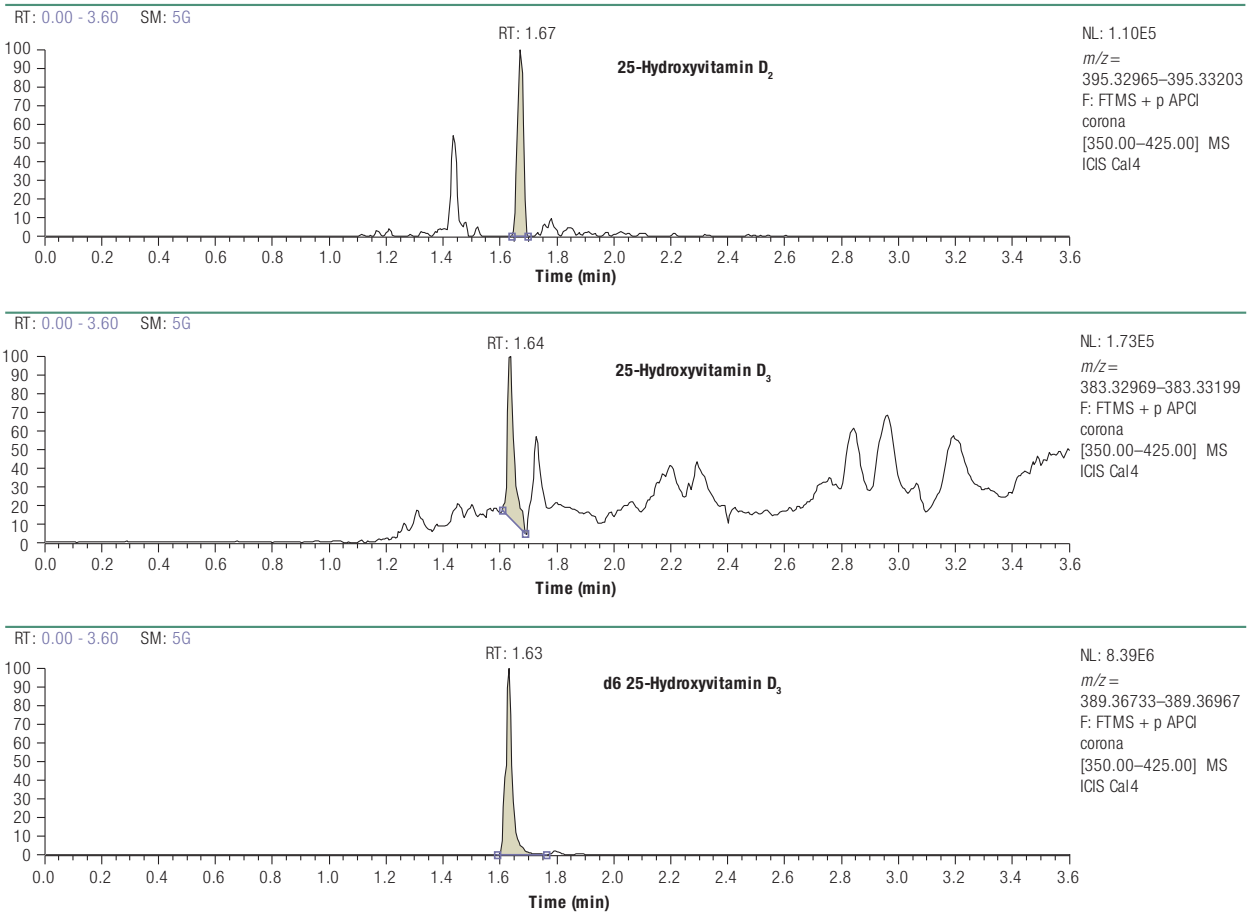


Figure 3. Chromatograms at LLOQ of 2 ng/mL with 50 ng/mL internal standard

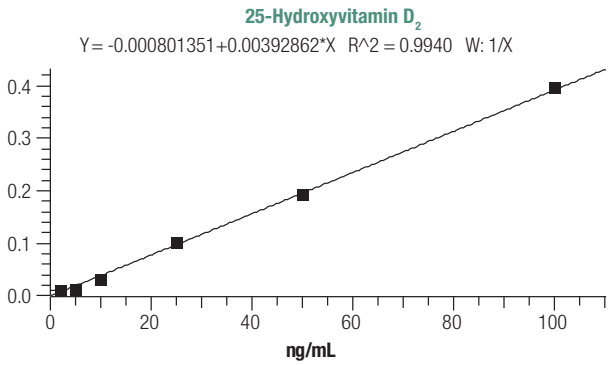


Figure 4. Calibration curve of 25-hydroxyvitamin D_2 in BSA

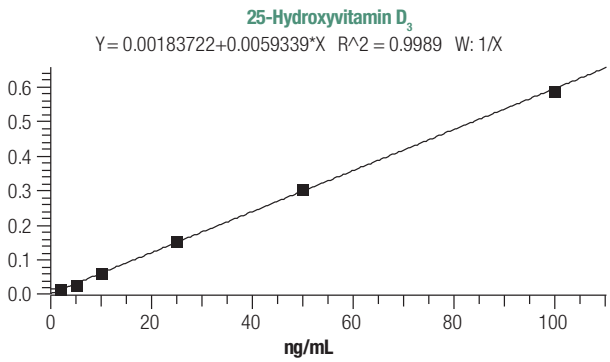


Figure 5. Calibration curve of 25-hydroxyvitamin D_3 in BSA

Table 1. 2 ng/mL replicate LLOQ injections

25-hydroxyvitamin D_2	2 ng Area
Replicate 1	134195
Replicate 2	162585
Replicate 3	148309
Mean	148363
SD	14195.1
%CV	9.6

25-hydroxyvitamin D_3	2 ng Area
Replicate 1	201766
Replicate 2	242186
Replicate 3	212094
Mean	218682
SD	20999.9
%CV	9.6

Table 2. 20 ng/mL serum injection replicates

D_2 20 ng Serum	Area
Replicate 1	4464244
Replicate 2	3757594
Replicate 3	4544819
Replicate 4	4332109
Replicate 5	3857037
Replicate 6	4581097
Replicate 7	5148234
Replicate 8	4704084
Replicate 9	4319873
Replicate 10	4175023
Mean	4388411
SD	405245.1
%CV	9.2

D_3 20 ng Serum	Area
Replicate 1	11759664
Replicate 2	10759647
Replicate 3	10886536
Replicate 4	10825748
Replicate 5	12543252
Replicate 6	12223745
Replicate 7	11278373
Replicate 8	11445949
Replicate 9	12537176
Replicate 10	11033701
Mean	11529379
SD	698829.3
%CV	6.1

Conclusion

An Exactive Plus high-resolution Orbitrap mass spectrometer with TurboFlow automated on-line sample extraction technology provides reliable detection for clinical researchers of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in serum.

In addition, the Exactive Plus MS offers higher resolving power and easier initial method setup than triple quadrupole mass spectrometers. Throughput can be increased to a sample every 3.7 minutes by using a Transcend TLX-2 multiplexed UHPLC system or a sample every 1.9 minutes with a Transcend TLX-4 system.

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Plasma Free Metanephrines Quantitation with Automated Online Sample Preparation and Liquid Chromatography—Tandem Mass Spectrometry

Xiang He and Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Application Note 563

Key Words

TSQ Vantage, Clinical Research, TurboFlow Technology, Metanephrine, MN, Normetanephrine, NMN, Pmets, Pheochromocytoma

Goal

To develop an automated method to quantitate plasma free metanephrines reducing method time while maintaining analytical performance compared to the original offline SPE method.

Introduction

Plasma free metanephrine (MN) and normetanephrine (NMN), collectively known as Pmets, are preferred biomarkers for pheochromocytoma for clinical research. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become widely used to measure Pmets because of its high analytical specificity.

Recently, we reported an LC-MS/MS method for measuring Pmets using ion-pairing solid phase extraction (IP-SPE) and porous graphitic carbon (PGC) column chromatography[1,2]. Although the method is fast and analytically sensitive, it can be further improved by automating the offline sample preparation with online sample preparation technology, which is more time- and cost-effective.

Thermo Scientific TurboFlow technology is an automated online sample preparation technology that has been coupled to LC-MS/MS for the quantitative analysis of a variety of biological samples.

To date, its use has been reported in clinical research, pharmaceutical analysis, bioanalysis, environmental testing, food safety, and forensic toxicology.

Methods

Sample Preparation

The 0.5-mL samples of human plasma and of charcoal stripped serum (CSS) were spiked with internal standards (IS) and then mixed with 0.25 mL of 10% tricholoacetic acid (w/v) in water. The mixtures were vortexed and stored at –30 °C for 30 minutes. Then, the mixtures were centrifuged at 16,000 g for 10 minutes, and 100 µL of the supernatants were injected for LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Transcend TLX-1 system. The TurboFlow™ method with automated online sample preparation was performed with a TurboFlow Cyclone MCX-2 column. Perfluoroheptanoic acid (PFHA) was used as the ion-pair during the sample preparation.

		Loading								Eluting					
Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
00:00	30	2.00	Step	100.0	-	-	-	====	out	1.00	Step	98.0	2.0	-	-
00:30	30	2.00	Step	90.0	10.0	-	-	====	out	0.50	Step	90.0	-	-	10.0
01:00	90	0.10	Step	90.0	10.0	-	-	T	in	1.00	Step	90.0	-	-	10.0
02:30	1	0.10	Step	90.0	10.0	-	-	====	in	0.50	Step	98.0	2.0	-	-
02:31	300	0.50	Step	-	-	-	100.0	====	in	0.50	Ramp	60.0	40.0	-	-
07:31	60	2.00	Step	-	-	100.0	-	====	in	1.00	Step	-	100.0	-	-
08:31	60	2.00	Step	70.0	30.0	-	-	====	in	1.50	Step	-	-	100.0	-
09:31	150	2.00	Step	100.0	-	-	-	====	out	1.00	Step	98.0	2.0	-	-

Figure 1. TurboFlow and LC method

Loading:
A: 0.1% PFHA in water
B: 60% ACN in water
C: Mixture of isopropanol, ACN and acetone (1:1:1 v/v/v) with 0.3% formic acid
D: 5 mM NH_4Ac and 50% ACN in water

Eluting:
A: 50 mM NH_4FA and 1% formic acid in water
B: 0.1% formic acid in ACN
C: Mixture of isopropanol, ACN and acetone (9:9:2 v/v/v)
D: 0.1% PFHA in water.

Eluting LC column temperature: 70 °C

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Analytical separation was carried out on a Thermo Scientific Hypercarb column (50×3 mm, 5.0-µm particle size) at 70 °C. The total LC runtime was 12 minutes (Figure 1). The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. Data was acquired in selected-reaction monitoring (SRM) mode.

Validation

The validation procedure included tests for 1) recovery; 2) lower limit of quantitation (LLOQ), dynamic range, accuracy; 3) precision; 4) ion suppression; 5) carryover; and 6) interferences.

Results and Discussion

Charcoal stripped serum (CSS) was first evaluated by comparing it to human plasma using a generally adopted mixing study[3]. It was determined that CSS is an appropriate matrix to conduct the validation experiments.

Recovery

The extraction recovery was assessed by comparing the direct injection to the TurboFlow method injection of MN, NMN, MN-d3 and NMN-d3 spiked in mobile phase (n=2). The absolute recovery of MN, NMN and their IS ranged from 56.4% to 62.4%, and the relative recovery of MN and NMN was 90.9% and 97.8%, respectively (Table 1).

Determination of LLOQ, Linearity and Accuracy

CSS was spiked with MN and NMN to achieve final concentrations of 500 and 1000 pg/mL, respectively. A serial two-fold dilution with CSS was performed to make eight levels of linearity samples with concentration ranges of 500 to 3.9 pg/mL and 1000 to 7.8 pg/mL for MN and NMN, respectively. Linearity samples were analyzed in triplicate along with one set of calibrators. The calibration curve was constructed by plotting the analyte:IS peak area ratio vs. analyte concentration.

The linearity was determined to be 6.3 to 455.4 pg/mL for MN and 12.6 to 954.5 pg/mL for NMN. Within the linear range, the accuracy ranged from 80.6% to 93.5% for MN, and from 80.9% to 101.7% for NMN. The CV (n=3) from all linearity levels ranged from 3.1% to 13.7% for MN, and from 1.6% to 10.7% for NMN (Table 1 and Figures 2 and 3). The determined LLOQ was 6.3 pg/mL for MN and 12.6 pg/mL for NMN (Table 2).

Table 1. Recovery

	Online Extraction (mean ± CV)[b]	Direct Injection (mean ± CV)	Absolute Recovery (%)	Relative Recovery (%)
MN (500 pg/mL)[a]	60281 ± 2.7%	106866 ± 10.5%	56.4	90.9
NMN (250 pg/mL)[a]	32186 ± 5.6%	51878 ± 9.4%	62.0	97.8
MN-d3 (500 pg/mL)[a]	40716 ± 1.1%	66790 ± 11.4%	61.0	N/A
NMN-d3 (500 pg/mL)[a]	28983 ± 3.7%	46482 ± 11.8%	62.4	N/A

[a] MN, NMN, MN-d3 and NMN-d3 were spiked to mobile phase at specified concentration levels.

[b] Measured peak area with CV (n=2)

Table 2. LLOQ, dynamic range and accuracy

	MN				NMN			
Dilution factor	**Expected (pg/mL)**	**Measured (pg/mL)**	**CV of triplicates (%)**	**Accuracy (%)**	**Expected (pg/mL)**	**Measured (pg/mL)**	**CV of triplicates (%)**	**Accuracy (%)**
128	3.91	5.5	17.2	71.1	7.8	7.4	35.3	94.9
64	7.81	6.3	13.7	80.6	15.6	12.6	10.7	80.9
32	15.6	13.9	7.2	88.8	31.3	30.8	1.6	98.7
16	31.3	27.5	4.9	88.0	62.5	61.0	6.0	98.1
8	62.5	56.6	10.3	90.6	125.0	121.2	9.2	96.9
4	125.0	112.2	4.0	89.8	250.0	254.2	9.4	101.7
2	250.0	233.7	3.1	93.5	500.0	496.9	2.7	99.4
1	500.0	455.4	4.0	91.1	1000.0	954.5	3.3	95.5
Mean (%)				**88.9**				**95.9**
Stdev (%)				**4.1**				**6.9**

Precision

Precision was assessed with spiked CSS. Inter- and intra-assay CV values at low and high quality control concentrations of both analytes varied between 2.0% and 10.5% (Table 3).

Table 3. Precision data

	MN		NMN	
Charcoal Stripped Serum	**31.3 pg/mL**	**250.0 pg/mL**	**62.5 pg/mL**	**500.0 pg/mL**
Intra 1 (%) n=5	6.7	4.2	4.5	5.4
Intra 2 (%) n=5	4.9	3.0	10.5	4.2
Intra 3 (%) n=5	7.3	4.7	10.0	2.0
Inter-assay (%) n=15	8.4	7.7	8.9	4.8

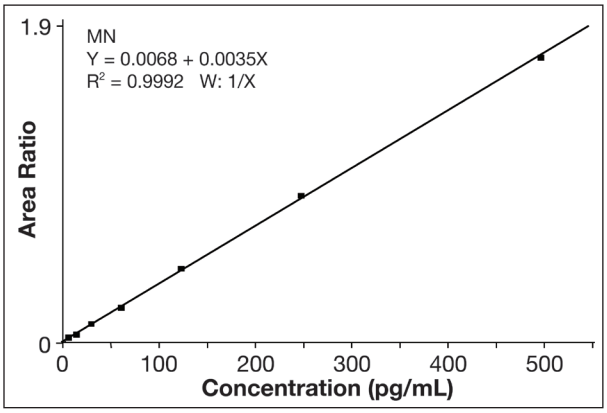

Figure 2. Calibration curve of MN in CSS

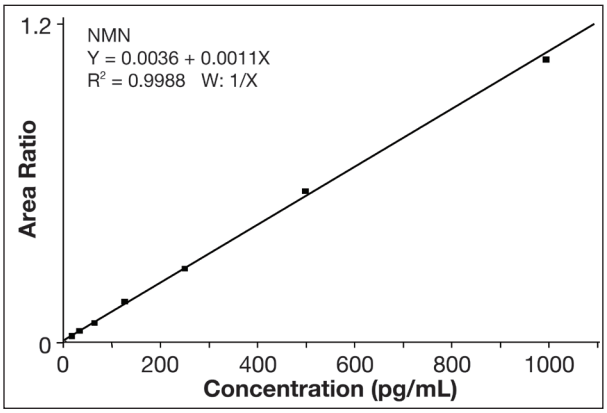

Figure 3. Calibration curve of NMN in CSS

Ion Suppression

The MS responses of MN-d3 and NMN-d3 in solvent (n=4) and individual human plasma samples (n=4) at the same concentrations (400 pg/mL for both MN-d3 and NMN-d3) were measured with LC-MS/MS analysis. The average MS responses (integrated area) of MN-d3 and NMN-d3 from solvent and real human plasma samples were calculated. The intensity ratios with standard deviations between human plasma (n=4) and solvent (n=4) were 113.3% ± 18.4% and 126.4% ± 18.0% for MN-d3 and NMN-d3, respectively. This indicated that this method has no obvious ionization suppression or enhancement.

Carryover

No carryover was observed.

Interferences

Epinephrine (EPI) and NMN share the same SRM transitions and could not be differentiated just by MS/MS analysis. Using the Hypercarb™ analytical column, the EPI peak was baseline resolved from the NMN peak (0.3 min apart, data not shown).

Data Examples of Clinical Research Samples

Figure 4 shows the SRM chromatograms of MN and NMN in an individual plasma sample. Figure 5 shows the SRM chromatograms of MN and NMN in a CSS sample.

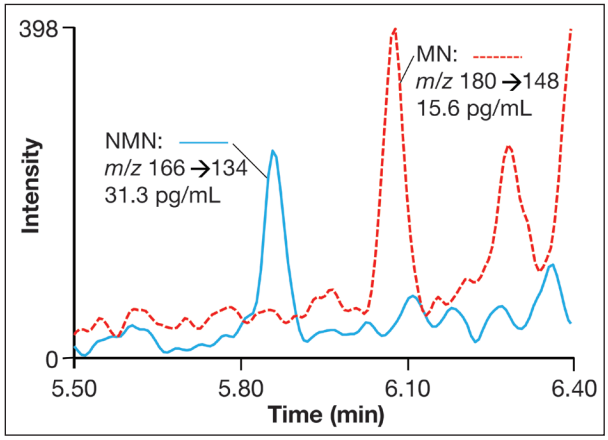

Figure 4. SRM chromatograms of MN and NMN in human plasma sample

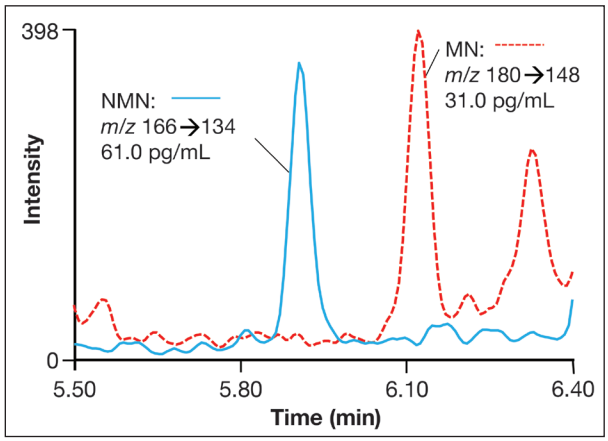

Figure 5. Representative SRM chromatograms of MN (31.0 pg/mL) and NMN (61.0 pg/mL) in CSS sample

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Conclusion

A fast, automated and analytically sensitive LC-MS/MS method was developed to quantify plasma metanephrines for clinical research purposes[4]. By using TurboFlow technology, the sample preparation procedure was significantly simplified compared to a previously reported offline IP-SPE method. The presence of PFHA during the online sample preparation was critical to the success of this method. A PGC column was used for chromatographic separation of metanephrines. The total online extraction and analytical LC runtime was 12 minutes. This method was linear from 6.3 to 455.4 pg/mL for metanephrine and 12.6 to 954.5 pg/mL for normetanephrine, with an accuracy of 80.6% to 93.5% and 80.9% to 101.7%, respectively. The lower limit of quantitation was 6.3 pg/mL for metanephrine and 12.6 pg/mL for normetanephrine. Inter-assay and intra-assay precision for metanephrine and normetane-phrine at low and high concentration level ranged from 2.0% to 10.5%.

Overall, the analytical performance achieved with this automated online TurboFlow method is consistent with the previously reported offline SPE method[2]. More importantly, the online method significantly saved sample preparation time by more than 50% and eliminated the expense of SPE cartridges with an offline approach.

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AN63539_E 04/12S

Application Note: 558

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Quantitation of Estrone and Estradiol with Automated Online Sample Preparation and LC-MS/MS

Xiang He and Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Vantage
- Transcend TLX System
- Accucore RP-MS Column
- Clinical Research
- TurboFlow Technology

Introduction

Estrone (E1) and estradiol (E2) are two major biologically active estrogens. Quantitative measurements of these two estrogens are important in clinical research.

Quantitation of serum estrogens has been performed with immunoassay and gas chromatography-mass spectrometry (GC-MS). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is preferred over immunoassay and other analytical techniques because it is more analytically specific. Recently, we developed a simple, fast and analytically sensitive method for measuring underivatized E1 and E2 in serum or plasma by LC-MS/MS using atmospheric pressure chemical ionization (APCI).[1]

Thermo Scientific TurboFlow technology is an automated online sample preparation technology that has been coupled to LC-MS/MS for the quantitative analysis of a variety of biological samples. To date, its use has been reported in the fields of clinical research, pharmaceutical analysis, bioanalysis, environmental testing, food safety, and forensic toxicology.

Goal

To develop a fast and analytically sensitive LC-MS/MS method with automated online sample preparation for simultaneous quantitation of underivatized E1 and E2 in serum using TurboFlow™ technology.

Methods

Sample Preparation

Briefly, 0.5 mL of sample was mixed with 0.5 mL of working internal standard (E2-d5, IS) solution in methanol. The mixture was vortexed, kept at -30 °C for 30 min and then centrifuged at 16,000 g for 3 min at room temperature. This process was repeated once for complete protein precipitation. The supernatant (300 µL) was directly injected for TurboFlow LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Transcend TLX-1 system equipped with Accela 1250 pumps. The online sample preparation was performed with TurboFlow Cyclone-P polymer-based columns. Analytical high-performance liquid chromatography (HPLC) was carried out on a Thermo Scientific Accucore RP-MS solid core column (100 × 3 mm, 2.6 µm particle size) at room temperature using water and methanol as mobile phases (Figure 1). The total runtime was 10 min. The mass spectrometer was operated with an APCI source in negative ion mode. Data was acquired in selected reaction monitoring (SRM) mode.

	Loading										Eluting					
Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
1	0.00	45	2.00	Step	100.0	-	-	-	====	out	0.60	Step	100.0	-	-	-
2	0.75	60	0.10	Step	100.0	-	-	-	T	in	0.60	Step	100.0	-	-	-
3	1.75	60	2.00	Step	-	100.0	-	-	====	in	0.60	Ramp	40.0	60.0	-	-
4	2.75	120	2.00	Step	100.0	-	-	-	====	in	0.60	Ramp	20.0	80.0	-	-
5	4.75	60	2.00	Step	-	100.0	-	-	====	in	0.60	Step	-	100.0	-	-
6	5.75	90	2.00	Step	100.0	-	-	-	====	out	0.60	Step	100.0	-	-	-

Loading: A: water; B: methanol. **Eluting:** A: water; B: methanol.

Figure 1. TurboFlow and LC method

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Validation

The validation procedure included tests for 1) recovery of sample preparation; 2) lower limit of quantitation (LLOQ), dynamic range, accuracy; 3) precision; 4) ionization suppression; and 5) carryover.

Results and Discussion

Human plasma has endogenous E1 and E2 so it was not suitable for validation experiments except the precision study. Therefore, charcoal stripped serum (CSS) is used to conduct the validation experiments.

Recovery

The absolute recoveries of E1, E2 and IS from CSS samples compared to spiked neat solutions ranged from 61.2% to 65.6%. The relative recoveries of E1 and E2 against IS ranged from 99.0% to 107.1% at the two spiked concentration levels (20 and 100 pg/mL).

Determination of LLOQ, Linearity and Accuracy

A stock solution of E1 and E2 at 1000 pg/mL was prepared in CSS. A serial 2-fold dilution with blank CSS was performed to make 9 levels of linearity samples with concentrations from 1000 to 3.9 pg/mL for both E1 and E2. Linearity samples were analyzed in triplicate. The calibration curve was constructed by plotting the analyte:IS peak area ratio vs. expected analyte concentration.

The method was linear between 3.8 and 1000.9 pg/mL with accuracy (n=3) from 95.5% to 103.2% for E1, and between 3.7 and 993.1 pg/mL with accuracy (n=3) from 92.7% to 112.3% for E2 (Table 1, Figures 2 and 3). The LLOQ for E1 and E2 are 3.8 and 3.7 pg/mL, respectively (Table 1 and Figure 4).

Table 1. LLOQ, dynamic range and accuracy

		E1			E2		
Dilution Factor	**Expected (pg/mL)**	**Measured (mean, pg/mL)**	**CV (n=3 %)**	**Accuracy (n=3, %)**	**Measured (mean, pg/mL)**	**CV (n=3, %)**	**Accuracy (n=3, %)**
256	3.9	**3.8**	5.0	97.8	**3.7**	11.7	94.6
128	7.8	8.0	9.0	102.9	8.8	13.9	112.3
64	15.6	16.1	5.1	102.8	15.7	7.4	100.4
32	31.3	32.2	8.4	103.2	29.0	7.6	92.7
16	62.5	59.7	0.8	95.5	62.7	4.4	100.3
8	125.0	123.3	9.9	98.7	129.4	9.8	103.5
4	250.0	245.9	7.0	98.4	253.1	3.7	101.2
2	500.0	503.5	2.3	100.7	478.9	4.1	95.8
1	1000.0	**1000.9**	4.5	100.1	**993.1**	5.3	99.3
Mean				**100.0**			**100.0**

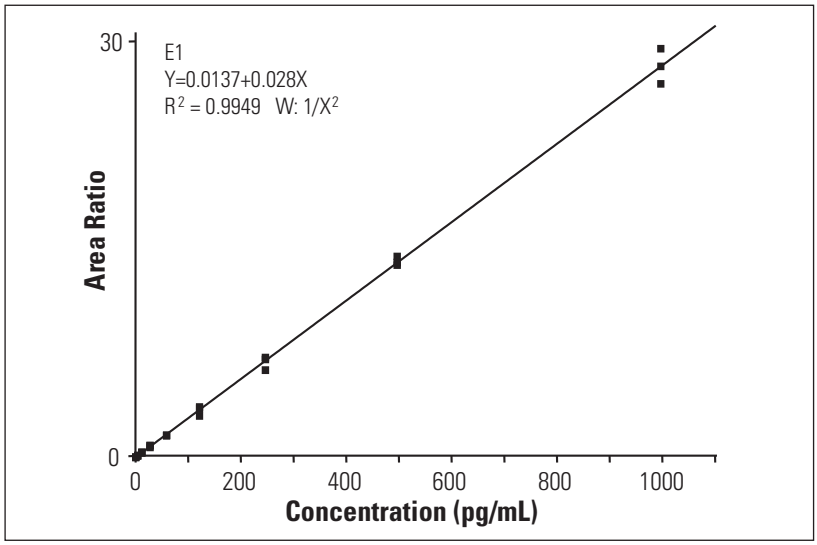

Figure 2. Calibration curve of E1 in CSS

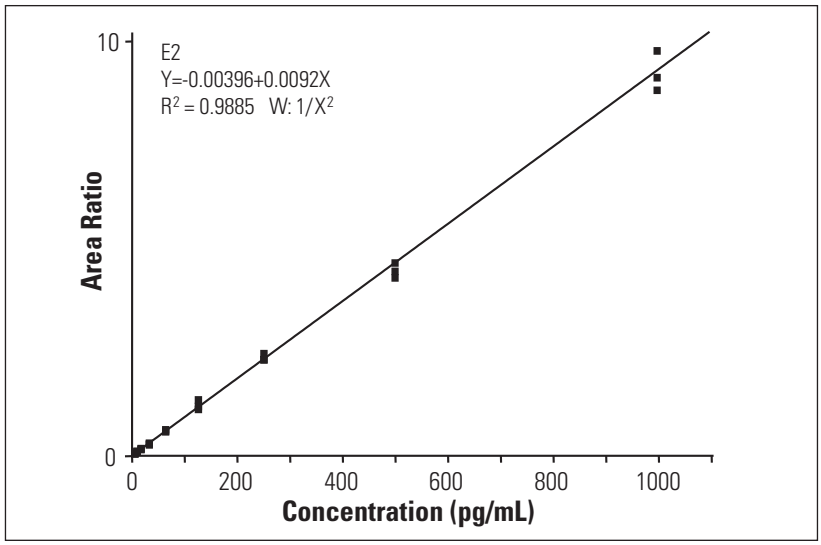

Figure 3. Calibration curve of E2 in CSS

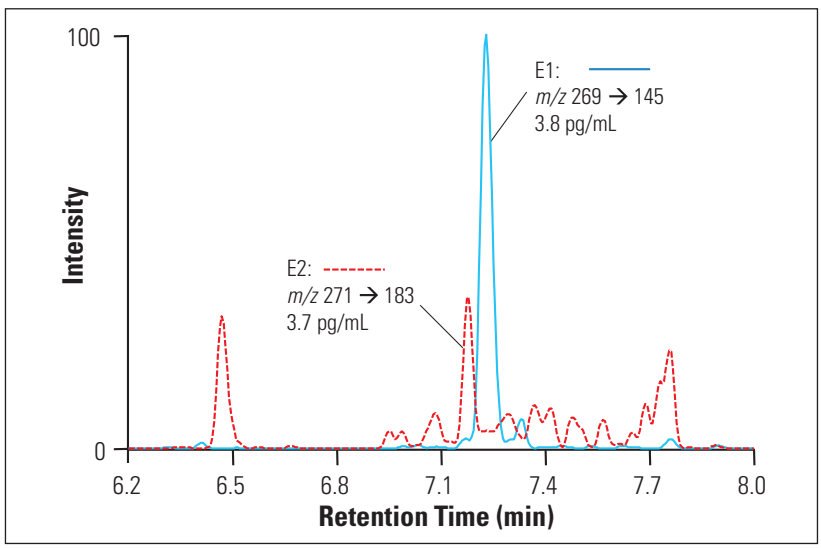

Figure 4. SRM chromatograms of E1 and E2 at their LLOQ in spiked CSS

Precision

Precision was assessed with spiked CSS and human plasma at low and high concentration levels. Inter- (n=15) and intra-batch (n=5) coefficient of variation (CV) values ranged between 3.5% and 18.0% (Table 2).

Table 2. Precision data

		E1		E2	
	Charcoal Stripped Serum	**Low (15 pg/mL)**	**High (364 pg/mL)**	**Low (15 pg/mL)**	**High (357 pg/mL)**
Batch 1	Intra-assay Precision (n=5, %)	9.9	9.6	13.5	11.5
Batch 2	Intra-assay Precision (n=5, %)	17.1	3.5	12.4	4.3
Batch 3	Intra-assay Precision (n=5, %)	14.6	7.2	17.2	4.8
Batch 1-3	Inter-assay Precision (n=15, %)	13.1	8.1	14.0	8.4
	Spiked Pooled Plasma	**Low (12 pg/mL)**	**High (239 pg/mL)**	**Low (11 pg/mL)**	**High (227 pg/mL)**
Batch 1	Intra-assay Precision (n=5, %)	5.3	5.8	18.0	7.9
Batch 2	Intra-assay Precision (n=5, %)	12.9	7.1	16.3	4.3
Batch 3	Intra-assay Precision (n=5, %)	10.0	6.8	12.3	9.0
Batch 1-3	Inter-assay Precision (n=15, %)	9.3	6.3	17.3	7.1

Ionization Suppression

In this test, a constant flow (5 µL/min) of E2-d5 (100 ng/mL) was infused post-column into the mobile phase using a T-junction while protein-crashed human plasma (without internal standards) or mobile phase buffer (blank) were injected. An SRM transition of the infused E2-d5 was monitored for the entire LC gradient. Compared to the solvent blank (60% methanol in water), no obvious ionization suppression was detected in the SRM chromatogram of infused E2-d5 (Figure 5).

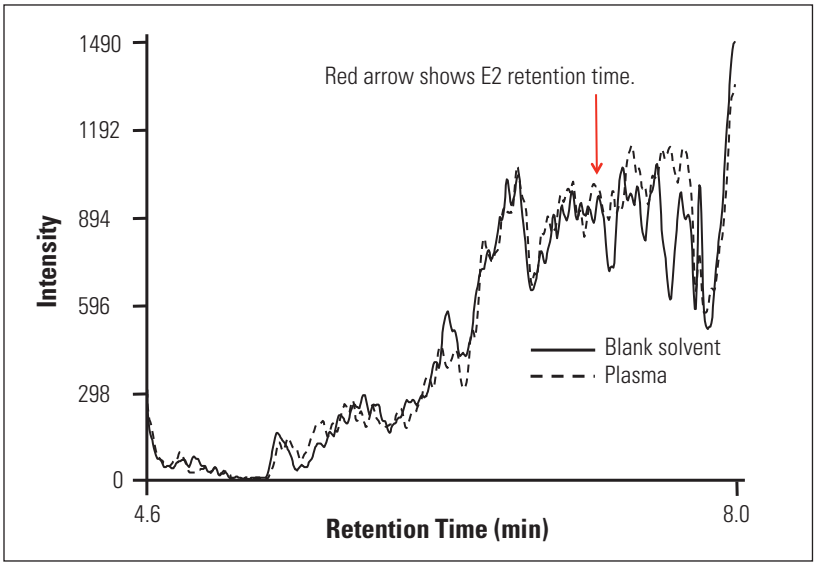

Figure 5. Ionization suppression test

Carryover

CSS was spiked with E1 and E2 to create a high-level sample (>500 pg/mL) and a low-level sample (8 pg/mL). The low-level sample was injected first (Low1) for LC-MS/MS analysis followed by the injection of the high-level sample (High). Immediately afterward, another low-level sample was injected (Low2). No carryover was observed by testing the spiked CSS samples with Low1 (9.9 pg/mL)-High (556.0 pg/mL)-Low2 (9.1 pg/mL) for E1 and Low1 (10.0 pg/mL)-High (582.5 pg/mL)-Low2 (8.9 pg/mL) for E2.

Data examples of clinical research samples

Figures 6 and 7 show the SRM chromatograms of E1 and E2 in two individual plasma samples.

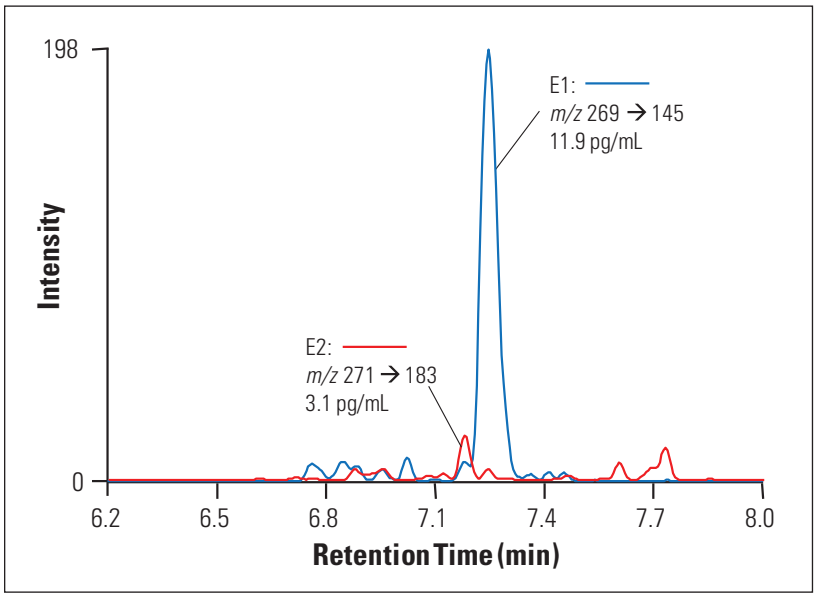

Figure 6. SRM chromatograms of E1 and E2 in human plasma sample 1 (female)

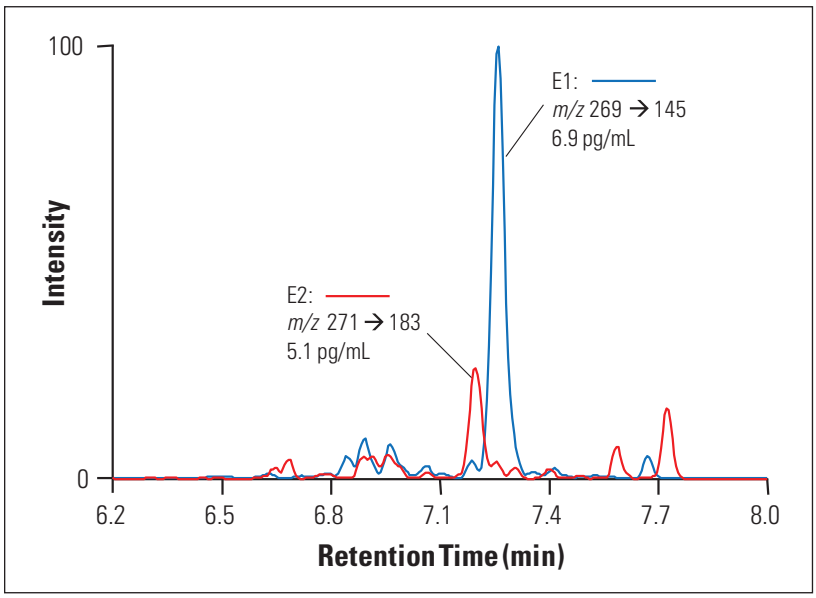

Figure 7. SRM chromatograms of E1 and E2 in human plasma sample 2 (male)

Conclusion

We have developed a novel 10-min LC-MS/MS method for quantitation of E1 and E2 in serum using TurboFlow technology for clinical research laboratories. This method is fast and analytically sensitive and sample preparation effort is significantly reduced. The Accucore HPLC column was used for analytical LC separation because of its superior performance. The lower limit of quantitation was 3.8 pg/mL for estrone and 3.7 pg/mL for estradiol. This method was linear from 3.8 to 1000.9 pg/mL for estrone and 3.7 to 993.1 pg/mL for estradiol with accuracy from 95.5% to 103.2% for estrone and from 92.7% to 112.3% for estradiol, respectively. Inter-assay and intra-assay CV for estrone and estradiol at low and high concentration levels in both spiked charcoal stripped serum and pooled human plasma ranged from 3.5% to 18.0%.

Reference

1. Xiang He and Marta Kozak, Fast and Sensitive LC-APCI-MS/MS Quantitative Analysis of Estrone and Estradiol in Serum without Chemical Derivatization, Thermo Fisher Scientific Application Note 530.

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Quantitative Analysis of Serum 1α,25-dihydroxyvitamin D by APPI-LC-MS/MS

Xiang He, Marta Kozak; Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- TSQ Vantage
- Clinical Research
- Endocrine Analysis

Introduction

Quantitation of 1α,25-dihydroxyvitamin D_2 and D_3 (1,25D) in serum is very important in clinical research but is challenging because of the low circulating serum concentration of 1,25D. Due to its high analytical specificity and sensitivity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been used for quantitation of 1,25D.

We have previously reported the use of immunoextraction and atmospheric pressure chemical ionization (APCI) for LC-MS/MS analysis of 1,25D in human serum[1]. Immunoextraction greatly simplifies the sample preparation and efficiently removes interferences. In addition, while APCI is good for this analysis, atmospheric pressure photoionization (APPI) is a more specific ionization technique than APCI and, therefore, further improves the analytical sensitivity of 1,25D detection.

Goal

To develop a highly sensitive LC-MS/MS analytical method to quantitate 1,25D with APPI using immunoextraction that provides better sensitivity than an APCI method.[1]

Methods

Sample Preparation

Serum 1,25D was purified with an immunoextraction method using an ImmunoTube® immunoextraction tube (Immundiagnostik AG, Bensheim, Germany). Briefly, samples were mixed with immobilized 1,25D antibody slurry and incubated at room temperature for 1 hour before the 1,25D-antibody beads were washed with aqueous buffer. Then, $1,25D_2$ and $1,25D_3$ were eluted with ethanol, dried, and reconstituted for LC-MS/MS injection.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Accela UHPLC system. A Thermo Scientific Hypersil GOLD column (150 × 1 mm, 3 µm particle size) was used. The column temperature was maintained at 50 °C. Mobile phases were 70% methanol in water and methanol from Fisher Chemical brand. The LC method used a 10-minute gradient, and the LC flow was diverted to the mass spectrometer between 2 and 5 minutes.

The mass spectrometer was equipped with an APPI probe and operated in the positive ion mode. Selected reaction monitoring (SRM) transitions of $1,25D_2$, $1,25D_3$, d6-$1,25D_2$ and d6-$1,25D_3$ were monitored (see Table 1).

Table 1. SRM transitions

	Q1 *(m/z)*	Q3 *(m/z)*	CE (V)	S-Lens (V)
$1,25D_2$	411.3	135.0	19	87
		151.0	20	87
$1,25D_3$	399.2	135.0	21	90
		151.0	22	90
d6-$1,25D_2$	417.3	151.0	19	95
d6-$1,25D_3$	405.3	151.0	20	90

Validation

The validation procedure included tests for 1) recovery, linearity, and lower limit of quantitation (LLOQ) and 2) precision.

Results and Discussion

1. Sample Preparation

The immobilized 1,25D antibody used in this study was highly specific and had no cross-reactivity from other vitamin D derivatives. Serum samples processed with immunoextraction showed no matrix effects or ionization suppression.

2. Recovery, Linearity, and LLOQ

Two sets of calibrators were prepared in ethanol (solvent) and pooled human plasma sample. Human plasma contains endogenous 1,25D, so it is not an appropriate choice to be used as the matrix for calibrators. Different levels of 1,25D were spiked into both solvent and human plasma to evaluate the feasibility of using solvent as the calibrator matrix. Solvent calibrators were prepared without immunoextraction, but with drying and reconstituting steps. Endogenous concentrations of 1,25D in pooled plasma were determined with solvent calibrators first. The pooled human plasma samples were then spiked with increasing levels of 1,25D and processed with immunoextraction. Concentrations of total 1,25D (endogenous and spiked concentration) in plasma were determined against solvent

calibrators and compared to expected concentrations to calculate recovery (Table 2).

Table 2. Recovery

$1,25D_2$			$1,25D_3$		
Expected (pg/mL)	Measured (pg/mL)	Recovery (%)	Expected (pg/mL)	Measured (pg/mL)	Recovery (%)
43.7	45.0	103.0	11.1	11.1	100.0
48.7	47.3	97.2	16.1	17.4	108.5
58.7	57.0	97.1	26.1	27.7	106.5
88.7	99.5	112.2	56.1	57.6	102.7
238.7	235.9	98.8	206.1	203.2	98.6

The slopes of the calibration curves of $1,25D_2$ and D_3 in both solvent and pooled human plasma calibrators were compared and found to be nearly identical (Figures 1 and 2). This indicated that 1,25D originated from spiked solvent and 1,25D originated from human plasma behaved similarly relative to their corresponding IS during the whole process of immunoextraction and LC-MS/MS.

The method was linear between 5 and 200 pg/mL for both $1,25D_2$ and $1,25D_3$. The LLOQ was 5 pg/mL for both $1,25D_2$ and D_3. Figure 3 shows the representative SRM chromatograms of $1,25D_2$ and $1,25D_3$ of the lowest calibrator in solvent and pooled human plasma.

3. Precision

Precision was determined with spiked charcoal stripped serum at both 10 and 20 pg/mL, which are close to the LLOQ (Table 3).

Table 3. Precision

$1,25D_2$	Measured (pg/mL)	Accuracy (%)	Precision (%)
10 pg/mL	9.1	90.8	8.4
20 pg/mL	19.8	99.2	7.4
$1,25D_3$			
10 pg/mL	9.9	98.8	12.5
20 pg/mL	20.9	104.4	11.1

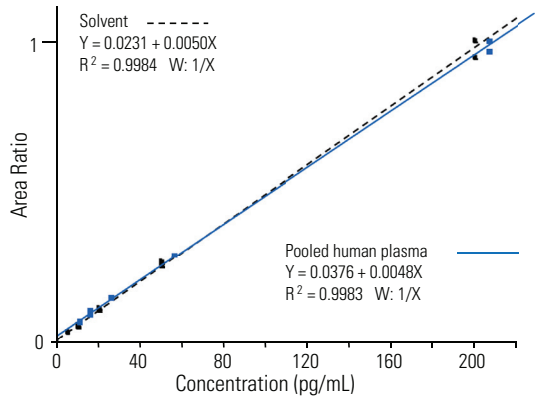

Figure 1. Calibration curves of $1,25D_2$ in solvent (dotted line, black) and pooled human plasma (solid line, blue)

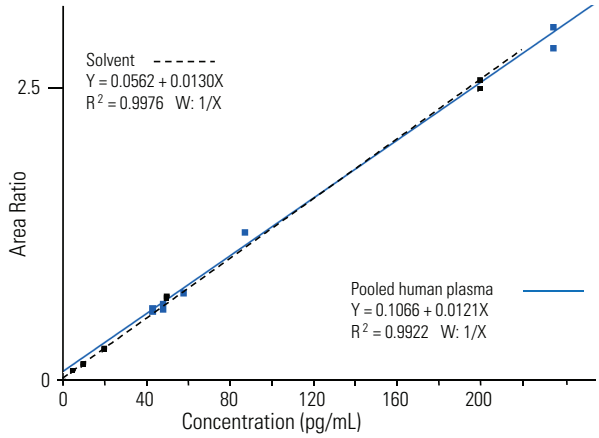

Figure 2. Calibration curves of $1,25D_3$ in solvent (dotted line, black) and pooled human plasma (solid line, blue)

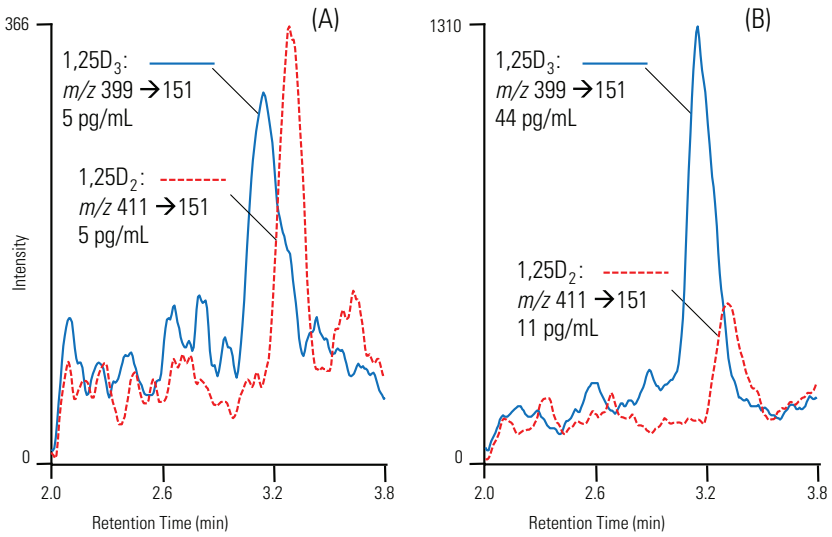

Figure 3. Representative SRM chromatograms of $1,25D_2$ and $1,25D_3$ of the lowest calibrator in solvent (A) and in pooled human plasma (B)

Conclusion

A fast and analytically sensitive LC-MS/MS method for quantitation of 1,25D in human plasma was developed for clinical research laboratories. Sample preparation was done with immunoextraction. APPI ionization was used for its ionization specificity and sensitivity. The LLOQ of this method was 5 pg/mL for both $1,25D_2$ and $1,25D_3$.

Reference

1. He, X; Damkroger, G; Kozak, M. *Quantitative Analysis of 1,25-dihydroxyvitamin D_2 and D_3 using Immunoaffinity Extraction with APCI-LC-MS/MS*, Thermo Fisher Scientific Application Note: 522.

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Quantitative Measurement of Plasma Free Metanephrines by Ion-Pairing Solid Phase Extraction and LC-MS/MS with Porous Graphitic Carbon Column

Xiang He, Marta Kozak; Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- TSQ Vantage
- Hypercarb HPLC column
- Clinical Research
- LC-MS/MS

Introduction

Plasma free metanephrine (MN) and normetanephrine (NMN), collectively known as Pmets, are important molecules for clinical research. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become widely used to measure Pmets because of its high analytical sensitivity and specificity.

Because Pmets are very polar, special solid phase extraction (SPE) and chromatographic methods have been developed for their analysis. Ion-paring (IP)-SPE, which has been used to purify a wide range of polar compounds, is well suited for the purification of Pmets.

Goal

To develop an LC-MS/MS method for measuring Pmets using IP-SPE and porous graphitic carbon (PGC) column chromatography.

Methods

Sample Preparation

Thermo Scientific HyperSep C-18 cartridges (1 mL) were preconditioned with acetonitrile and 0.1% perfluoroheptanoic acid (PFHA) before samples were loaded. After sample loading, cartridges were washed with 0.1% PFHA and eluted with 60% acetonitrile. The eluate was dried and reconstituted for LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Accela UHPLC system. A Thermo Scientific Hypercarb column (50 × 2.1 mm, 5 µm particle size) was used. This PGC-based column is highly durable and ideal for retaining and resolving very polar and hydrophilic molecules. The column temperature was maintained at 70 °C. Mobile phases were 1% formic acid in water with ammonium formate, and 0.1% formic acid in acetonitrile. The LC gradient was 7 minutes long.[1]

The mass spectrometer was equipped with a heated electrospray ionization probe (HESI-II) and operated in the positive electrospray ionization mode. MN-d3 and NMN-d3 were used as the internal standards for MN and NMN.

Validation

The validation procedure included tests for 1) interference; 2) SPE recovery; 3) ion suppression; 4) lower limit of quantitation (LLOQ), dynamic range, accuracy; 5) precision; and 6) carryover.

Results and Discussion

1. Interference

Epinephrine (EPI) and NMN share the same selected reaction monitoring (SRM) transitions and could not be differentiated by MS/MS analysis alone. With Hypercarb™ column chromatography, the EPI-d3 peak was baseline resolved from the NMN-d3 peak (Figure 1).

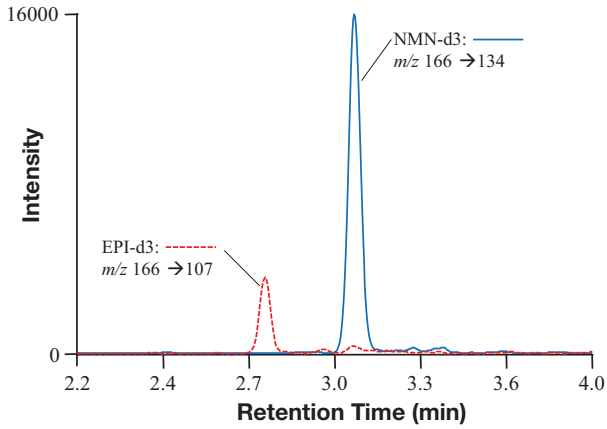

Figure 1. SRM chromatograms of EPI-d3 and NMN-d3 in a processed CSS sample

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2. SPE Recovery

Extraction efficiency was assessed in charcoal stripped serum (CSS, n=3). Absolute recovery of Pmets and IS ranged from 86.4% to 97.5%, and the relative recovery of MN and NMN was 97.7% and 113.5%, respectively (Table 1).

Table 1. SPE Recovery

In Charcoal Stripped Serum	Spiked before SPE[a] (mean ± CV)	Spiked after SPE[b] (mean ± CV)	Absolute Recovery (%)	Relative Recovery (%)
MN (n=3)	22865 ± 13.9%	25265 ± 9.3%	90.5	97.7
NMN (n=3)	11165 ± 11.1%	11453 ± 12.5%	97.5	113.5
MN-d3 (n=3)	27809 ± 7.2%	30140 ± 12.9%	92.3	n/a
NMN-d3 (n=3)	22627 ± 9.2%	26192 ± 4.5%	86.4	n/a

[a] *Measured peak area of charcoal stripped serum spiked with 100, 400, 400, and 1600 pg/mL of MN, NMN, MN-d3, and NMN-d3, respectively, before SPE*

[b] *Measured peak area when equivalent amounts of above compounds were spiked after SPE*

3. Ion Suppression

Results from the post-column infusion experiments are shown in Figure 2. Compared to injections of blanks, no obvious ion suppression was detected in the SRM chromatograms of MN-d3 and NMN-d3 using processed human plasma samples.

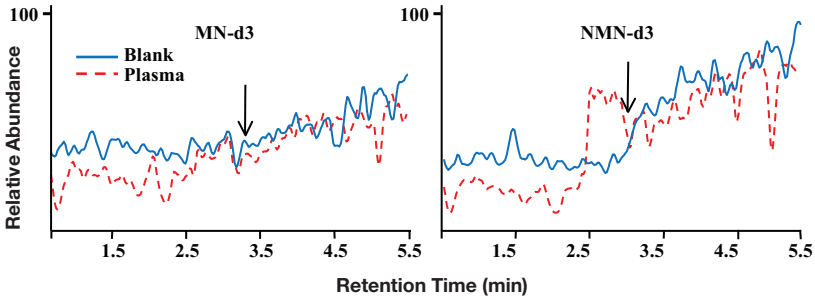

Figure 2. Representative SRM chromatograms of post-column infusion of 100 ng/mL MN-d3 (left) and NMN-d3 (right) after injections of buffer blanks (solid lines) and processed human plasma samples (dashed lines). No internal standards were added to human plasma samples. Arrows indicate retention times of MN and NMN.

4. LLOQ, Linearity and Accuracy

It was determined that CSS is a suitable matrix to conduct this part of validation (mixing study, data not shown). CSS samples with progressively lower concentrations of MN and NMN were prepared in triplicate along with one set of CSS calibrators.

The linearity range was determined to be 7.2 - 486.8 pg/mL for MN and 18.0 - 989.1 pg/mL for NMN (Figure 3). Accuracy ranged from 92.2% to 118.0% for MN, and from 92.1% to 115.0% for NMN. The determined LLOQ was 7.2 pg/mL for MN and 18.0 pg/mL for NMN.

Figures 3 and 4 show the calibration curves for MN and NMN. Figure 5 shows the representative SRM chromatograms of MN and NMN at their LLOQ in CSS.

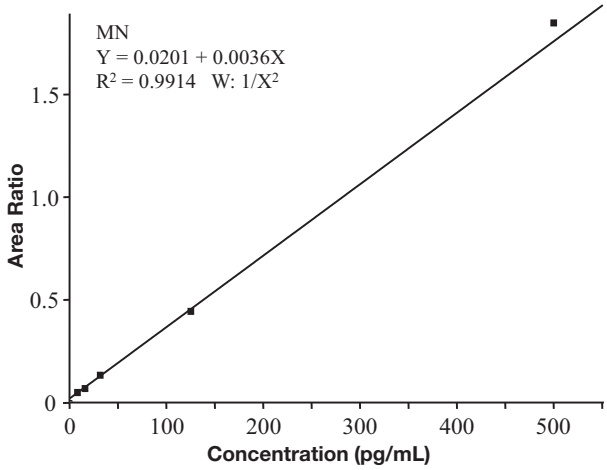

Figure 3. Calibration curve of MN in CSS

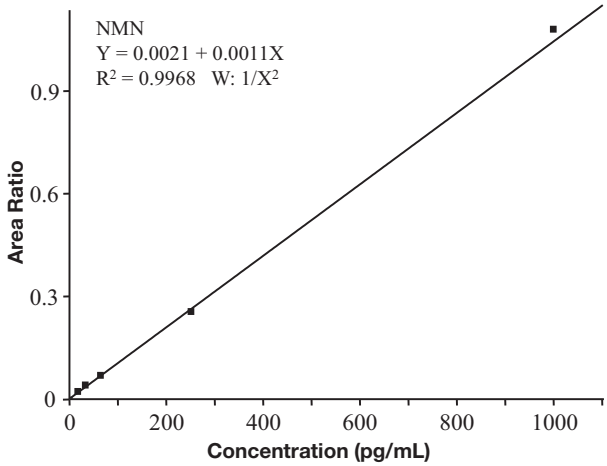

Figure 4. Calibration curve of NMN in CSS

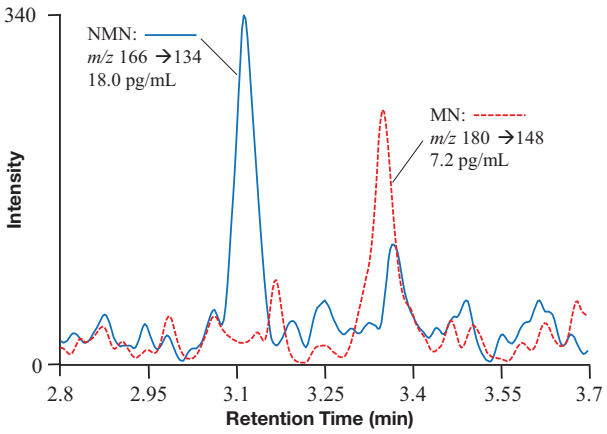

Figure 5. Representative SRM chromatograms of MN and NMN at their LLOQ in a spiked CSS sample.

5. Precision

Precision results are summarized in Table 2.

A) CSS samples: Precision was first assessed with spiked CSS at two concentration levels (25 and 250 pg/mL for MN, and 50 and 500 pg/mL for NMN). Inter- (n=15) and intra-batch (n=5) CV values ranged from 2.1% to 10.9%.

B) Pooled human plasma samples: Precision was also assessed with a spiked human plasma pool (35.6 pg/mL of MN and 53.1 pg/mL of NMN, n=5). The determined intra-assay CV (n=5) was 6.3% and 7.8% for MN and NMN, respectively.

Table 2. Precision Data in Spiked CSS

	MN		NMN	
	25 pg/mL	250 pg/mL	50 pg/mL	500 pg/mL
Intra-assay Precision (%) n=5	10.9	4.6	9.6	2.1
Accuracy (%)	98.9	96.9	110.2	90.9
Inter-assay Precision (%) n=15	10.3	6.5	10.6	5.6
Accuracy (%)	100.6	102.7	108.7	97.4

Figure 6 shows representative SRM chromatograms of MN and NMN using a processed human plasma sample.

6. Carryover

No carryover was observed up to 500 and 1000 ng/mL for MN and NMN, respectively.

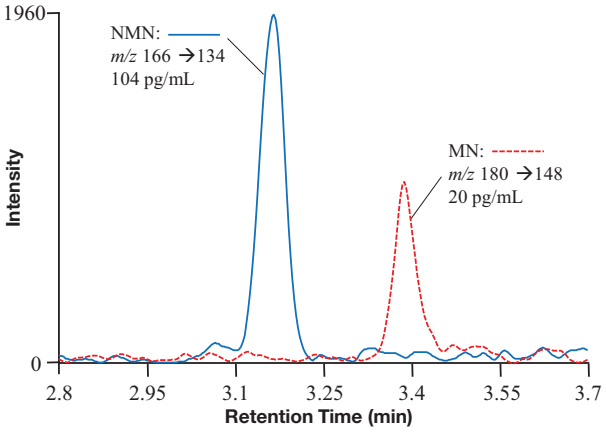

Figure 6. Representative SRM chromatograms of MN and NMN using a processed human plasma sample

Conclusion

A sensitive LC-MS/MS method was developed to quantify plasma free metanephrines in clinical research laboratories. This method has an LLOQ of 7.2 and 18.0 pg/mL for metanephrine and normetanephrine, respectively. Method precision ranged from 2.0% to 10.9%. Ion-pairing SPE was used for sample preparation, and a Hypercarb column was used for chromatographic separation of metanephrines.

Reference

1. He, X.; Gabler, J.; Yuan, C.; Wang, S.; Shi, Y.; Kozak, M. Quantitative Measurement of Plasma Free Metanephrines by Ion-pairing Solid Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry with Porous Graphitic Carbon Column *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2011**, 879(23), 2355-2359.

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Fast and Sensitive LC-APCI-MS/MS Quantitative Analysis of Estrone and Estradiol in Serum without Chemical Derivatization

Xiang He and Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Vantage
- Accela UHPLC
- Clinical Research

Introduction

In the clinical research setting, quantitative measurements of estrone (E1) and estradiol (E2) in serum typically have been done with immunoassay or liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS is preferred over immunoassay and other analytical techniques because of its high sensitivity.

E1 and E2 are usually chemically derivatized before they are detected by mass spectrometry for enhanced sensitivity. The derivatization step extends the sample preparation procedure and usually involves chemicals/reagents that might compromise the performance of the mass spectrometer in the long term.

Goal

To develop and validate a simple, fast and sensitive analytical method for measuring E1 and E2 in serum or plasma by LC-APCI-MS/MS.

Methods

Sample Preparation

Serum was spiked with internal standard (IS, deuterated E2) and underwent liquid-liquid extraction (LLE) with methyl tert-butyl ether (MTBE). After extraction, the MTBE layer was dried under nitrogen and re-suspended with 60% methanol. The reconstituted sample was centrifuged to remove particulates and the supernatant was injected for LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Accela UHPLC system. UHPLC was carried out on a Thermo Scientific Hypersil GOLD column (150 × 2.1 mm, 3 µm) at room temperature using water and methanol as mobile phases. The total LC run time was 6 minutes. The mass spectrometer was operated with an atmospheric pressure chemical ionization (APCI) source in negative ion mode. Data was acquired in selected reaction monitoring (SRM) mode.

Validation

The validation procedure included tests for 1) recovery of sample preparation; 2) calibration range; 3) lower limit of quantitation (LLOQ), dynamic range, accuracy; 4) precision; 5) ion suppression; and 6) carryover.

Results and Discussion

Sample Preparation

LLE was used to extract E1 and E2 from serum/plasma and was found to be efficient. MTBE was selected as the extraction solvent for its excellent recovery and ease of handling.

Validation

1. Recovery for LLE Sample Preparation

The absolute recovery of E1, E2 and their internal standard from liquid-liquid extraction ranged from 70% – 115% (n=4).

2. Calibration Range

Calibration curves (Figures 1 and 2) using calibrators in charcoal stripped serum (CSS) showed excellent linearity (R^2 > 0.998) between 5 and 1000 pg/mL.

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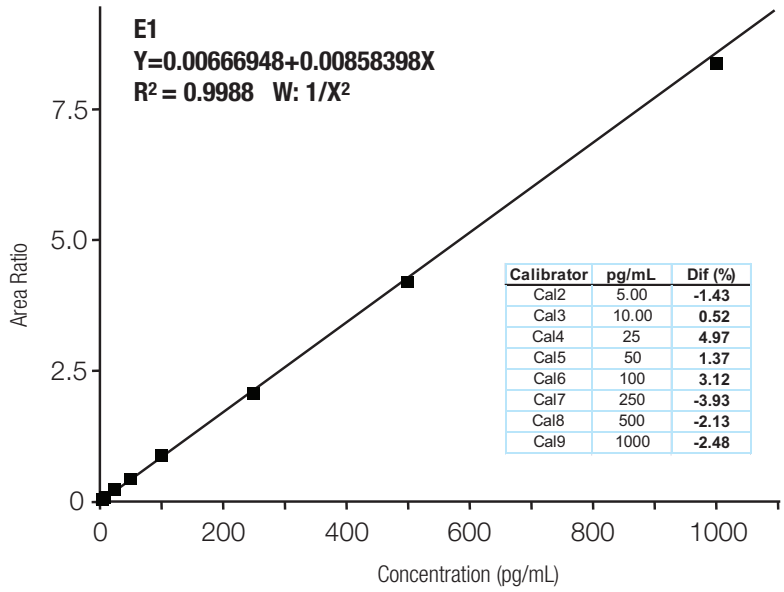
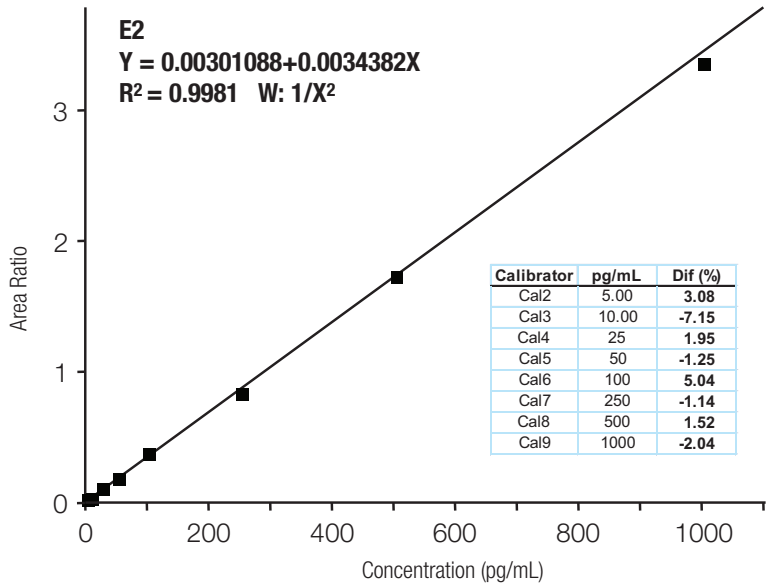


Calibrator	pg/mL	Dif (%)
Cal2	5.00	**-1.43**
Cal3	10.00	**0.52**
Cal4	25	**4.97**
Cal5	50	**1.37**
Cal6	100	**3.12**
Cal7	250	**-3.93**
Cal8	500	**-2.13**
Cal9	1000	**-2.48**

Figure 1: Calibration curve of E1 in CSS



Calibrator	pg/mL	Dif (%)
Cal2	5.00	**3.08**
Cal3	10.00	**-7.15**
Cal4	25	**1.95**
Cal5	50	**-1.25**
Cal6	100	**5.04**
Cal7	250	**-1.14**
Cal8	500	**1.52**
Cal9	1000	**-2.04**

Figure 2. Calibration curve of E2 in CSS

3. Determination of LLOQ, Linearity and Accuracy

CSS was first evaluated by comparing it to human plasma to determine if it was suitable. During this stage of the validation, CSS samples with progressively lower concentrations of E1 and E2 were prepared in triplicate along with one set of CSS calibrators.

The method was linear between 3.5 and 1019.3 pg/mL with accuracy (n=3) from 85.8% to 107.0% for E1, and between 4.4 and 1032.5 pg/mL with accuracy (n=3) from 92.9% to 112.8% for E2 (Table 1 and Figure 3). The LLOQ for E1 and E2 are 3.5 and 4.4 pg/mL, respectively (Table 1 and Figure 4).

Table 1. LLOQ, dynamic range and accuracy

		E1			E2		
Dilution factor	**Expected (pg/mL)**	**Measured (mean, pg/mL)**	**CV of Triplicates (%)**	**Accuracy (%)**	**Measured (mean, pg/mL)**	**CV of Triplicates (%)**	**Accuracy (%)**
256	3.91	3.5	18.8	90.5	4.4	7.1	112.8
128	7.81	8.4	4.5	107.0	8.0	9.0	102.2
64	15.63	15.8	9.4	101.2	18.0	5.1	115.2
32	31.25	28.7	0.6	92.0	31.0	8.8	99.1
16	62.50	56.7	4.8	90.7	60.8	6.7	97.2
8	125.00	107.2	3.9	85.8	116.1	6.8	92.9
4	250.00	224.2	7.4	89.7	242.2	4.4	96.9
2	500.00	484.2	3.5	96.8	492.2	2.4	98.4
1	1000.00	1019.3	8.9	101.9	1032.5	9.1	103.2
Mean				**95.1**			**102.0**

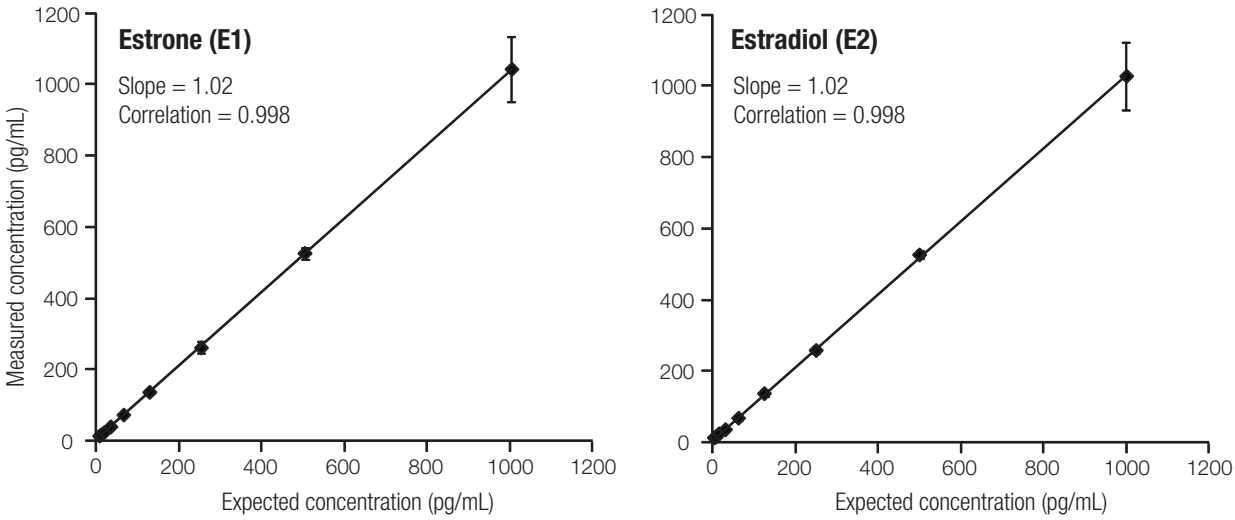


Figure 3: Linearity (Deming regression)

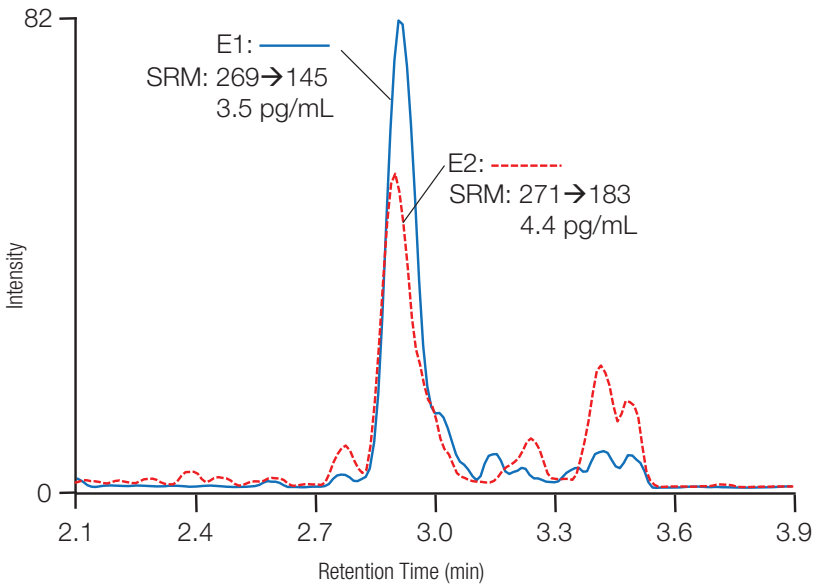


Figure 4. SRM chromatograms of E1 and E2 at their LLOQ in spiked CSS

4. Precision

A) CSS samples: Precision was first assessed with spiked CSS at two concentration levels (12 and 300 pg/mL). Inter- (n=15) and intra-batch (n=5) CV values ranged between 1.6% to 12.5% (Table 2).

B) Pooled human plasma samples: Precision was also assessed with a spiked human plasma pool (35.4 pg/mL of E1 and 18.1 pg/mL of E2, n=5) and the determined intra-batch CV was 2.2% and 3.6% for E1 and E2, respectively.

Table 2. Precision data

		E1		E2	
	Charcoal Stripped Serum	**Low (12 pg/mL)**	**High (300 pg/mL)**	**Low (12 pg/mL)**	**High (300 pg/mL)**
Batch 1	Intra-assay Precision (n=5, %)	7.1	6.9	9.4	6.7
Batch 2	Intra-assay Precision (n=5, %)	5.5	1.6	12.5	3.0
Batch 3	Intra-assay Precision (n=5, %)	7.2	4.9	8.0	3.1
Batch 1-3	Inter-assay Precision (n=15, %)	7.3	4.7	10.9	4.4

	Spiked Pooled Plasma	E1 (35.4 pg/mL)	E2 (18.1 pg/mL)
	Precision (n=5, %)	2.2	3.6

5. Ion Suppression

Results from the post-column infusion experiments are shown in Figure 5. Compared to solvent blank (60% methanol), no obvious ion suppression was detected in the SRM chromatograph of IS using a processed human plasma sample without IS. The red arrow indicates where E1 and E2 elute during the LC gradient.

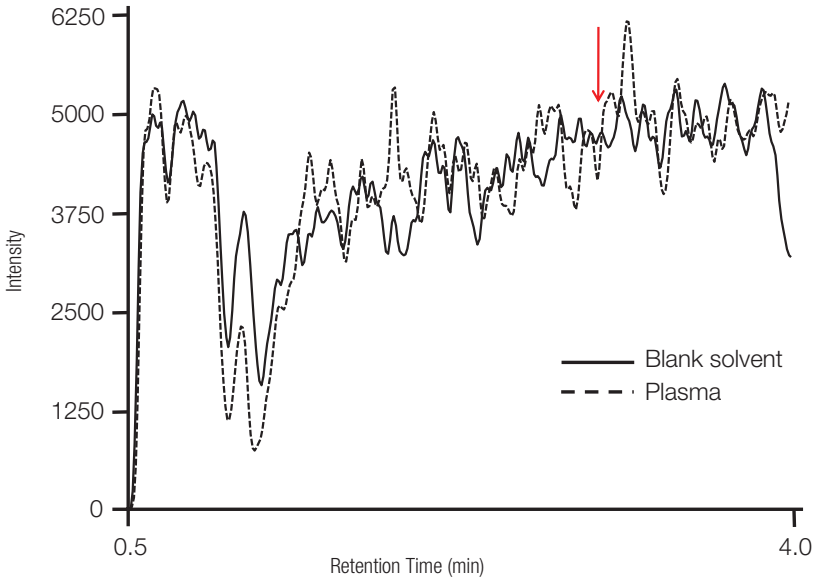


Figure 5: Ion suppression test

6. Carryover

No carryover was observed in the solvent blank injection that was right after a processed spiked CSS sample with E1 and E2 concentration at 300 pg/mL.

Figures 6 and 7 show the SRM chromatograms of E1 and E2 in two individual plasma samples.

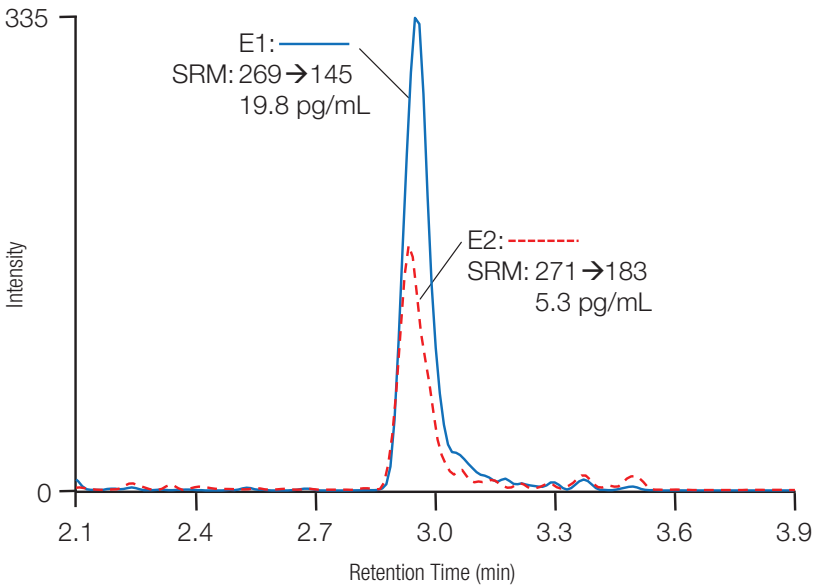


Figure 6: SRM chromatograms of E1 and E2 in human plasma sample 1 (male)

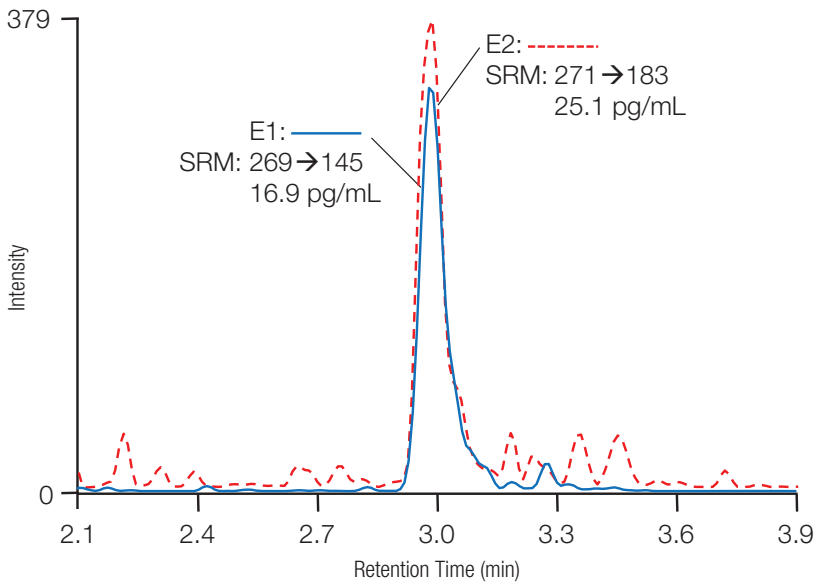


Figure 7: SRM chromatograms of E1 and E2 in human plasma sample 2 (female)

Conclusion

We have developed and fully validated a simple, fast and sensitive LC-APCI-MS/MS method for measurement of E1 and E2 in serum/plasma without derivatization. The LLOQ for E1 and E2 are 3.5 and 4.4 pg/mL, respectively. The method was linear between 3.5 and 1019.3 pg/mL for E1, and 4.4 and 1032.5 pg/mL for E2. No ion suppression or carryover was observed. In addition, for clinical research laboratories, this method offers high precision and recovery.

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Quantitative LC-MS/MS Analysis of 25-OH Vitamin D_3/D_2 Comparing 1D Chromatography, 2D Chromatography and Automated Online Sample Preparation

Neil Leaver[1], Bevean Chihoho[1], Sarah Robinson[2]; [1]Royal Brompton & Harefield NHS Foundation Trust, Harefield Hospital, Harefield, UK; [2]Thermo Fisher Scientific, Hemel Hempstead, UK

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Key Words

- Transcend TLX-1
- TurboFlow Technology
- TSQ Quantum Ultra
- Endocrinology

Introduction

High performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) is now widely accepted for measurement of vitamin D metabolites. Many clinical research laboratories use 1-dimensional (1D) chromatography (for example, a single HPLC pump and chromatography column) with a triple stage quadrupole mass spectrometer. Various sample cleanup protocols, such as solid phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PPT), have been applied in these analyses. Frequently, interfering peaks are seen in 25-OH vitamin D3 chromatograms, adversely affecting peak integration and leading to poor accuracy and reproducibility. Here we investigate the use of 2-dimensional chromatography using TurboFlow technology to remove all interfering peaks and significantly improve data quality.

Goal

Compare three methods for the quantitative analysis of 25-OH vitamin D_3/D_2: a validated, online TurboFlow™ method; a commercially available 2D-SPE-LC-MS/MS kit method (Chromsystems MassChrom® 25-OH Vitamin D_3/D_2); and a 1D chromatography method.

Experimental Conditions

Sample Preparation

A 100 µL sample of plasma was mixed with 200 µL internal standard (IS) in acetonitrile, vortexed, and centrifuged. For analysis, 50 µL of supernatant was injected onto the column. Details of the commercial calibrator and QC values (Chromsystems) used in each assay are provided in Tables 1 and 2. (Please note that the control product has since been reformulated to validate borderline D_3 insufficiency and normal levels.) These commercial products were validated against in-house calibration and control material over a wider dynamic range.

HPLC

HPLC analysis was performed using the Thermo Scientific Transcend TLX-1 system powered by TurboFlow™ technology. For analysis, a TurboFlow XL C18 extraction column (50 x 0.5 mm) and a Thermo Scientific Hypersil GOLD analytical column (50 x 2.1 mm, 1.9 µm) were used. For 1D analysis, the analytical column alone was used. For the commercial 2D set up, columns provided within the 2D-SPE-LC-MS/MS kit were used. Eluents for the TurboFlow method were 0.1% formic acid, methanol + 0.1% formic acid, and acetonitrile/IPA/acetone blend (wash solution).

Table 1. Calibrator levels.

Calibrator	Cal 1 (nmol/L)	Cal 2 (nmol/L)	Cal 3 (nmol/L)	Cal 4 (nmol/L)
25-OH Vitamin D_3	9.9	47.8	86.2	174.0
25-OH Vitamin D_2	0.0	37.5	72.3	146.0

Table 2. Quality control levels.

	Mean 25-OH vitamin D_3 (nmol/L)	Mean 25-OH vitamin D_2 (nmol/L)
QC1	77.1	72.7
QC2	167	150

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Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer. Atmospheric pressure chemical ionization (APCI) was used to generate the [M-H_2O]+ ion for 25-OH vitamin D_3, D_2 and the IS.

Results and Discussion

Example calibration lines for the D_3 and D_2 metabolites analyzed by the TurboFlow LC-MS/MS method are presented in Figures 1A and 1B.

Examples of a plasma sample analyzed by the 1D LC-MS/MS method and by the TurboFlow method are provided in Figures 2A and 2B, respectively. There is an interference peak observed in the LC-MS/MS 25-OH-D_3 selected reaction monitoring (SRM) extracted ion chromatogram (XIC). This is commonly observed in analyses where only 1D LC-MS/MS is utilized. When using the TurboFlow method, the interference is removed and larger peak areas with better signal-to-noise ratios are achieved.

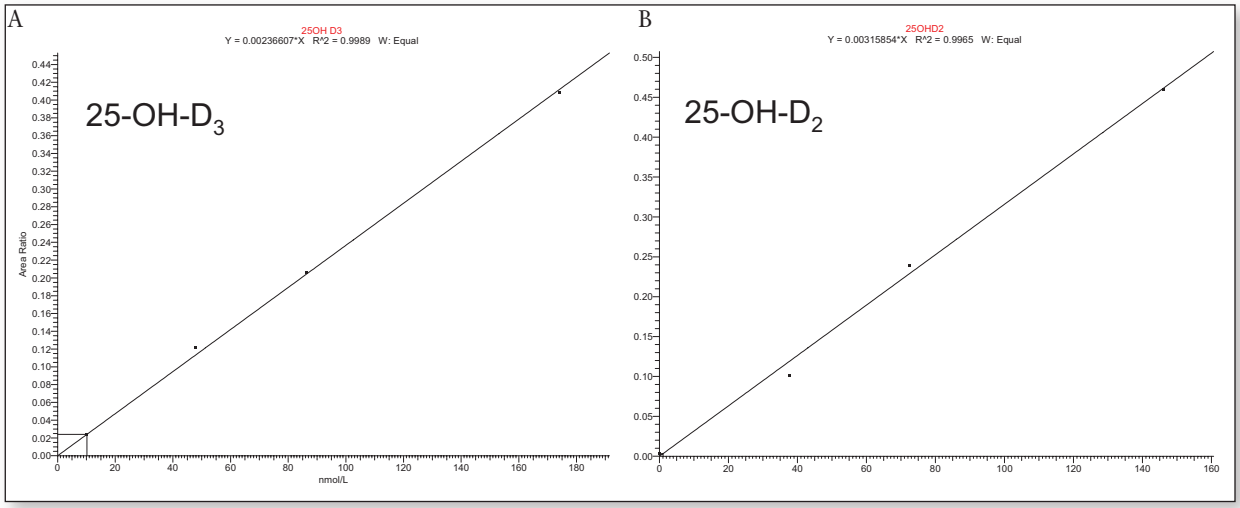


Figure 1. Calibration curves for 25-OH vitamin D_3 (A) and 25-OH vitamin D_2 (B) by TurboFlow LC-MS/MS.

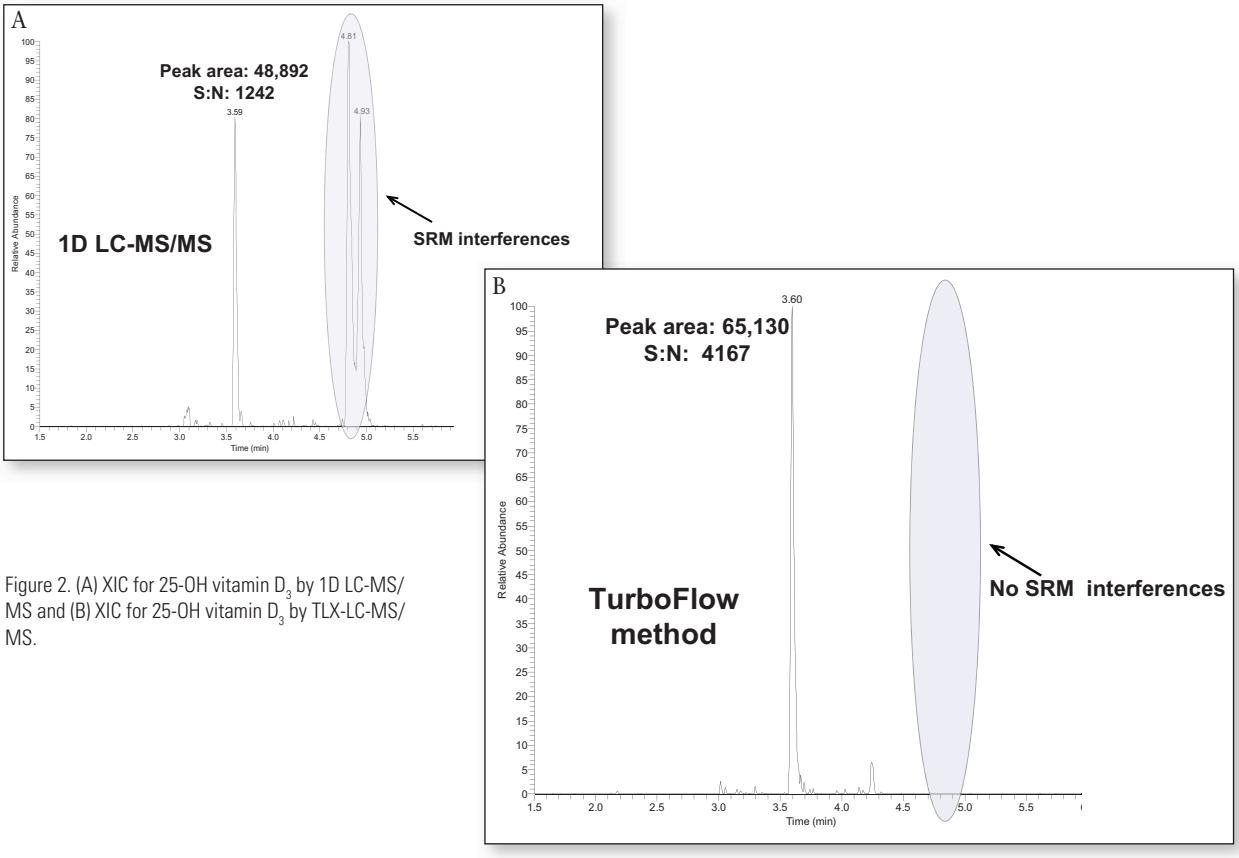


Figure 2. (A) XIC for 25-OH vitamin D_3 by 1D LC-MS/MS and (B) XIC for 25-OH vitamin D_3 by TLX-LC-MS/MS.

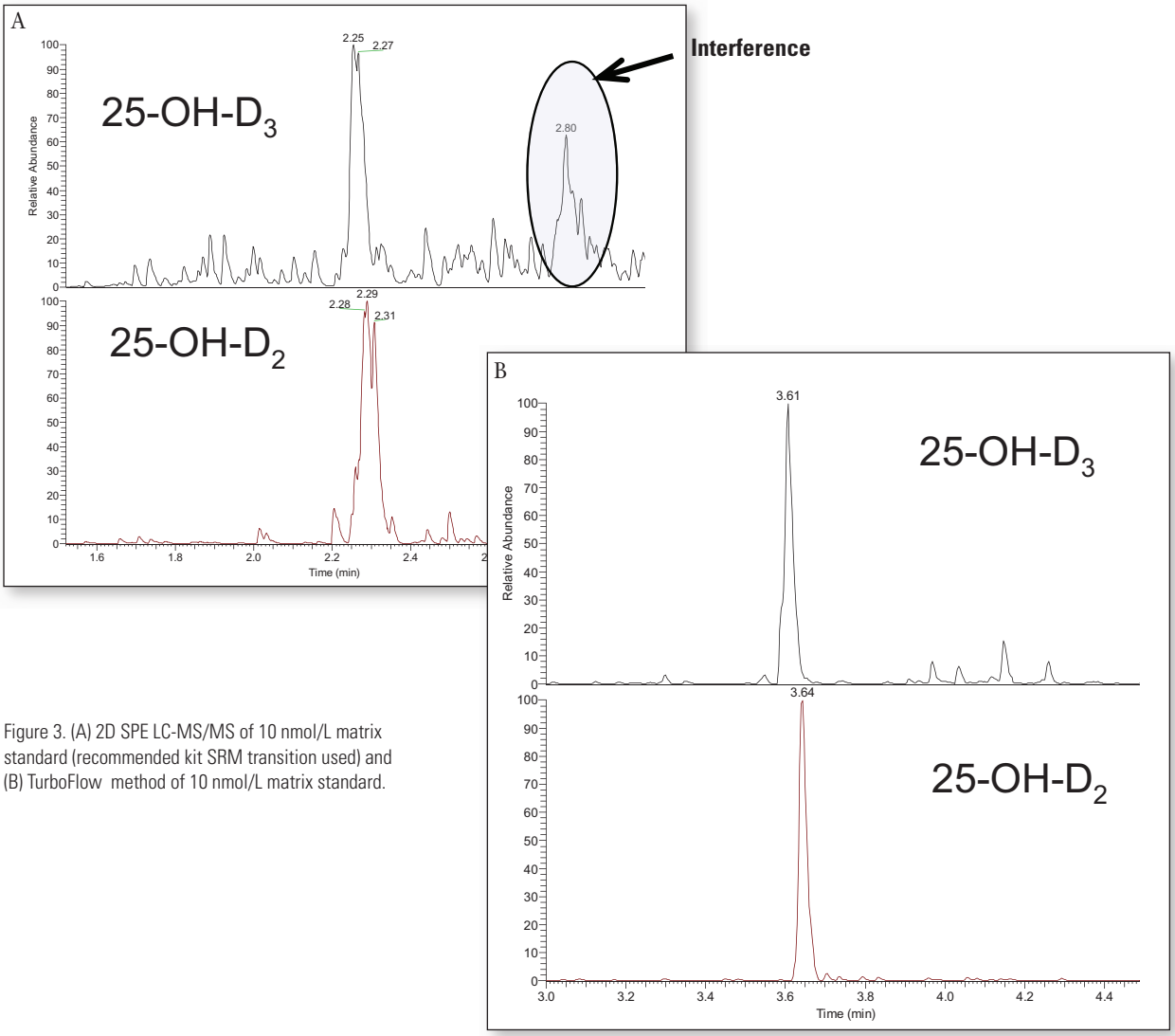


Figure 3. (A) 2D SPE LC-MS/MS of 10 nmol/L matrix standard (recommended kit SRM transition used) and (B) TurboFlow method of 10 nmol/L matrix standard.

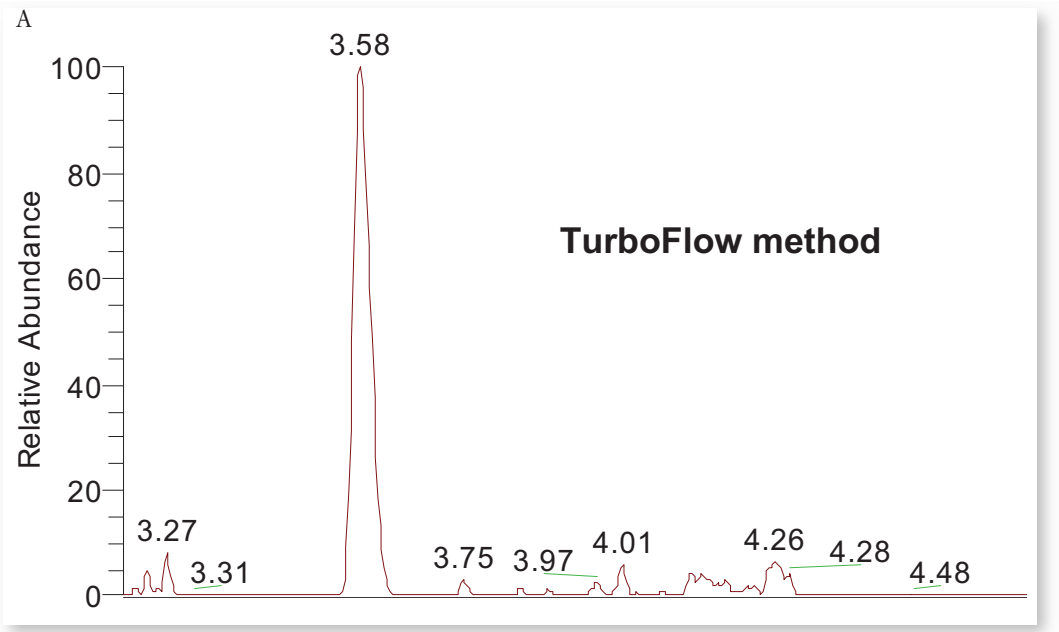
Although cleanup is improved when using other 2D LC-MS/MS methods, interferences are still observed in the 25-OH-D_3 XIC (Figure 3A). Furthermore, at the bottom of the range for 25-OH-D_3 (~10 nmol/L), is detected with greater analytical sensitivity and less noise when analyzed using the TurboFlow method versus a 2D SPE cleanup procedure (Figure 3B).

The 2D-LC-MS/MS approach reduces SRM interferences in the 25-OH-D_3 XICs because the integration of the analyte peak is easier and more accurate. An example of the impact of these interferences on peak integration is shown in Figures 4A and 4B. Here, the result for an individual with normal levels of 25-OH-D_3 would be reported incorrectly due to the high level of interference merging with the analyte peak, and thus, affecting the peak integration.

Conclusion

The TurboFlow method described here has been developed and validated to industry recommended guidelines for clinical laboratories.

Isobaric interferences observed with a 1D LC-MS/MS method at low 25-OH D_3 metabolite concentrations were much reduced by using a 2D-LC-MS/MS approach, and even further improved by using TurboFlow technology. The Transcend™ TLX-1 LC-MS/MS with TurboFlow technology improved the sensitivity and the signal-to-noise ratio.



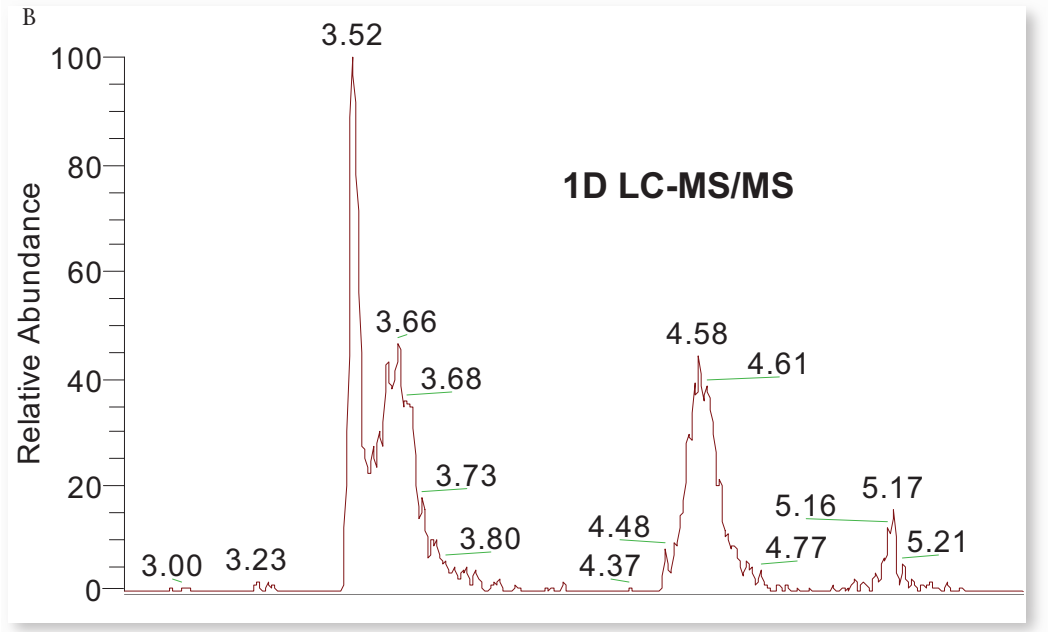


Figure 4. XICs for 25-OH vitamin D_3 by (A) TurboFlow method and (B) 1D LC-MS/MS analysis of a sample at normal levels of analyte (83 nmol/L).

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Quantitative Analysis of 1,25-dihydroxy-vitamin D_2 and D_3 using Immunoaffinity Extraction with APCI-LC-MS/MS

Xiang He[1], Glenn Damkroeger[2], and Marta Kozak[1]
[1]Thermo Fisher Scientific, San Jose, CA , USA
[2]Thermo Fisher Scientific, Dreieich, Germany

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Key Words
- Endocrine Testing
- TSQ Vantage
- Accela U-HPLC

Introduction

1,25-dihydroxyvitamin D (1,25D) tests are important in conducting clinical research in chronic renal failure and hypoparathyroidism. Circulating 1,25D levels are a thousand-fold less than 25-hydroxyvitamin D levels, making it a challenging test that benefits from immunoaffinity purification prior to analysis with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In this work, both $1,25D_2$ and $1,25D_3$ were extracted from human plasma using immunoaffinity extraction and quantified with LC-MS/MS.

Goal

To validate a very sensitive LC-MS/MS method to quantify 1,25-dihydroxyvitamin D by combining immunoaffinity extraction and highly selective atmospheric pressure chemical ionization (APCI).

Materials

ImmunoTube® kits (KM1000) were purchased from Immundiagnostik AG (Bensheim, Germany). Immunoextraction tubes, washing and eluting buffers, and calibrators (CAL1 and CAL2) and controls (CTRL1 and CTRL 2) were provided in the KM1000 kit. The concentrations of the calibrators and controls are specified in Table 1.

Table 1: Calibrators and controls in KM1000 kit

Standards	$1,25D_2$ (pg/mL)	$1,25D_3$ (pg/mL)
CAL1	33	26
CAL2	350	250
CTRL1	63-105	49-81
CTRL2	203-348	146-244

Sample Preparation

Five hundred (500) µL of plasma were spiked with deuterated $1,25D_3$ and processed with the ImmunoTube kit. The immunoaffinity method for processing plasma was provided in the kit.

Instrument Method

A Thermo Scientific Accela UHPLC pump and Accela autosampler were used as the front end system. The detector was a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer run in selected reaction monitoring (SRM) mode and equipped with an APCI probe. The LC gradient consisted of a fast, 5-minute method at a flow rate of 500 µL/min.

Results and Discussion

Figures 1 and 2 display the data collected for $1,25D_2$ and $1,25D_3$ using the calibrators and controls provided in the ImmunoTube kit. Calibration curves were plotted without weighting and set to include the origin of the coordinate (x, y = 0,0).

Conclusion

In this research, ImmunoTube immunoaffinity extraction was used to prepare human plasma prior to LC-MS/MS to quantify $1,25D_2$ and $1,25D_3$. Immunoaffinity extraction allows for the efficient extraction of target compounds from biological samples and almost completely eliminates matrix effects and interferences in LC-MS/MS analysis. The sample preparation is fast, simple, and does not require chemical derivatization. These features make it an ideal method in clinical research for the quantitation of $1,25D_2$ and $1,25D_3$. APCI was used for the method validation with an ImmunoTube kit, and the lowest concentrations tested for $1,25D_2$ and $1,25D_3$ in the kit were 26 and 33 pg/mL, respectively. Based on the S/N ratios at these concentrations, the limit of quantitation (LOQ) of this method was estimated to be around 15 pg/mL.

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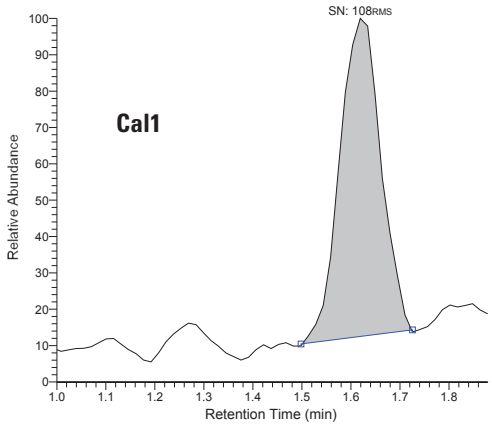
1,25D$_2$	Measured (pg/mL)	Specified (pg/mL)
Cal1	34.8	33
Cal2	349.8	350
Ctrl1	94.3	63-105
Ctrl2	311.6	203-348



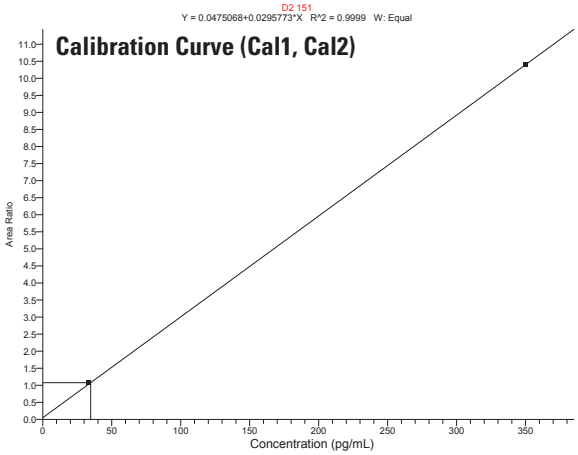
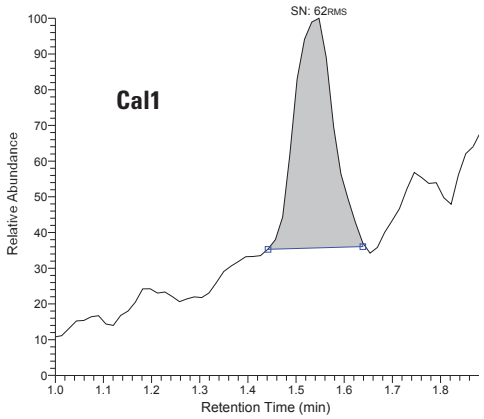


Figure 1: Data for 1,25D$_2$ (Transition 411→151)

1,25D$_3$	Measured (pg/mL)	Specified (pg/mL)
Cal1	21.7	26
Cal2	250.4	250
Ctrl1	59.9	49-81
Ctrl2	200.8	146-244



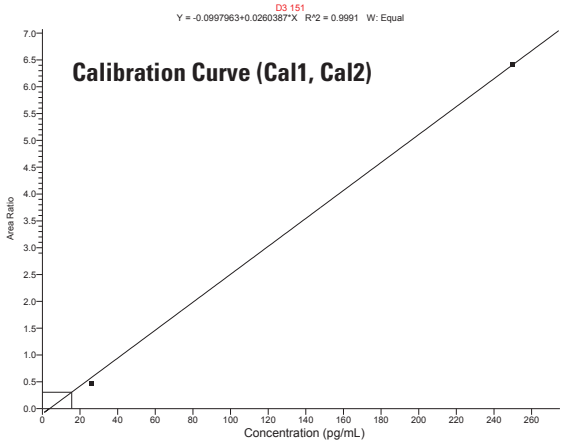


Figure 2: Data for 1,25D$_3$ (Transition 399→151)

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Quantitative Determination of Testosterone in Plasma Using Unique Automated Online Sample Preparation and LC-MS/MS

Marta Kozak, Thermo Fisher Scientific, San Jose, CA, USA

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Key Words

- Transcend System
- TSQ Vantage
- Endocrinology
- Clinical Research
- TurboFlow Technology

Introduction

Testosterone quantitative methods require a limit of quantitation of 10 pg/mL in plasma. The commonly-used liquid-liquid extraction (LLE) technique for sample preparation consists of multiple steps (including evaporation). It is neither time- nor cost-efficient. We have developed a fast and cost-efficient online sample preparation method implementing Thermo Scientific TurboFlow technology for clinical research purposes.

Goal

The goal is to develop an automated, interference-free LC-MS/MS method to quantitate testosterone with a low limit of quantitation (LOQ) in plasma. The method utilizes the analytical speed of the Thermo Scientific Transcend system, powered by TurboFlow™ automated, online sample preparation technology, coupled with a triple stage quadrupole mass spectrometer.

Experimental

Sample Preparation

A 100 µL aliquot of plasma was mixed with 100 µL of methanol containing testosterone-d3 (internal standard) and precipitated in ice for 10-15 minutes. The resulting plasma was centrifuged at 12,000 rpm for 10 minutes at 4 °C. Calibrators were prepared in double charcoal-stripped plasma at six concentration levels from 10 pg/mL to 500 pg/mL.

HPLC

HPLC analysis was performed using the Transcend™ TLX-1 system. Plasma samples were extracted using a TurboFlow™ Cyclone P column (1 x 50 mm). Chromatographic separation was performed using a Thermo Scientific Hypersil GOLD aQ column (100 x 2.1 mm, 5µm). A gradient liquid chromatography method was used.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer with a Thermo Scientific Ion MAX source and atmospheric pressure chemical ionization (APCI) probe in the positive ionization mode. The selective reaction monitoring (SRM) mode was used for mass spectrometry detection.

Results and Discussion

Figure 1 shows the linear calibration curve for testosterone. The R^2 value is 0.9999, which indicates an excellent linear fit over the dynamic range of 10 – 500 pg/mL. Figure 2 shows the results for four unknown samples. Table 1 compares these results to the averaged results from three other laboratories. The outside labs used either SPE or LLE sample preparation. Ion ratios were used for confirmation.

Conclusion

An automated method utilizing online sample preparation coupled with a triple stage quadrupole mass spectrometer met analytical requirements. The results correlated well with conventional (LLE) sample preparation methods. The method is interference-free and robust. The entire analysis in plasma samples can be done in 10 minutes, with a quantitation limit of 10 pg/mL and linearity range from 10 to 500 pg/mL. Analytical throughput can be increased by implementing a 2-channel (TLX-2) or 4-channel (TLX-4) column multiplexing system in clinical research.

Method performance summary

Target Analyte	Testosterone
Matrix	Plasma
LOD	3 pg/ mL
LOQ	10 pg/mL
Recovery	> 90%
Assay Linearity	10 – 500 pg/mL
Precision (%CV)	1% to 7% (10% at LLOQ)
Carryover at LLOQ	< 20%
Sample Volume	100 µL
Analysis Time	10 minutes

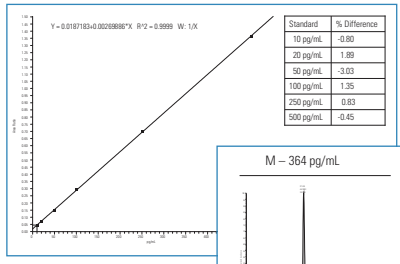

Figure 1: Testosterone calibration curve

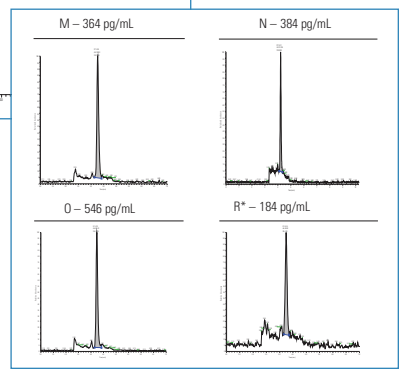

Figure 2: SRM chromatograms for four unknown samples

Table 1: Comparison of results for four unknown samples

Sample	Current Method (pg/mL)	Other Laboratories (pg/mL)
M	36	32
N	38	36
O	55	61
R*	18	13

R is a gel-tube sample.*

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Selective Testosterone Analysis in Human Serum by LC-FAIMS-MS/MS

Jonathan McNally and Michael Belford, Thermo Fisher Scientific, San Jose, CA
James Kapron, Thermo Fisher Scientific, Ottawa, ON, Canada

Key Words

- TSQ Quantum Ultra
- Clinical analysis
- High-throughput
- Selectivity

Overview

Current high throughput clinical assays utilizing triple quadrupole mass spectrometry for the quantitation of testosterone can be further enhanced through the use of FAIMS (high-Field Asymmetric waveform Ion Mobility Spectrometry) coupled to a TSQ Quantum triple quadrupole mass spectrometer. This application note describes how a FAIMS-enabled TSQ Quantum improves the performance of a testosterone assay.

Introduction

Testosterone is the androgenic hormone primarily responsible for normal growth and development of male reproductive organs. Although testosterone production declines naturally with age, testosterone production may be compromised by diseased or damaged organs.

Women biosynthesize very low levels of testosterone, which makes quantitation extremely difficult. Estrogen replacement therapy may further reduce testosterone production, resulting in additional complications in its quantitation. In addition, endogenous interferences may prevent accurate and precise testosterone measurement.

In this study, an LC-MS/MS method was used together with the selectivity offered by FAIMS to quantify testosterone in human serum. FAIMS acts to remove chemical background and endogenous interferences resulting in more accurate and precise determinations for clinical samples than LC-MS/MS alone.

Experimental Conditions

Chemicals and Reagents

Testosterone and testosterone-d_3 (internal standard-IS) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol and formic acid were acquired from Burdick and Jackson (Muskegon, MI). All chemicals were used as received.

Sample preparation: Stripped human serum (Golden West Biologicals, Temecula, CA) was fortified with testosterone at the following concentrations: 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, and 2500 pg/mL. Internal standard in 5% formic acid was added to a final concentration of 500 pg/mL. No further sample preparation was required.

Sample analysis: LC-MS/MS analyses were performed on a Thermo Scientific Surveyor LC system. The method used mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in methanol) at a flow rate of 0.5 mL/min. Serum samples (90 µL) were injected onto an LC-MS/MS extraction column. The analyte was back-flushed to the 2.1×50 mm, 3 µ, Thermo Scientific Hypersil GOLD analytical column. The entire LC effluent from the sample injections was directed to the Ion Max source on a Thermo Scientific TSQ Quantum Ultra.

Additional gas-phase separation prior to entry of ions into the mass spectrometer was achieved by including FAIMS in the analysis.

FAIMS Conditions

Dispersion voltage	–5000 V
Outer bias voltage	35 V
Compensation voltage	–12.5 V
Temperature (inner electrode)	60 °C
Temperature (outer electrode)	60 °C
FAIMS gas composition	50% He in N_2
FAIMS gas flow rate	3.8 L/min

MS Conditions

Ionization mode and source	Positive APCI
Spray current	1.0 µA
Vaporizer temperature	400 °C
Sheath gas	35
Transfer tube temperature	250 °C
Transfer tube offset	35 V
Tube lens offset	100 V
Collision energy	22 eV
Scan time	50 ms
Q1 Resolution	0.7 Da FWHM
Q3 Resolution	0.7 Da FWHM
Testosterone	*m/z* 289.2 → *m/z* 97.1, 109.1
Testosterone-d_3	*m/z* 292.2 → *m/z* 97.1, 109.1

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Results and Discussion

LC-MS/MS is a highly selective technique for analyzing drugs from biological matrices. As shown in Figure 1, samples are loaded to the extraction column via an autosampler and LC pump combination. After a short washing time, the central valve is rotated 60° to the injection position, which allows a second pump to elute the analytes from the extraction column onto the analytical column and into the FAIMS-enabled mass spectrometer. In cases where background or co-eluting interferences appear, the limiting factor is selectivity.

FAIMS is a unique selectivity enhancing tool for LC-MS. With FAIMS, gas-phase ions are purified after LC analysis but before they are mass analyzed. The waveform shown in Figure 2 separates the gas-phase ions as they are transferred into the high vacuum region of the MS. The interference ions in orange are filtered out from the ion beam, while the analyte ions in blue pass into the MS.

The basic experiment that establishes FAIMS conditions is shown in Figure 3. While infusing a reference standard of testosterone into the mobile phase, the CV is scanned over a specific range. The black trace appears for the transition due to testosterone, and a maximum signal for testosterone appears at CV –12.5 V. To ensure that mobile phase components with the same transition will be eliminated, stop the infusion of the reference standard and repeat the CV scan over the same specified range. The red trace indicates that chemical background in the mobile phase emerges from FAIMS not at the CV for testosterone, but rather in a broad range between CV –15 and –25 V. For subsequent LC-FAIMS-MS/MS assays, the CV set to –12.5 V will exclude mobile phase contributions to the analysis of testosterone. Other endogenous components present in the human serum samples may also be excluded if their FAIMS behavior is different from that of testosterone.

Regression analysis based on a linear model with 1/[concentration]^2 weighting was used. The average accuracy, as deviation from theoretical is less than 5% at all concentrations. The precision of the standards, as relative standard deviation (%RSD), is less than 17% at the lower limit of quantitation and less than 11% at all other concentrations.

Despite the excellent performance of LC at cleaning up samples, many interferences are still present from the matrix. Other extraction techniques might remove these

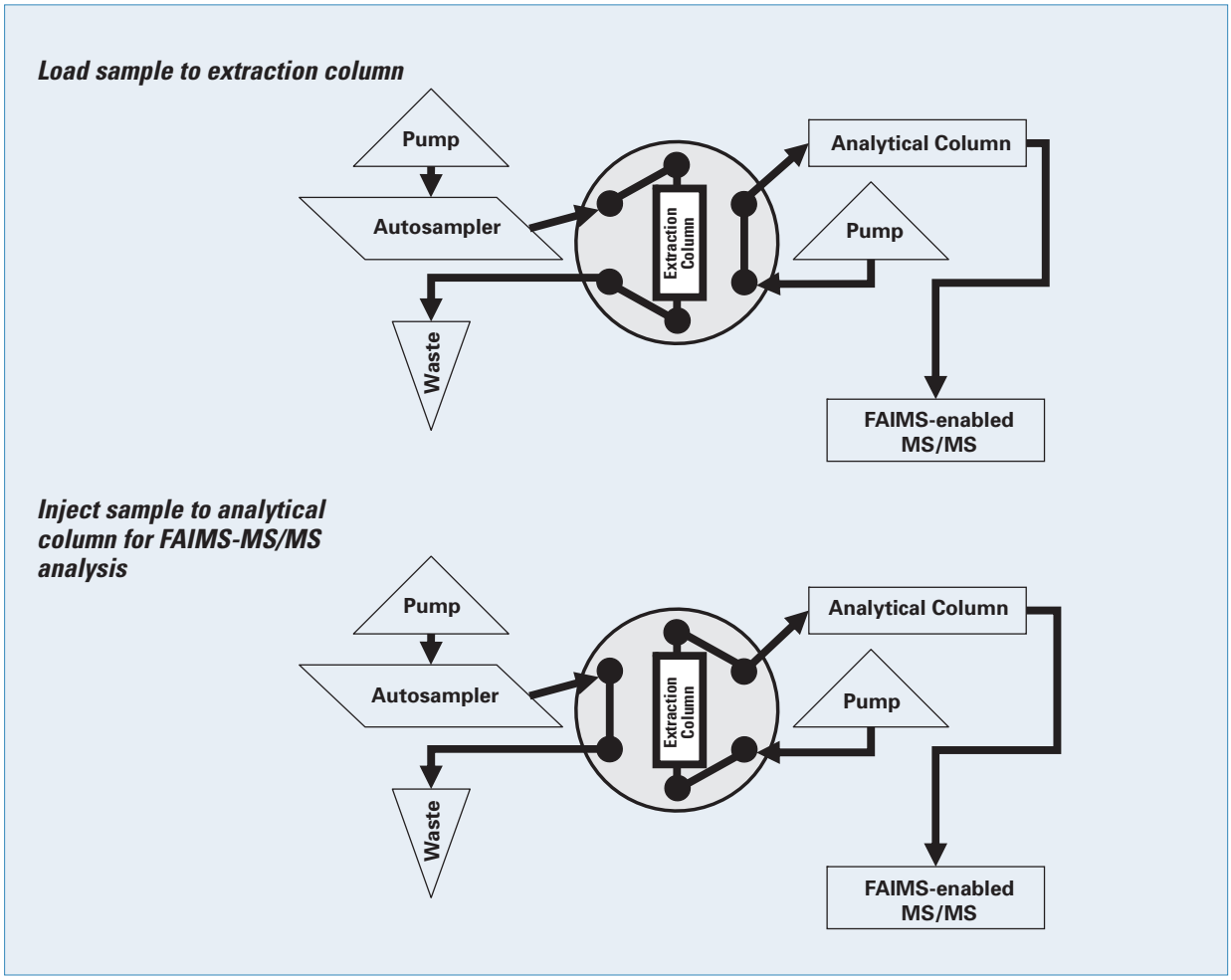

Figure 1: Schematic diagram of LC-FAIMS-MS/MS system

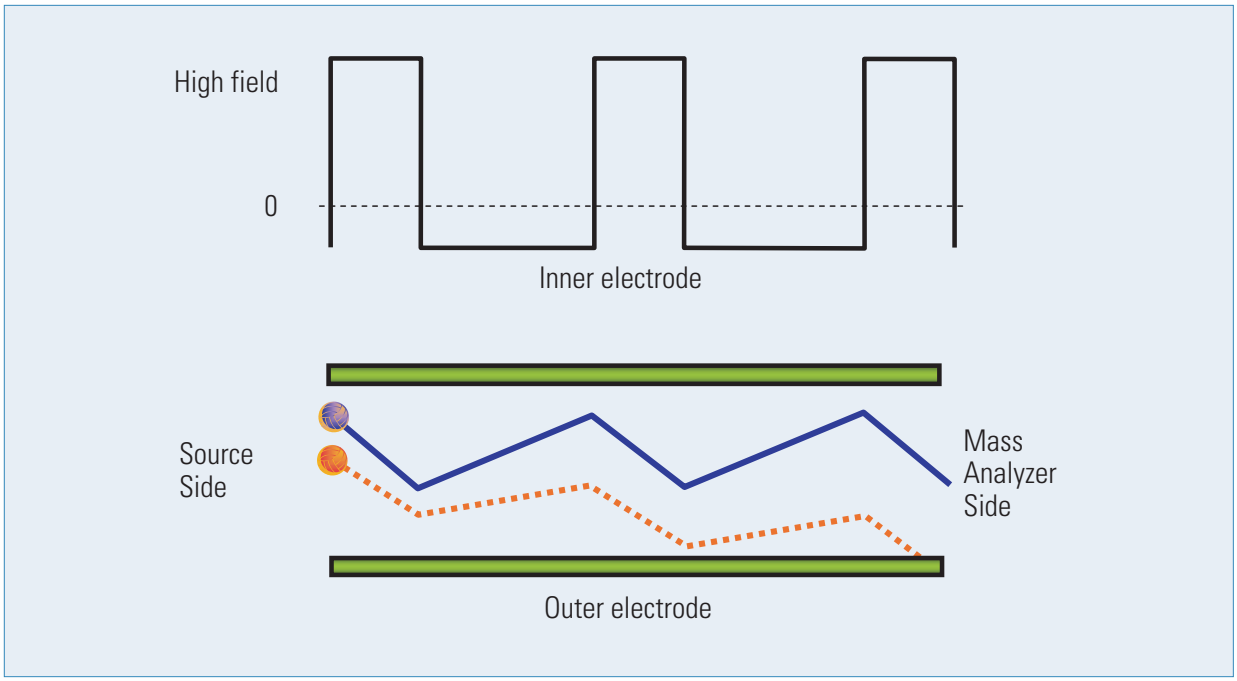

Figure 2: Compensation voltage set to transmit testosterone (blue ion, solid line). Testosterone and some of the interferences behave like the blue ion, while most of the interferences behave like the orange ion.

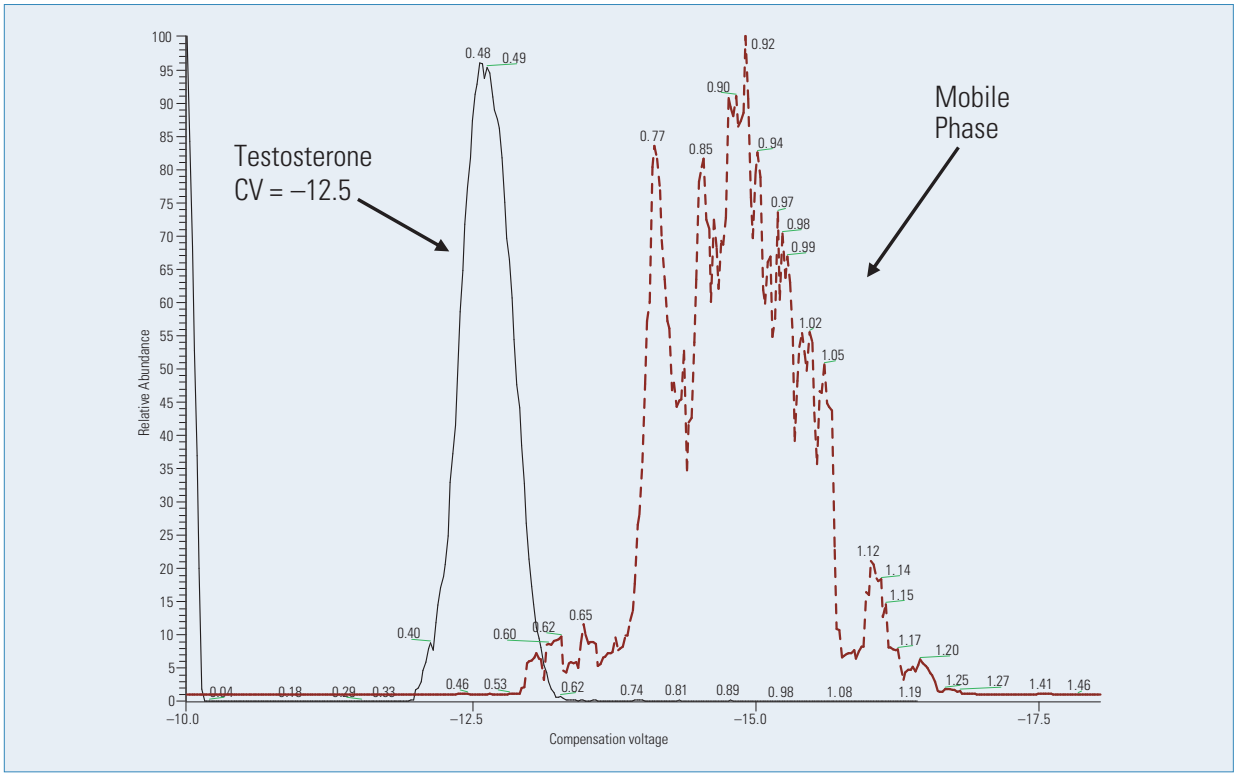

Figure 3: Compensation voltage scan for the infusion of testosterone reference standard in black. The red overlaid trace represents the CV scan from mobile phase alone.

interferences, but much time can be spent changing extraction selectivity. A representative LC-MS/MS chromatogram for testosterone in a human serum clinical sample is shown in the upper trace of Figure 4. Multiple interferences prevent accurate integration of the analyte. Note that the peak(s) at retention time 2.7 are not due to testosterone but rather multiple interferences. If this were the true concentration of testosterone the patient would not be able to survive having such a high endogenous level. The lower trace of Figure 4 shows the IS in human serum. Multiple interferences and an elevated baseline due to chemical background make peak integration difficult.

Figure 5 shows the same sample analyzed with FAIMS included in the method. LC-FAIMS-MS/MS of testosterone in the upper trace shows that many of the interferences of Figure 4 are removed. Correct peak integration for testosterone is now possible. The lower trace for the IS shows that the chemical background and interferences were eliminated. The use of FAIMS together with LC and tandem MS has improved the selectivity of the assay, resulting in a very accurate and precise method. The lower level of quantitation was improved four-fold more than the LC-MS/MS method.

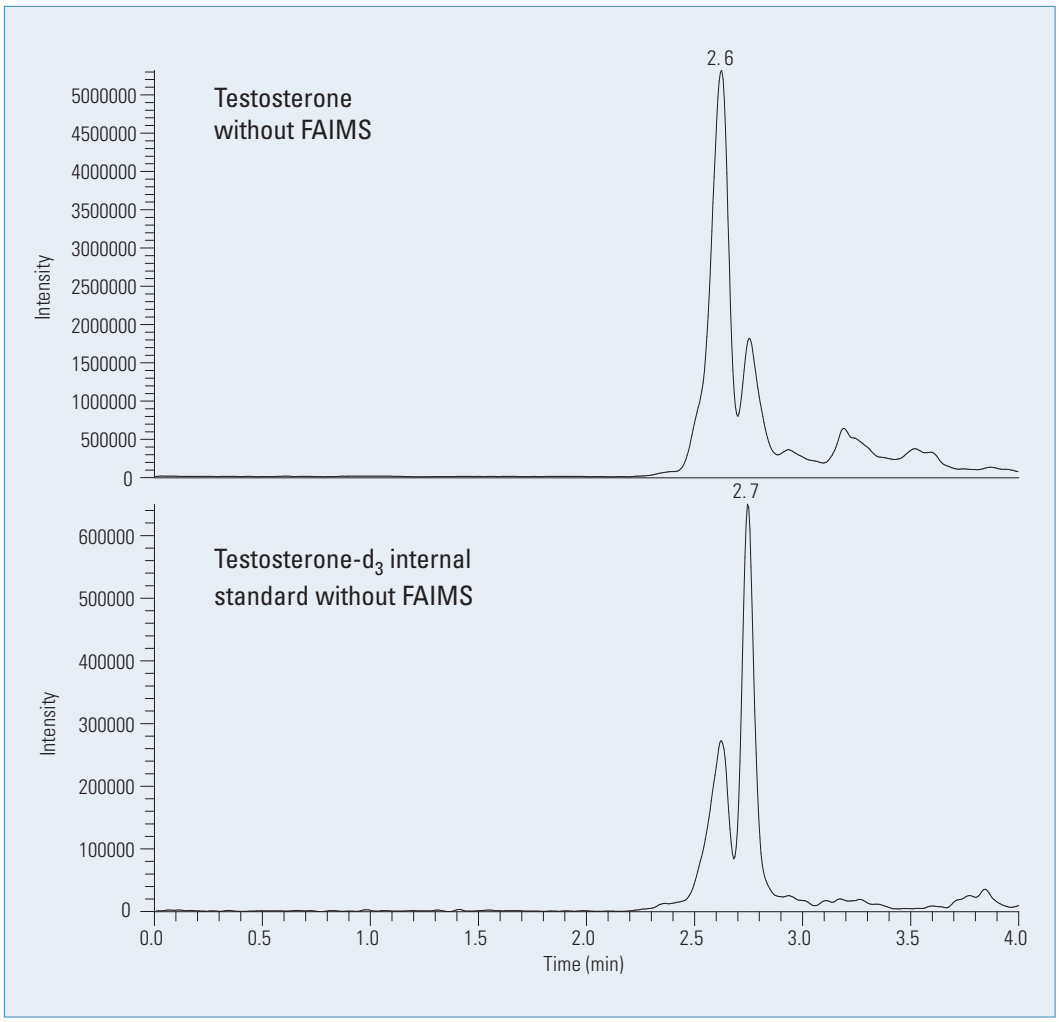

Figure 4: Representative LC-MS/MS chromatogram for testosterone in a human serum clinical sample. The upper trace is testosterone (retention time 2.7 min), the lower trace is IS (testosterone-d_3).

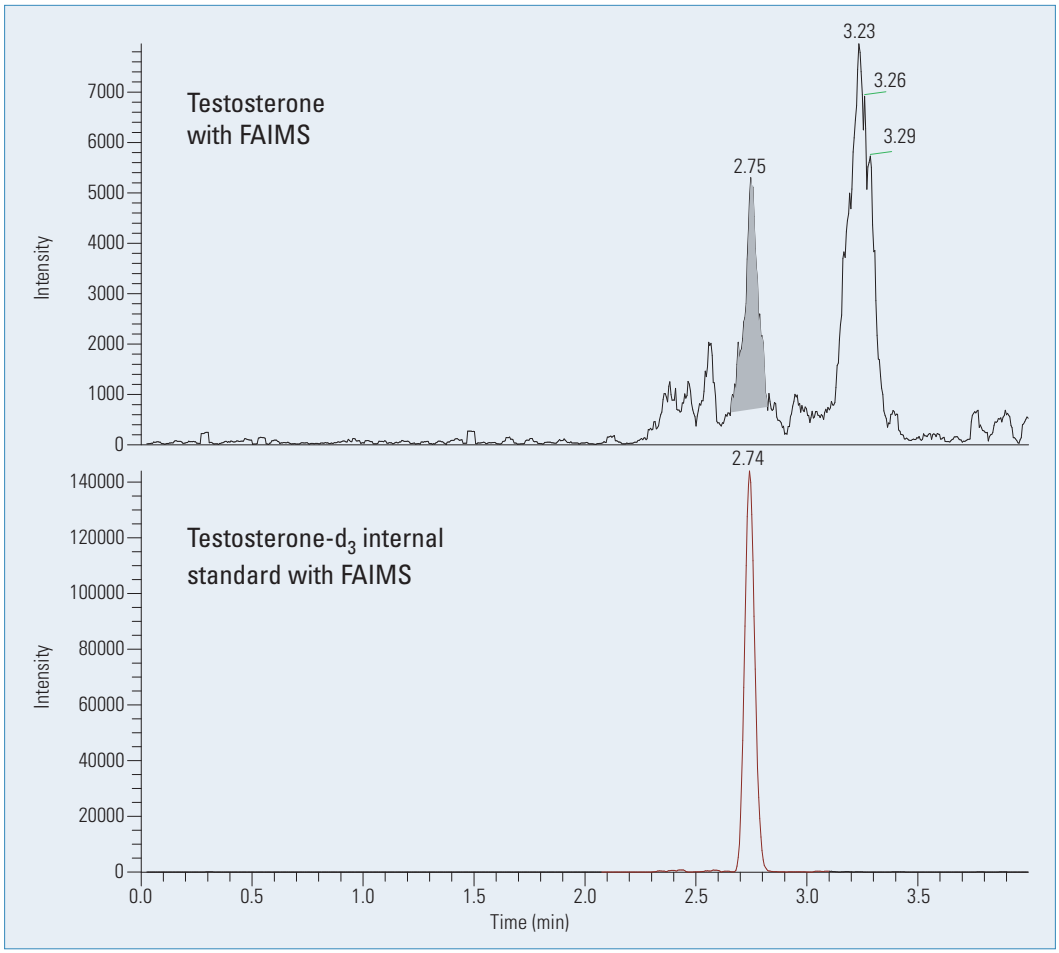

Figure 5: Using FAIMS. Representative LC-FAIMS-MS/MS chromatogram for testosterone and the lower trace is for the internal standard. Note that the absolute signal from FAIMS analysis is lower than without FAIMS because of the removal of interferences.

Conclusions

A FAIMS-equipped TSQ Quantum Ultra™ triple quadrupole mass spectrometer provides more accurate results by eliminating the chemical noise that arises from mobile phase and sample matrix. In cases where interferences prevent accurate and precise determination, FAIMS removes interferences. The resulting chromatograms accurately represent the concentration of testosterone in the samples. The LLOQ was improved four-fold compared to the LC-MS/MS method.

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Quantitative Analysis of Testosterone in Serum by LC-MS/MS

Ravinder J. Singh, Ph.D., James L. Bruton, Mayo Clinic, Rochester, MN, USA
Taha Rezai, Ph.D., Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- TSQ Quantum Ultra
- Clinical Research
- LC-MS/MS
- Transcend TLX-2

Introduction

Testosterone is the major androgenic hormone. It is responsible for the development of the male external genitalia and secondary sexual characteristics. In females, its main role is as an estrogen precursor. In both genders, it also exerts anabolic effects and influences behavior. In men, testosterone is secreted by the testicular Leydig cells and, to a minor extent, by the adrenal cortex. In premenopausal women, the ovaries are the main source of testosterone with minor contributions by the adrenals and peripheral tissues. After menopause, ovarian testosterone production is significantly diminished. Testosterone production in testes and ovaries is regulated via pituitary-gonadal feedback involving luteinizing hormone (LH) and, to a lesser degree, inhibins and activins.

Most circulating testosterone is bound to sex hormone-binding globulin (SHBG), which in men also is called testosterone-binding globulin. A lesser fraction is albumin bound and a small proportion exists as free hormone. Historically, only the free testosterone was thought to be the biologically active component. However, testosterone is weakly bound to serum albumin and dissociates freely in the capillary bed, thereby becoming readily available for tissue uptake. All non-SHBG-bound testosterone is therefore considered bioavailable.

For adults, the normal values for testosterone are 240-950 ng/dL for males and 8-60 ng/dL for females.

Goal

To develop a sensitive, quantitative LC-MS/MS assay for testosterone in serum for research laboratories.

Experimental Conditions/Methods:

Chemicals and Reagents

Testosterone standard was purchased from Steraloids, Inc. in the powder form and is stored at room temperature. The internal standard, Testosterone 16,16,17-d_3, was purchased from CDN Isotopes in the powder form and is also stored at room temperature. Bovine serum albumin and PBS buffer were purchased from Sigma-Aldrich and stored in a refrigerator. Bovine serum was used because human serum with undetectable levels of testosterone was not commercially available.

Sample Preparation

0.025 mL deuterated stable isotope internal standard (d_3-testosterone) is added to a 0.1 mL serum sample as internal standard. Protein is precipitated from the mixture by the addition of 0.25 mL acetonitrile. The testosterone and internal standard are extracted from the resulting supernatant by an on line extraction. This is followed by conventional liquid chromatography and analysis on a tandem mass spectrometer equipped with a heated nebulizer ion source.

Calibration Curve Standards Preparation

A standard stock solution of 1 mg/mL of testosterone was prepared in methanol. Standard spiking solutions of testosterone in methanol/water at concentrations of 1000 ng/mL and 100 ng/mL were prepared by dilution of the stock standard solution. The appropriate amount of standard spiking solution was added to 100 mL of 5% BSA in 0.01M PBS (pH 7.4) to prepare calibration standards at the following concentrations: 5 ng/dL, 10 ng/dL, 20 ng/dL, 50 ng/dL, 100 ng/dL, 200 ng/dL, 500 ng/dL, 1000 ng/dL, and 2000 ng/dL. The standards were processed with the sample preparation procedure described above. The standard stock solution and the standard spiking solutions were stored at -20 °C.

HPLC

HPLC analysis was performed using the Thermo Scientific Transcend TLX-2 System. The 0.1 mL samples were injected onto a 4 x 2 mm C18 Guard cartridge that served as an extraction column. The analyte was directly transferred from the extraction column and focused onto the 33 x 4.6 mm analytical column which was packed with 3 micron particles. Loading and Eluting Mobile phase A was water. Loading phase B was methanol. Loading phase C was a solution containing 45% acetonitrile, 45% isopropanol, and 10% acetone which is used to wash the extraction column. Eluting Mobile phase B was a 50/50 solution of water and acetonitrile. The appropriate gradients and flow rates are described in Table 1.

MS/MS

MS/MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer with an atmospheric pressure chemical ionization (APCI) probe.

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Time (min)	Loading Flow (µL/min)	Loading A%	Loading B%	Loading C%	Eluting Flow (µL/min)	Eluting A%	Eluting B%
0.00	1.0	100			0.5	60	40
0.67	1.0	90	10		0.5	60	40
1.67	0.20	90	10		0.3	60	40
1.77	0.15	20	80		0.6	60	40
2.77	1.00	20	80		1.00		100
5.02	2.00			100	1.20		100
6.02	2.00	100			0.80	60	40

Table 1: HPLC Method

The MS/MS conditions were as follows:

Ion Polarity: Positive Ion Mode
Vaporizer Temperature: 525 °C
Capillary Temperature: 350 °C
Discharge Current: 5.0 uA
Sheath Gas Pressure (N_2): 35 units
Auxiliary Gas Pressure (N_2): 10 units
Scan Type: Unit Resolution
Scan Time: 0.050 s

Analyte	Parent Ion (Q1)	Product Ion (Q3)	Collision	Tube Lens
Testosterone	289.201	97.118	23	113
Testosterone	289.201	109.114	25	113
Testosterone IS	292.216	97.111	21	92
Testosterone IS	292.216	109.097	26	92

Table 2: List of SRM transitions and their parameters

Results and Discussion

Representative-SRM chromatograms for Testosterone at 5 ng/dL and 200 ng/dL are shown in Figures 1 and 2, respectively. Clearly identifiable and quantifiable peaks were observed.

The method precision was evaluated by analyzing patient sample pools at concentrations of 20 ng/dl, 50 ng/dL, 140 ng/dL and 1000 ng/dL. Intra-assay variability was determined by processing and analyzing twenty replicates of the lowest two QC sample pools. Inter-assay variability was determined by processing and analyzing two replicates of each QC sample pools in six different batches. Intra-assay and inter-assay precision results are displayed in Table 3 as % CV.

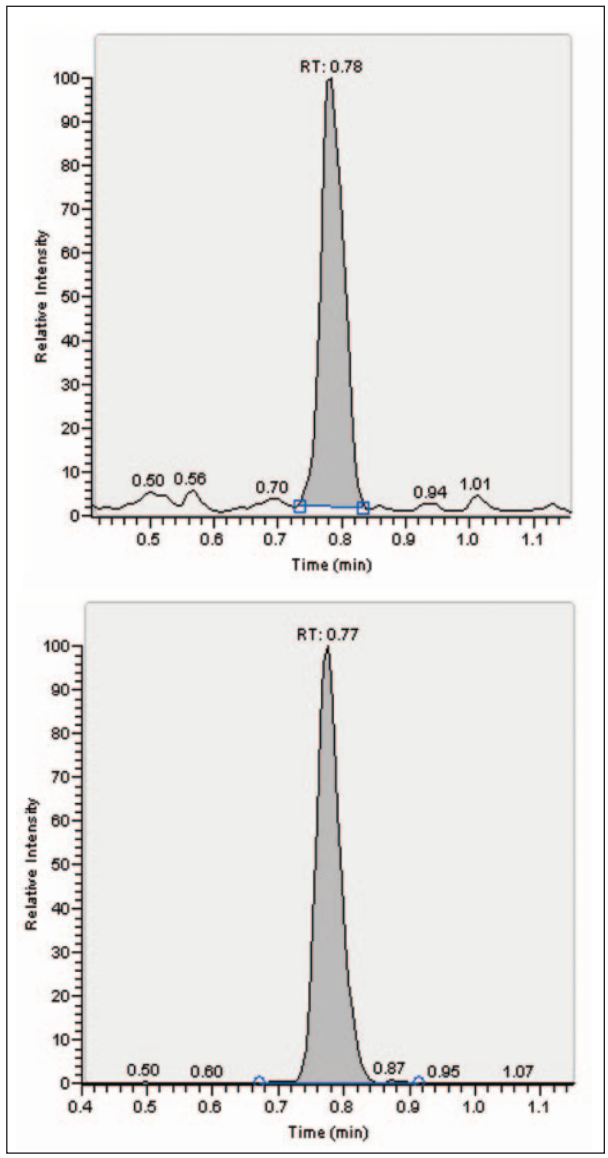

Figure 1: 5 ng/dL Testosterone standard with deuterated internal standard

	Precision	
	Intra-assay	Inter-assay
QC Level 1 (n=20)	8.43%	N/A
QC Level 2 (n=20)	7.33%	N/A
QC Level 1 (n=12)	N/A	15.70%
QC Level 2 (n=12)	N/A	12.29%
QC Level 3 (n=12)	N/A	2.91%
QC Level 4 (n=12)	N/A	5.71%

Table 3: Intra and Inter assay precision

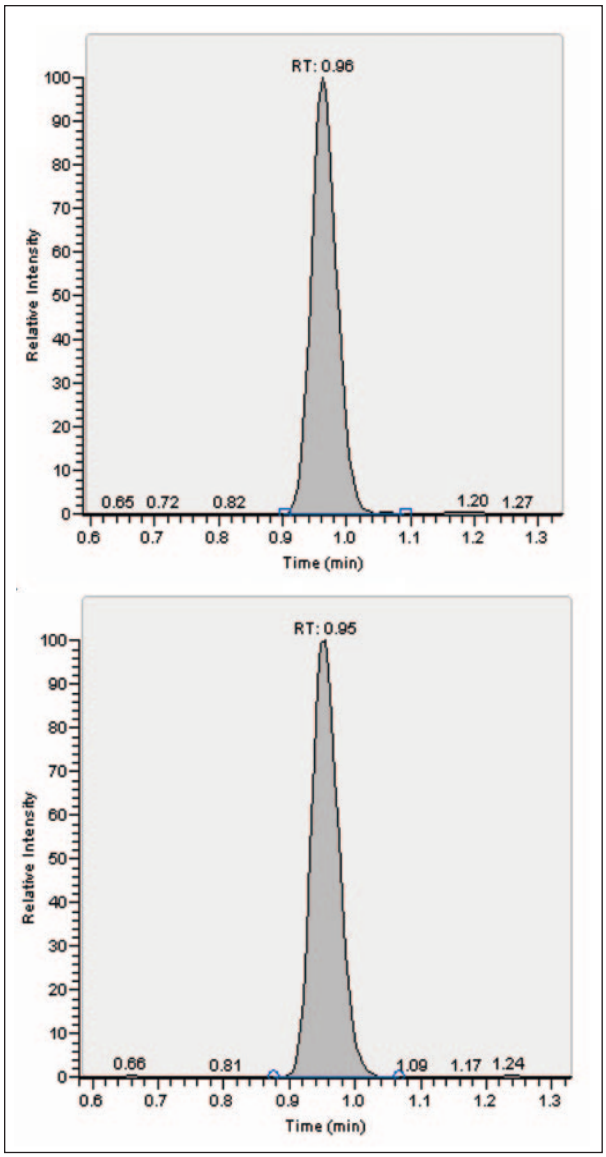

Figure 2: 200 ng/dL Testosterone standard with deuterated internal standard

SST Interferent Peak on Thermo Ultra

It had been observed in our clinical laboratory that specimens acquired with a serum separator tube had an interferent that eluted immediately in front of testosterone containing the same *m/z* transition. The presence of this interferent precluded baseline resolution and was very troublesome when integrating low levels of testosterone. Initially, this interferent was observed on the TSQ Quantum Ultra.

However, it was determined that by raising the capillary temperature of the TSQ Quantum Ultra up to 350 °C this interferent disappears without diminishing the testosterone response.

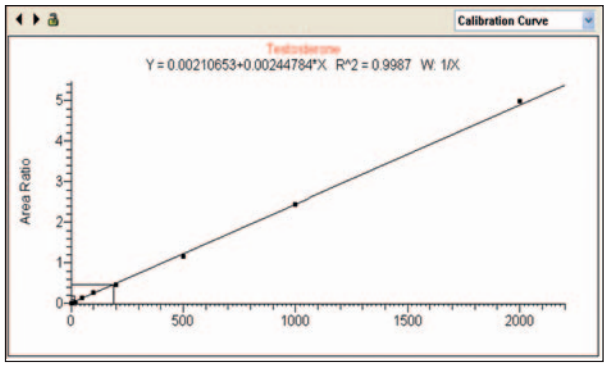

Figure 3: The linear fit calibration curve for testosterone. The calibration curve has R^2 values greater than 0.99, which indicate excellent linear fits over the dynamic range of 11-2000 ng/dL for testosterone. The LOQ value is 11 ng/dL with LOD values approximately 3 times lower.

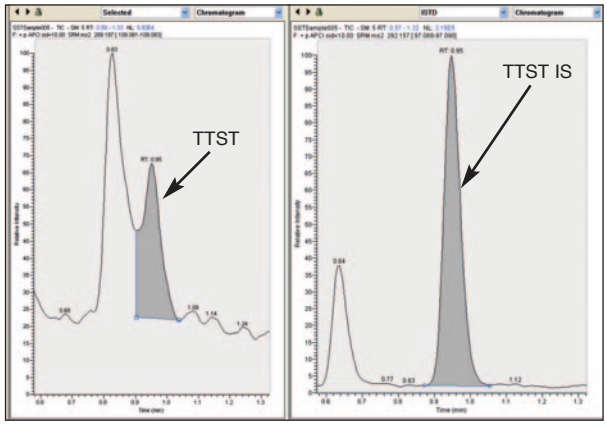

Figure 4: Testosterone sample injection with Interferent present from Serum separator Tube

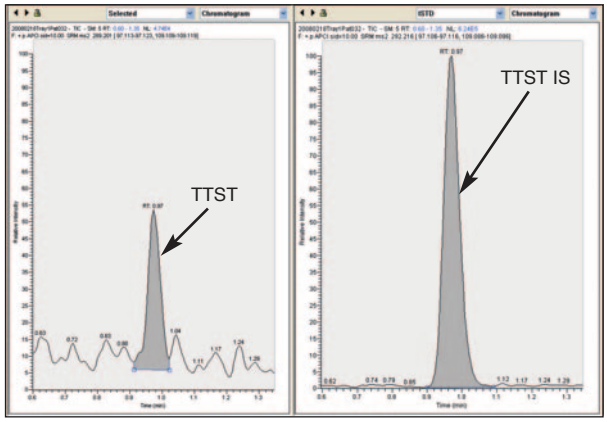

Figure 5: Testosterone sample injection with Interferent from Serum separator Tube eliminated

Conclusion

A fast, sensitive and reliable LC-MS/MS SRM method has been developed for the determination of testosterone in serum. Sample analysis was performed with a run time of 7 minutes with a quantitation limit of 11 ng/dL and a linearity range of 11-2000 ng/dL. The low intra-assay and inter-assay variability of the results demonstrates the reliability of the method for research laboratories.

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Quantitative Analysis of Cortisol and Cortisone in Urine by LC-MS/MS

Ravinder J. Singh, Ph.D., James L. Bruton, Mayo Clinic, Rochester, MN

Key Words

- Aria TLX-2 System
- TSQ Quantum Ultra
- Clinical Research

Introduction

Cortisol is a steroid-hormone synthesized from cholesterol by a multienzyme cascade in the adrenal glands. It is the main glucocorticoid in humans and acts as a gene-transcription factor influencing a multitude of cellular responses in virtually all tissues. Its production is under hypothalamic-pituitary feedback control.

Only a small percentage of circulating cortisol is biologically active (free), with the majority of cortisol inactive (protein bound). As plasma cortisol values increase, free cortisol (i.e., unconjugated cortisol and hydrocortisone) increases and is filtered through the glomerulus. Urinary free cortisol (UFC) in the urine correlates well with the concentration of plasma free cortisol. UFC represents excretion of the circulating, biologically active, free cortisol.

Goal

To develop a sensitive quantitative LC-MS/MS method for measuring cortisol and cortisone in urine for research applications.

Experimental Conditions/Methods

Chemicals and Reagents

Cortisol standard was purchased from the National Bureau of Reference Materials in the powder form and is stored at room temperature. Cortisone standard was purchased from Sigma in the powder form and is stored at room temperature. The internal standard, Cortisol 9,12,12-d_3, was purchased from Cambridge Isotope Laboratory in the powder form and is also stored at room temperature. Stripped urine was purchased from SeraCare Life Sciences and is stored at -20 °C.

Sample Preparation

0.050 mL deuterated stable isotope (d3-cortisol) is added to a 0.1 mL urine sample as internal standard. The cortisol, cortisone and internal standard are extracted by an online extraction utilizing high-throughput liquid chromatography (HTLC). This is followed by conventional liquid chromatography and analysis on a tandem mass spectrometer equipped with a heated nebulizer ion source.

Calibration Curve Standards Preparation

A standard stock solution of 1 mg/mL of cortisol and cortisone was prepared in methanol. Standard spiking solutions of cortisol and cortisone in methanol/water at concentrations of 5 µg/mL were prepared by dilution of the stock standard solution. The appropriate amount of standard spiking solution was added to 100 mL of stripped urine to prepare calibration standards at the following concentrations: 0.25 µg/dL, 1 µg/dL, 4 µg/dL, and 20 µg/dL. The standards were processed with the sample preparation procedure described above. The standard stock solution and the standard spiking solutions were stored at -20 °C.

HPLC

HPLC analysis was performed using the Thermo Scientific Aria TLX-2 System. The 0.1 mL samples were injected onto a Thermo Scientific 0.5 x 50 mm C18 HTLC Column that served as an extraction column. The analyte was directly transferred from the extraction column and focused onto the analytical column which was a C18, 30 x 4.6 mm, packed with 3 micron particles. Loading Mobile phase A was 95% water and 5% acetonitrile. Loading phase B was acetonitrile. Loading phase C was a solution containing 45% acetonitrile, 45% isopropanol, and 10% acetone. Loading phase D was water with 0.1% ammonium hydroxide. Eluting Mobile phase A was 90% acetonitrile and 10% water. Eluting Mobile phase B was 90% water and 10% acetonitrile. The appropriate gradients and flow rates are described in Table 1.

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Time (min)	Loading Flow (µL/min)	Loading A%	Loading B%	Loading C%	Loading D%	Eluting Flow (µL/min)	Eluting A%	Eluting B%
0.00	1.5	100				0.75		100
1.00	1.5	100				0.75		100
2.00	0.2	100				0.55		100
2.10	0.2	70	30			0.55		100
3.60	1.0			100		0.75	20	80
5.10	2.0				100	0.75	20	80
5.82	2.0			100		0.75	20	80
6.53	2.0				100	0.75	20	80
7.25	2.0			100		0.75	20	80
7.97	1.5				100	0.75	20	80
8.47	1.5	70	30			0.75	100	
9.47	1.5	100				0.75		100

Table 1: HPLC gradient

MS/MS

MS/MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer with an atmospheric pressure chemical ionization (APCI) probe.

The MS/MS conditions were as follows:

Ion Polarity:	Positive Ion Mode
Vaporizer Temperature:	475 °C
Capillary Temperature:	200 °C
Discharge Current:	5.0 µA
Sheath Gas Pressure:	60 units
Auxiliary Gas Pressure:	20 units
Scan Type:	Unit Resolution
Scan Time:	0.100 s

Analyte	Parent Ion (Q1)	Product Ion (Q3)	Collision Energy	Tube Lens
Cortisol	363.188	121.047	24	109
Cortisol	363.189	97.034	18	109
Cortisone	361.179	163.067	22	103
Cortisol IS	366.300	121.000	25	140

Table 2: SRM Transitions and their parameters

Results and Discussion

Representative-SRM chromatograms for cortisol and cortisone at 0.25 µg/dL and 20 µg/dL are shown in Figures 1 through 4. Clearly identifiable and quantifiable peaks were observed.

Figure 5 shows the linear fit calibration curve for cortisol. The calibration curve has an R^2 value greater than 0.99, which indicates an excellent linear fit over the dynamic range of 0.12 – 20 µg/dL. The LOQ value is 0.12 µg/dL with LOD values approximately 3 times lower.

Figure 6 shows the linear fit calibration curve for cortisone. The calibration curve has an R^2 value greater than 0.99, which indicates an excellent linear fit over the dynamic range of 0.20 – 20 µg/dL. The LOQ value is 0.20 µg/dL with LOD values approximately 3 times lower.

The method precision for cortisol was evaluated by analyzing urine cortisol pools at concentrations of 0.06, 0.15, 0.9, 4.1 and 10 µg/dL. For cortisone, precision was evaluated by analyzing urine cortisone pools at concentrations of 0.07, 0.29, 3.2, 5.1, and 12.1 µg/dL. Intra-assay variability was determined by processing and analyzing twenty replicates of one low urine pool and two quality control urine pools. Inter-assay variability was determined by processing and analyzing two replicates of the four urine quality pools in five different batches. Intra-assay and inter-assay precision results are displayed in Table 3 as % CV.

Conclusion:

A fast, sensitive and reliable LC-MS/MS SRM method has been developed for the determination of cortisol and cortisone in urine for use in clinical research. Sample analysis was performed with a runtime of 10 minutes with a quantification limit of 0.12 µg/dL for cortisol and a linearity range of 0.12 – 20 µg/dL for cortisol. The quantification limit for cortisone is 0.20 µg/dL and a linearity range of 0.20 – 20 µg/dL. The low intra-assay and inter-assay variability of the results demonstrates the reliability of the method.

References and Acknowledgements:

1. H.M. Dodds, P.J. Taylor, G.R. Cannell, S.M. Pond. A High Performance Liquid Chromatography-Electrospray–Tandem Mass Spectrometry Analysis of Cortisol and Metabolites in Placental Perfusate. Analytical Biochemistry 247, 342-347 (1997).
2. Lin CL, Wu TJ, Machacek DA, Jiang NS, Kao PC. Urinary free cortisol and cortisone determined by High Performance Liquid Chromatography in the Diagnosis of Cushing's syndrome. J Clin Endo Metab, 1997;82:151-155.
3. Taylor, RL, Machacek, D, Singh, RJ. Validation of a High-Throughput Liquid Chromatography–Tandem Mass Spectrometry Method for Urinary Cortisol and Cortisone. Clin. Chem., Sep 2002; 48: 1511 - 1519.

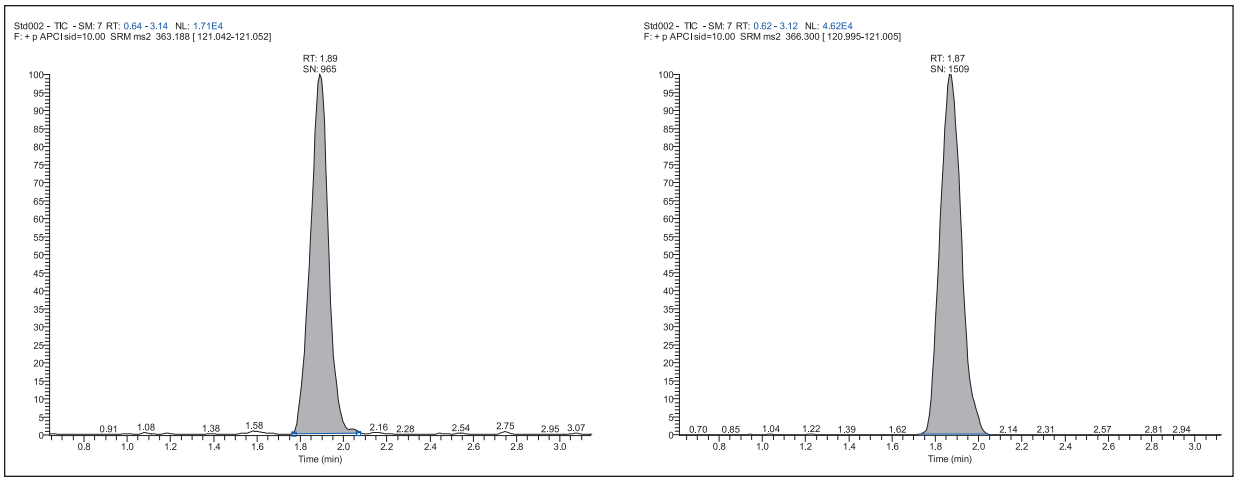

Figure 1: 0.25 µg/dL Cortisol Standard with deuterated internal standard (d3-cortisol)

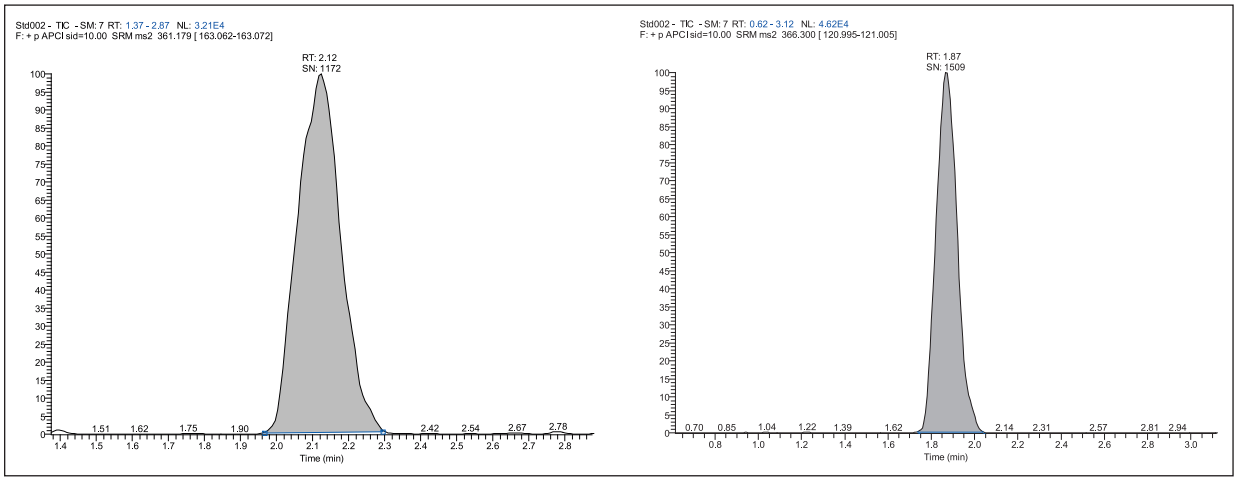

Figure 2: 0.25 µg/dL Cortisone Standard with deuterated internal standard (d3-cortisol)

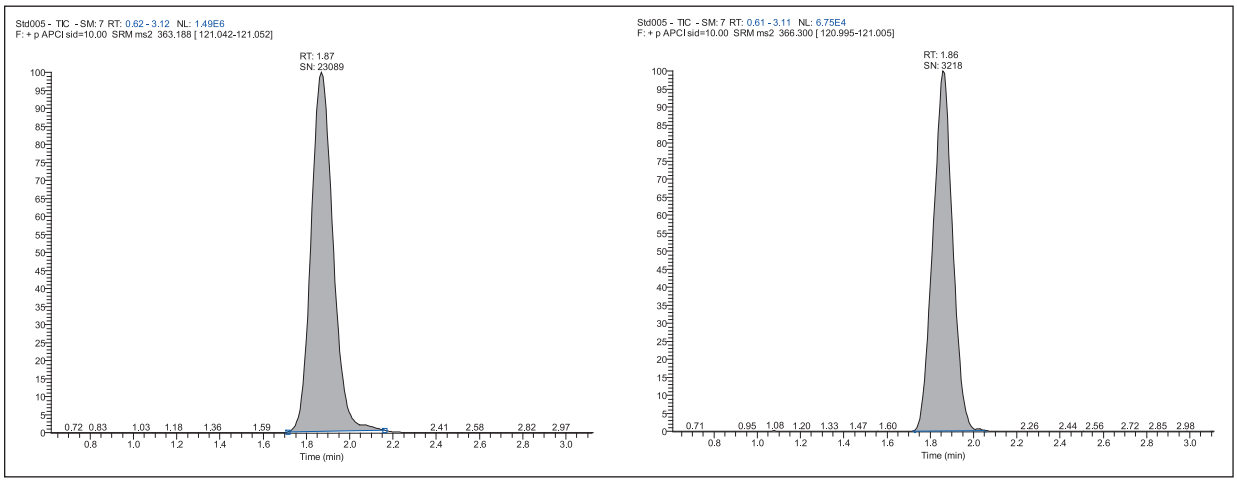

Figure 3: 20 µg/dL Cortisol Standard with deuterated internal standard (d3-cortisol)

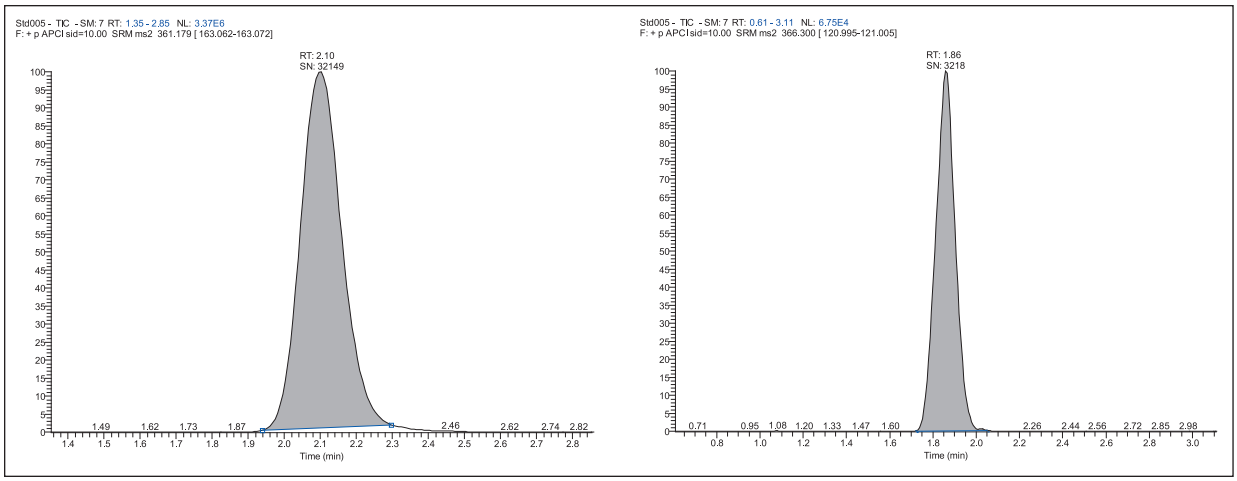

Figure 4: 20 µg/dL Cortisone Standard with deuterated internal standard (d3-cortisol)

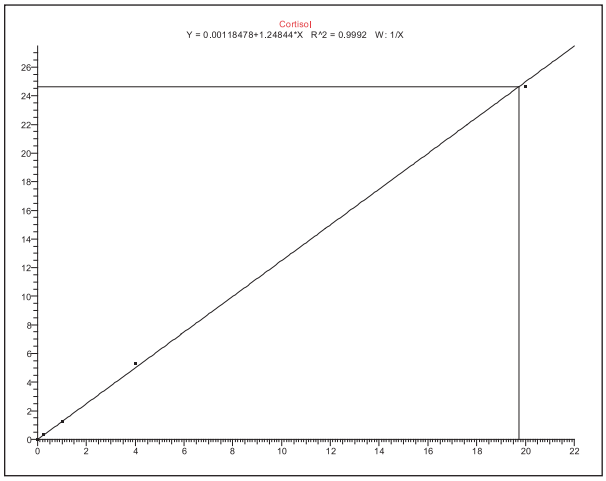

Figure 5: Urine cortisol calibration curve

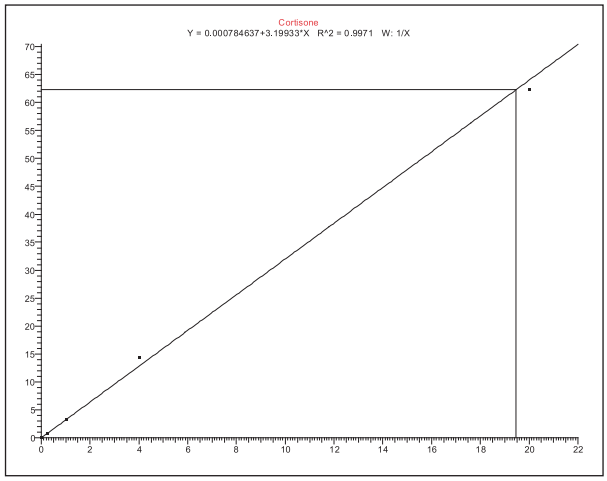

Figure 6: Urine cortisone calibration curve

	Cortisol		Cortisone	
	Intra-assay	**Inter-assay**	**Intra-assay**	**Inter-assay**
Urine Low Pool (n=20)	14.9%	N/A	6.9%	N/A
Urine QC Pool 2 (n=20)	8.6%	N/A	7.6%	N/A
Urine QC Pool 3 (n=20)	8.3%	N/A	7.1%	N/A
Urine QC Pool 1 (n=10)	N/A	20.2%	N/A	14.1%
Urine QC Pool 2 (n=10)	N/A	6.0%	N/A	8.7%
Urine QC Pool 3 (n=10)	N/A	7.5%	N/A	7.2%
Urine QC Pool 4 (n=10)	N/A	6.1%	N/A	6.2%

Table 3: Intra- and inter- assay precision

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Therapeutic Drug Analysis for Clinical Research

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Poster Note ASMS13_T545: An Improved Immunosuppressant Drug Research Method Based on a Novel SPLC-MS/MS System

Poster Note ASMS13_M503: Integrated Targeted Quantitation Method for Insulin and its Therapeutic Analogs

Poster Note ASMS13_M011: Development and Validation of Methods for Chemotherapy Drugs on the New Prelude SPLC LC-MS/MS System

Poster Note 63785: The Utilization of Novel Platform in a LC-MS/MS Workflow for the Analysis of Vitamin D, Testosterone, Immunosuppressants, Chemotherapeutics and Cortisol

Poster Note 63784: Therapeutic Drug Monitoring of 9 New Anticancer Agents by High-Performance Liquid Chromatography-Tandem Mass Spectrometry

Poster Note 63781: LC-MS Quantitative Screening Method for 18 Anabolic Steroids in Oral Fluid Using MS2 Spectra Data Collected with Q Exactive Orbitrap Mass Spectrometer

Poster Note 63780: A Novel On-Line Sample Cleanup and Liquid Chromatography Platform for LC/MS Analysis in the Clinical Research Laboratory

Note 577: Quantitation of Immunosuppressant Drugs in Blood Utilizing Second-Generation High-Resolution Accurate-Mass Mass Spectrometer

Note 518: Simultaneous Quantitative Analysis of Four Immunosuppressive Drugs Using High Resolution Accurate Mass LC-MS

Note 513: Validated LC-MS/MS Method for the Analysis of Immunosuppressant Drugs in Whole Blood Using the RECIPE ClinMass® Complete Kit

Note 505: Quantitative Analysis of Mevalonate in Plasma Using LC-MS/MS

Note 487b: Quantitative Analysis of Immunosuppressant Drugs in Whole Blood Using High Throughput LC-MS/MS

Note 472: Research Analysis of Clozapine and Norclozapine in Plasma Using Automated Sample Preparation and LC-MS/MS

Note 436: Bioanalytical Assay for Neurotransmitters in Whole Blood by LC-MS/MS

Note 384: Determination of Digoxin in Serum by Liquid Chromatography–Tandem Mass Spectrometry

Note 310: Improved Quantitative Selectivity of Clenbuterol in Human Urine Using High Resolution on the TSQ Quantum Mass Spectrometer

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An Improved Immunosuppressant Drug Research Method Based on a Novel SPLC-MS/MS System

Joseph Di Bussolo, Christopher Esposito and Francois Espourteille
Thermo Fisher Scientific, Franklin, MA, USA

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Overview

Purpose: Demonstrate robust and rugged method performance utilizing an automated two-channel sample preparation-liquid chromatography (SPLC) system that minimizes matrix interferences from whole blood when measuring immunosuppressant drugs (ISDs) for research purposes by tandem mass spectrometry (MS/MS) with electrospray ionization (ESI).

Methods: A 5 minute method involved automated clean up of whole blood preparations (cell rupture and protein precipitation by aqueous zinc sulfate and methanol) using TurboFlow technology followed by high-resolution liquid chromatography using a short Accucore C8, 2.6 µm HPLC column. Reversed-phase extraction, elution and final separations were done in a way that avoided the accumulation and co-elution of phospholipids, which would have suppressed ionization of ISDs in ESI sources. Quantitation of four ISDs was achieved by stable-isotope dilution using two internal standards (IS).

Results: Performance specifications were consistently reproduced within systems and across different laboratories as whole-blood levels were reliably measured: between 2.5 and 50 ng/mL for Everolimus, Sirolimus and Tacrolimus; and between 25 and 1,250 ng/mL for Cyclosporin A. A throughput of 21 samples per hour was achieved when multiplexing across both channels, which generated only 165 mL of solvent waste. No significant carryover between samples was detected.

Introduction

Immunosuppressant drugs (ISDs) are often analyzed in whole-blood using LC-MS with electrospray ionization, which is prone to interference by phospholipids. Although stable isotopes for each ISD are available to compensate, minimizing such interferences would improve data quality. The Thermo Scientific™ Prelude™ SPLC system—a novel dual-channel system that automates sample preparation and liquid chromatography (SPLC), was interfaced to the ESI of a tandem mass spectrometer (MS/MS) for the analysis of ISDs. The Prelude SPLC system incorporated Thermo Scientific™ TurboFlow™ technology and high-efficiency LC utilizing solid-core packing. Stable isotope derivatives D_{12}-Cyclosporin-A and Tacrolimus-$^{13}CD_2$ were used as internal standards in the whole-blood sample preparation procedure. The method was optimized to reliably minimize interferences from phospholipids to improve data quality. The method was also designed to minimize solvent waste.

FIGURE 1. Immunosuppressant Drugs Analysed

Cyclosporin A

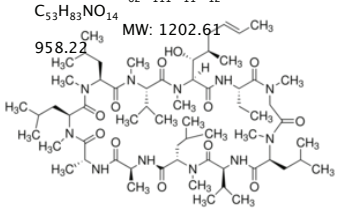
$C_{62}H_{111}N_{11}O_{12}$

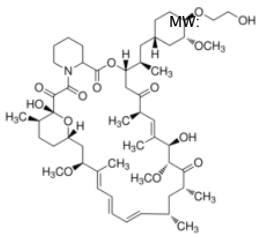
MW: 1202.61

Everolimus

$C_{53}H_{83}NO_{14}$

MW: 958.22



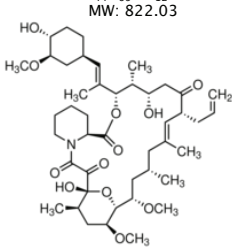


Tacrolimus (FK-506)

(Rapamycin)

$C_{44}H_{69}NO_{12}$

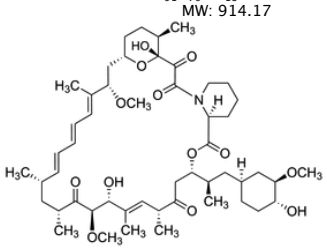
MW: 822.03



Sirolimus

$C_{51}H_{79}NO_{13}$

MW: 914.17



Methods

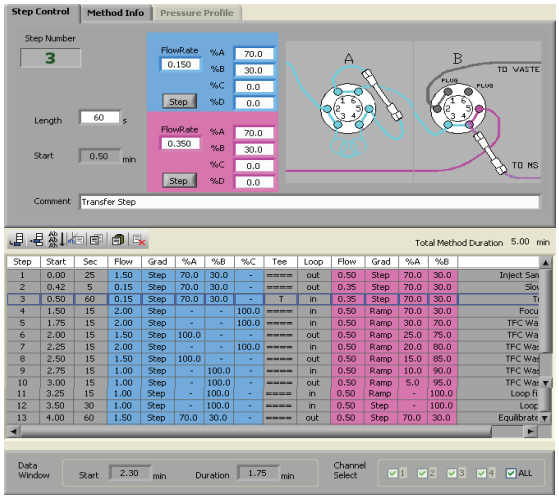
Off-Line Sample Preparation

ChromSystems 6PLUS1® ISD multilevel calibrator set and MassCheck® whole-blood controls as well as in-house test samples were mixed with aqueous zinc sulfate solution and then with methanol containing internal standards: Tacrolimus-$^{13}CD_2$ (Toronto Research Chemicals, Canada) and D_{12}-Cyclosporin A (Alsachim, France). After centrifugation, supernatants were harvested into glass autosampler vials.

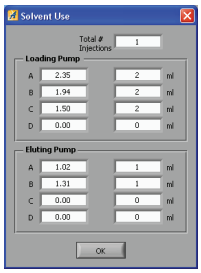
On-Line Sample Preparation & Liquid Chromatography (SPLC)

In each channel, 20 µL injections of supernatants were extracted with a Thermo Scientific™ TurboFlow™ Cyclone-P™ TurboFlow column (0.5 x 50mm) using a mobile phase mixture of 7:3 water:methanol containing 10 mM ammonium formate and 0.05% formic acid at 1.5 mL/min. A slow flow of methanol eluted extracted ISDs, which merged with a higher flow of a 7:3 water: methanol mixture, to transfer and focus the ISDs to an Accucore C8, 2.6 um, 3.0 x 30 mm HPLC column, which was maintained at 70 °C by the built-in heater. The ISDs were separated from matrix interferences and eluted to the heated electrospray ionization (HESI) source by a gradient of increasing methanol. Figure 2 shows this focus method.

FIGURE 2. Summary of SPLC Focus Method.



Solvents:
A: Water + 10mM NH_4OOCH + 0.05% HOOCH
B: Methanol + 10mM NH_4OOCH + 0.05% HOOCH
C: 45% Acetonitrile + 45% Isopropanol + 10% Acetone



Total solvent consumption is 3.37 mL A, 3.25 mL B, 1.5 mL C for each injection.

Mass Spectrometry

The Thermo Scientific™ TSQ Vantage™ triple-stage quadrupole system with heated electro-spray interface (HESI-II) was used to measure the transitions from ammonium-adduct precursor ions to product ions:

Everolimus: 975.7 > 908.4 Sirolimus: 931.6 > 864.6

Tacrolimus: 821.5 > 824.4 Tacrolimus IS: 824.4 > 771.0

Cyclosporin A: 1202.8 > 425.3 Cyclosporin A IS: 1214.9 > 437.2

During method development, the elution of phospholipids and dioctylphthalate were tracked by adding the following transitions:

Dioctylphthalate: 391 >149

Lyso-Phosphotidylcholine;16:0: 496 > 184

Lyso-Phosphotidylcholine;18:0: 524 > 184

Phosphotidylcholine;38:6: 806 > 184

Data Analysis

Thermo Scientific™ TraceFinder™ software with Aria MX was used for instrument control, data acquisition and data processing. The internal standards (IS) shown above were used for quantitation by stable-isotope dilution technique.

Results

Identifying the HPLC Column and Conditions that Minimize Interferences

Because ISDs are as hydrophobic as phospholipids and phthalates, all are extracted and transferred to the HPLC column during the TurboFlow process. Therefore, the HPLC conditions must be optimized to elute the ISDs to the detector in a reasonable timeframe while avoiding co-elution of interferences as well as buildup of interfering compounds in the HPLC column while processing many samples. Figure 3 shows buildup and co-elution from non-optimized conditions, which resulted in poor reproducibility (RSDs > 20%) of peak areas for internal standards in sample batches. Figure 4 shows results from optimized conditions, which resulted in improved IS peak area reproducibility (RSDs < 10%).

FIGURE 3. Non-Optimized HPLC Conditions

Elution from Accucore PFP, 2.6 µm, 3.0 x 50 mm column:

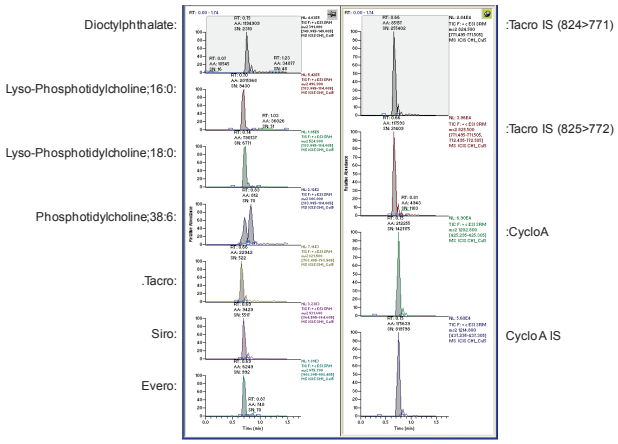
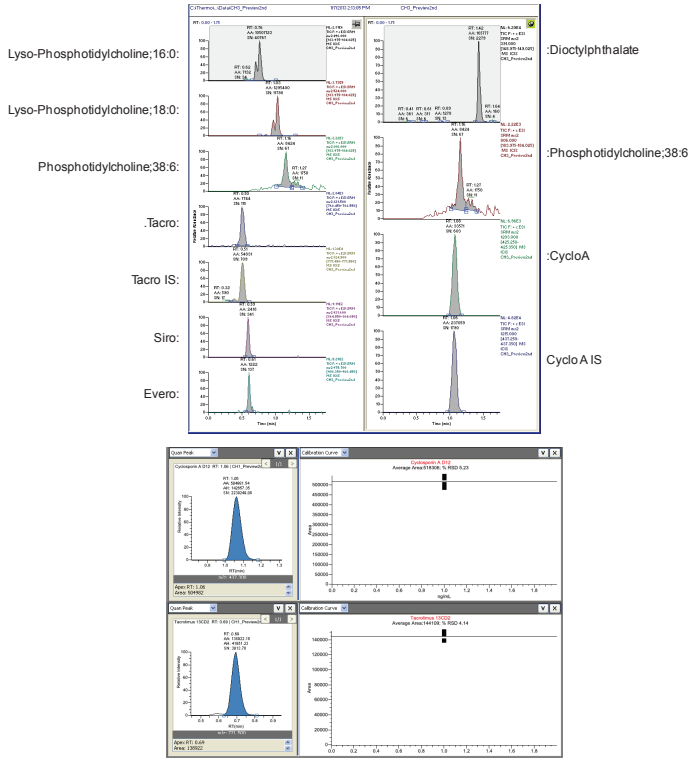


FIGURE 4. Optimized HPLC Conditions

Elution from Accucore C8, 2.6 µm, 3.0 x 30 mm column:



Achieving Required Linear Range with No Significant Carryover

As shown in Figures 5 and 6, the method consistently showed linear responses between 2.5 and 50 ng/mL for Everolimus, Sirolimus and Tacrolimus and between 25 and 1,250 ng/mL for Cyclosporin A. Weighting the data by 1/x minimized differences between expected and calculated concentrations in calibrators.

FIGURE 6. Everolimus Calibrators and QCs

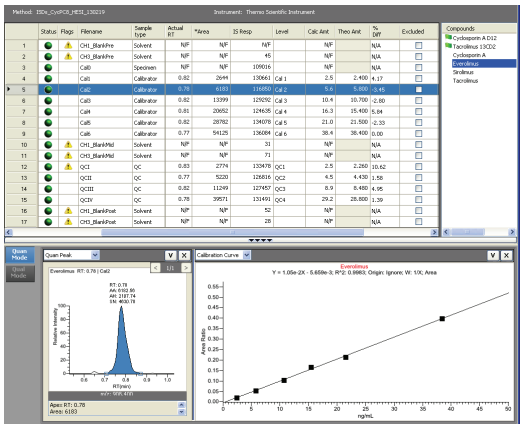


FIGURE 7. Cyclosporin A Calibrators and QCs

Reproducible QC Results were Reported Across 3 Different Test Sites.

As shown in Table 1, very similar results were reported from three different research test sites: Johns Hopkins University, Boston Children's Hospital and The Cleveland Clinic.

TABLE 1. Commercial Quality Control (QC) Reproducibility Results

	CyclosporinA			*Everolimus*		
Level	**Expected**	**Average**	**RSD%**	**Expected**	**Average**	**RSD%**
I	53	53	4.6	2.3	2.3	11.7
II	276	260	3.5	4.4	4.4	11.0
III	514	515	2.1	8.5	8.8	8.4
IV	1111	1172	6.4	28.8	28.6	6.1

	Sirolimus			*Tacrolimus*		
Level	**Expected**	**Average**	**RSD%**	**Expected**	**Average**	**RSD%**
I	2.9	2.9	8.5	2.6	2.8	5.3
II	10.1	10.0	4.6	7.3	7.1	6.1
III	20.4	20.6	5.2	16.7	16.4	4.1
IV	38.5	38.6	6.2	34.2	33.8	4.1

n=15 from 3 systems within 30 days

Matching Results from Legacy Method

As shown in Table 2, the Prelude method produced results that agreed with those produced by a legacy TurboFlow method for ISDs. Furthermore, the Prelude results were reproduced remarkably well from sample preparations that were almost 1 month old.

TABLE 2. Everolimus Calibrators and QCs

				Repeated						Repeated
		Ran on	1/9/2013	1/29/2013				Ran on	1/9/2013	1/29/2013
Test		Legacy	Prelude	Prelude		Test		Legacy	Prelude	Prelude
Sample	ISD	Method	Method	Method		Sample	ISD	Method	Method	Method
8KLE	Cyclosporin A:	86	105	103		120726-001	Everolimus:	3.5	3.0	4.5
8KBG	Cyclosporin A:	186	201	203		120726-002	Everolimus:	2.0	1.7	1.8
8KOU	Cyclosporin A:	84	99	93		120726-003	Everolimus:	2.0	1.8	2.0
8L20	Cyclosporin A:	80	81	75		120904-001	Everolimus:	4.0	4.3	3.9
8LB5	Cyclosporin A:	88	94	94		121227-001	Everolimus:	4.6	3.9	4.4
8JDF	Cyclosporin A:	168	176	176		121227-002	Everolimus:	2.3	2.2	2.5
8I6C	Cyclosporin A:	53	58	58		121227-003	Everolimus:	2.3	2.3	2.1
8KJNK	Sirolimus:	3.6	2.2	1.8		8LO5	Tacrolimus:	7.3	7.6	7.6
8KN6	Sirolimus:	3.0	1.2	2.0		8M3Y	Tacrolimus:	2.6	3.2	2.9
8L5K	Sirolimus:	8.4	9.5	7.3		8M4D	Tacrolimus:	12.5	11.1	12.5
8JB0	Sirolimus:	3.3	3.5	2.8		8M8F	Tacrolimus:	2.3	2.8	2.8
8GOC	Sirolimus:	14.4	12.5	10.9		8MI1	Tacrolimus:	16.2	15.0	17.9
8I27	Sirolimus:	3.2	2.5	1.9		8MDV	Tacrolimus:	8.9	8.8	9.6
86HF	Sirolimus:	5.7	5.5	4.2		8LRH	Tacrolimus:	20.0	17.7	19.0

Conclusion

Improved reliability and economy was achieved for ISD analysis for research purposes by using a novel SPLC-MS/MS system and method.

- Ion suppression of ISDs by co-eluting phospholipids was largely avoided by using the short Accucore C8 HPLC column.
- Using 1/x weighting, correlation coefficients (r2) > 0.995 were typical for:
  - Cyclosporin A, from 25 to 1250 ng/mL,
  - Everolimus, Sirolimus & Tacrolimus, from 2.5 to 50 ng/mL.
- Carryover, measured by peak areas corresponding to the ISDs from blank injections following the highest calibrators, was typically less than 0.1%.
- Reproducible ISD QC results were obtained from three research test sites evaluating this method with the PreludeSPLC-TSQ Vantage system.
- A reduction in solvent waste of about 40% was achieved, comparable to legacy TurboFlow methods for ISDs.

Acknowledgements

The authors thank the following who hosted our testing program at their laboratories:

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Dr. Mark Kellogg & Dr Roy Peake of Boston Children's Hospital and

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Integrated Targeted Quantitation Method for Insulin and its Therapeutic Analogs

Eric Niederkofler,[1] Dobrin Nedelkov,[1] Urban Kiernan,[1] David Phillips,[1] Kemmons Tubbs,[1] Scott Peterman,[2] Bryan Krastins,[2] Amol Prakash,[2] and Mary Lopez[2]
[1]Thermo Fisher Scientific, Tempe, AZ; [2]Thermo Fisher Scientific BRIMS, Cambridge, MA

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Overview

Purpose: Perform simultaneous qualitative measurements and quantitation on endogenous insulin and/or therapeutic analogs at biological levels for research.
Methods: Incorporate pan-insulin Ab in the Thermo Scientific™ MSIA™ (Mass Spectrometric Immunoassay) Tips for increased extraction efficiency of all insulin variants that are detected, verified, and quantified using HR/AM MS and MS/MS data on the Thermo Scientific™ Q Exactive™ mass spectrometer.

Results: Quantitation ranges reached 0.015 nM in plasma for all variants used in the experiment with linear regressions of 0.99 or better. In addition, robust qual/quan results were observed for multiple insulin variants spiked at different levels.

Introduction

The need to detect and quantify insulin and its therapeutic analogs has become paramount for many different research assays[1]. Insulin is typically present at sub ng/mL in the presence of complex biological matrices requiring extraction/enrichment protocols to be used prior to LC-MS detection and quantitation. In addition to endogenous insulin quantification, variants are also used to stimulate the same response and need to be quantified. Variants contain slight sequence variations to effect bioavailability and are generally administered at sub ng/mL levels. To reduce sample handling bias, a universal extraction method is required to facilitate simultaneous insulin variant extraction for targeted quantitation. In addition, the LC-MS detection method must be amenable to detection and quantification of known and unknown variants.

Methods

Sample Preparation
All samples were prepared from a stock solution of plasma. To each well a 500 µL aliquot of the plasma was added as well as 0.05 nM porcine insulin and used as an internal standard. Three different sets of samples were prepared in the wells. The first set had individual insulin variants spiked covering a range of 0.015 to 0.96 nM increasing in 2-fold steps. The second set of samples had one insulin variant spiked covering the same concentration range as that in sample set 1 except Humulin® S was spiked in at a constant concentration of 0.06 nM. The last set of samples spiked two different insulin variants over the expressed concentration range with Humulin S spiked in at a constant concentration of 0.06 nM. Each sample was extracted using a MSIA Thermo Scientific™ D.A.R.T. ™ (Disposable Automated Research Tips) loaded with 3 µg of pan-insulin Ab in an automated method using the Thermo Scientific™ Versette ™ Automated Liquid Handler[2]. Following insulin extraction, washing, and elution into a new well, the samples were dried down and then reconstituted in a 100 µL solution of 75:25:0.2% water/MeCN/formic acid with 15 mg/mL ACTH 1-24.

Liquid Chromatography (or more generically Separations)

An Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system was used for all experiments and 100 µL of each sample was separated on a 100 x 1 mm Thermo Scientific™ ProSwift™ RP-4H 1 x 250 mm monolithic column using a linear gradient (10-50% in 10 minutes) comprised of A) 0.1% formic acid in water and B) 0.1% formic acid in MeCN. The column was heated to a temperature of 50 ºC.

Mass Spectrometry

All experiments were acquired using a Q Exactive mass spectrometer operated in data-dependent/dynamic exclusion. A resolution setting of 70,000 (@*m/z* 200) was used for full scan MS and 17,500 for MS/MS events. Full scan MS data was acquired using a mass range of 800-2000 Da and a targeted inclusion list was used to trigger all data dependent events.

Data Analysis

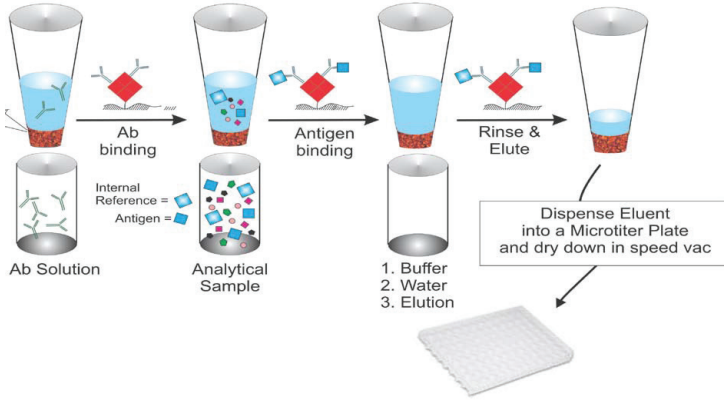
All data was processed using Thermo Scientific™ Pinpoint™ 1.3 software. HR/AM MS data extraction was used for quantitation. To provide additional levels of qualitative analysis, the three most abundant precursor charge states per insulin variant were used as well as the six most abundant isotopes per charge state. A mass tolerance of ±5 ppm was used for all data extraction. Qualitative scoring was based on mass error, precursor charge state distribution, and isotopic overlap as well as corresponding LC elution peak profiles measured for each sample. Product ion data was used for sequence verification. The measured AUC values for porcine insulin was used as an internal standard for all samples.

Results

The protocol for targeted detection and quantification of insulin and different insulin sequence variants must have specific attributes to be effective. The sensitivity and selectivity of extraction and detection methods must reach biological levels as well as provide qualitative measurements per target. A useful internal standard was included to normalize the entire method – from the sample preparation, LC-MS analysis, and data processing. Lastly, the protocol must be effective for most insulin variants to reduce cost and complexity for the workflow.

Our workflow has been shown to reach the required biological levels, facilitate a low-cost internal standard in porcine insulin, and automate the workflow to expedite sample analysis and data processing. The key aspect is based on effective targeted extraction using the Ab coated MSIA tips. Figure 1 shows the automated steps to first bind the insulin variants, wash off background compounds, and elution into a new plate. Once the extraction was performed, the plate was then prepped for LC-MS analysis. This process eliminates the two steps previously reported while increasing the detection/quantitative capabilities using the Q Exactive mass spectrometer.

FIGURE 1. Targeted extraction process using covalently bounded pan-insulin Ab to MSIA D.A.R.T tips. All samples were processed using the same protocol. Following automated extraction, washing, and elution, the samples were dried down prior to being reconstituted in an LC-MS solvent composition.



The subsequent LC-MS detection using HR/AM MS data enabled sufficient selectivity to distinguish insulin variants from the background signal using multiple precursor charge states and isotopes. The data extraction approach as shown in Figure 2 demonstrates multiple verification attributes from the LC and MS profiles. Data dependent MS/MS acquisition can also be used for specific variant determination as well. (data not shown) Decoupling the quantitative method (MS data) from sequence confirmation (MS/MS) enables the method to probe not only for known variants, but to perform significant post-acquisition processing as new variants become known, provided the b-chain epitope region remains consistent. Figure 3 shows the comparative extraction and detection efficiency of the workflow across five different insulin variants.

FIGURE 2. Targeted data extraction approach in the Pinpoint 1.3 software based on HR/AM MS data. Data from each targeted insulin variant was extracted based on isotopic *m/z* values from three precursor charge states. Integrated AUC values from each isotope was co-added to generate the reported values. In addition, qualitative analysis was performed to score each insulin variant based on 2A) comparative peak profiles (peak stop and stop, apex, and tailing factors) as well as 2b) isotopic distribution overlap.

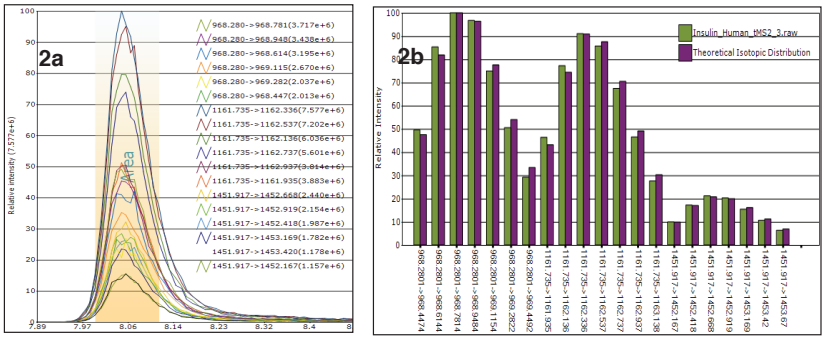


FIGURE 3. Comparative analysis of insulin variant extraction using a common workflow, including the same tips, LC separation, MS data acquisition, and data processing. Each sample was prepared by spiking 0.24 nM of each variant in different wells. The measured results are listed in each figure as well as the results for porcine (Figure 3e). The Pinpoint processing method included precursor *m/z* values for each variant.

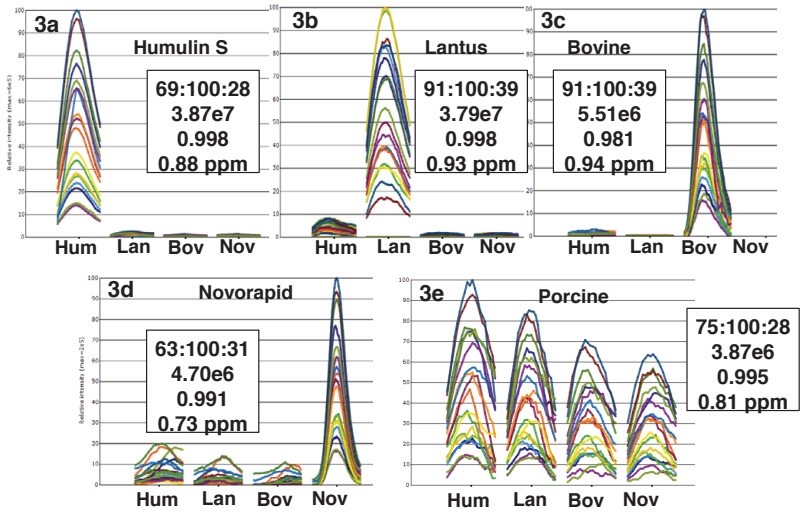


FIGURE 4. Targeted quantitation curve for Humulin S. The measured AUC values were summed from 18 isotopic *m/z* value across three charge states.

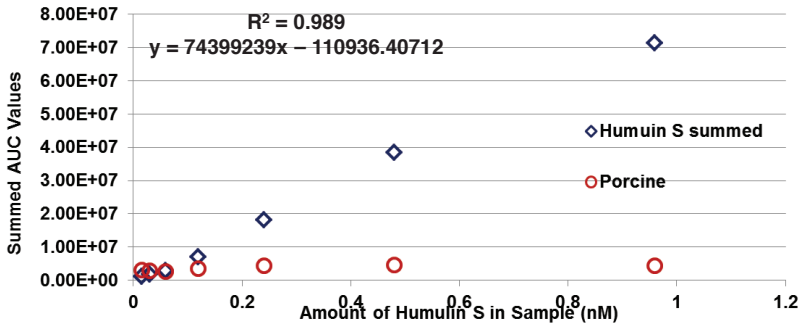


FIGURE 5. Qualitative output from Pinpoint software to evaluate 5a) precursor charge state and 5b) +5 isotopic distribution for each spiked Humulin S levels in plasma.

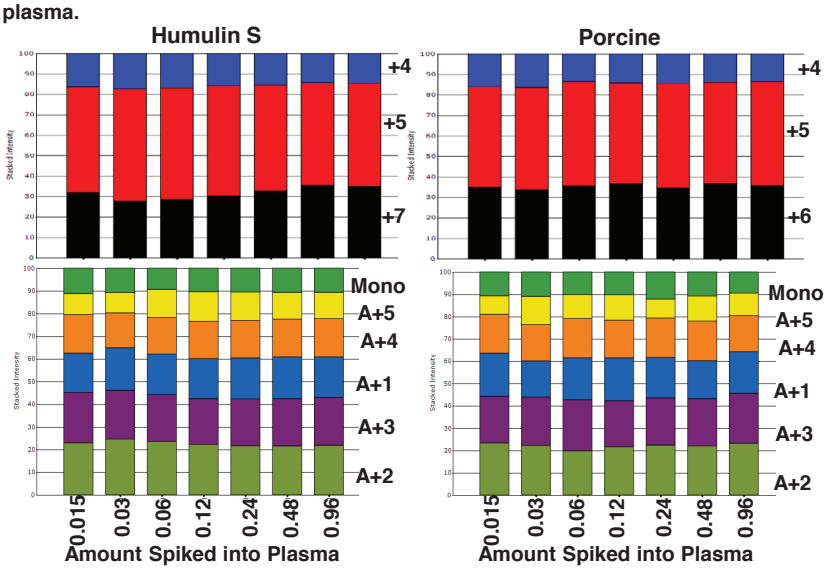


FIGURE 6. Normalized quantitative curve for Humulin S in plasma. The measured porcine response was used to normalize each level.

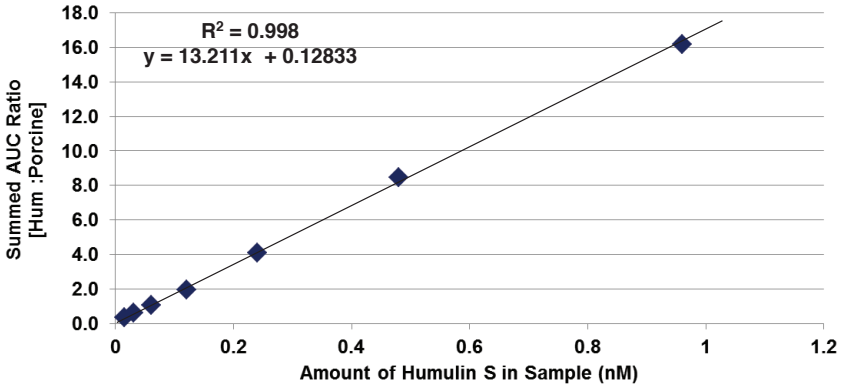


FIGURE 7. Normalized quantitative curves for bovine and Lantus insulin variants. Each variant was spiked into the plasma separately with a constant amount of porcine in all samples. The relative curves are reflective of the measured response shown in Figure 3 .

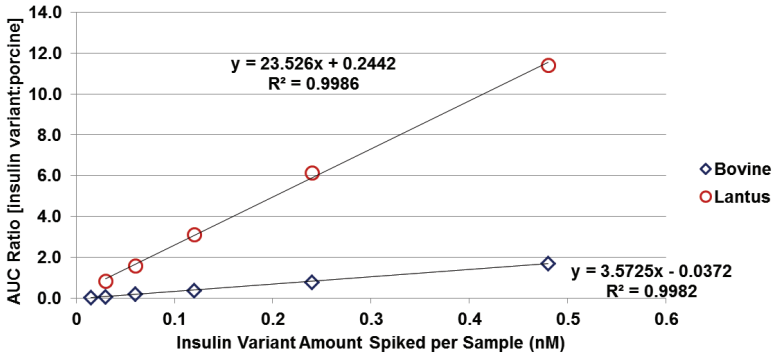
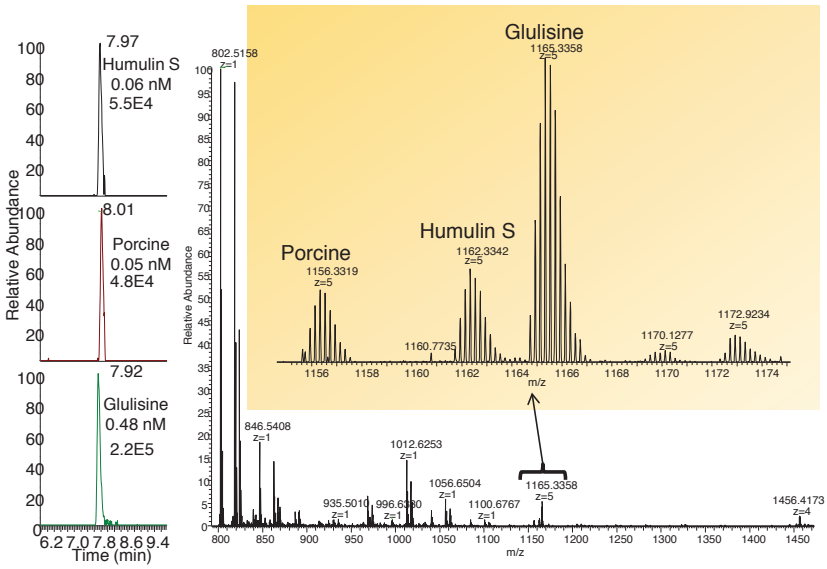
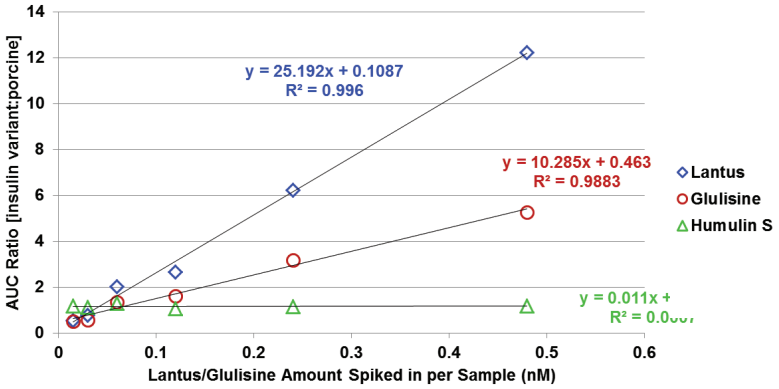


FIGURE 8. LC-MS data analysis of sample processing of four insulin variants spiked into plasma. The four samples are Lantus and Glulisine spiked at 0.48 nM, Humulin S (0.06 nM), and porcine as the internal standard. The resulting full scan spectrum was averaged across the three co-eluting variants. Lantus elutes 0.5 minutes prior to the three displayed insulin variants.



A secondary test was performed to evaluate the effects of multiple insulin variants spiked at different levels on targeted extraction and detection. Figure 8 shows the resulting full scan MS to demonstrate the Q Exactive data acquisition of the different insulin variants. The mass spectrum shows matrix from the MSIA elution solvents that formed predominantly singly charged ions compared to the targeted insulin variants at which are ca. 2-5% of the total signal and the resolution-facilitated peak detection and extraction. Figure 9 shows the quantitation for two different insulin variants spiked over same dynamic range as well as the porcine and Humulin S variants spiked at a constant level. Porcine insulin was used as the internal standard. Despite the difference in measured signal, each variant was detected at the lower levels and the resulting linear regression was 0.99 or better. In addition, the amount of Humulin S could be determined based on the individual quan curve presented in Figure 6. Using the y value of 1.1649 from Figure 9 and the linear equation in Figure 6, the calculated amount was 0.078 nM compared to the predicted amount of 0.06 nM.

FIGURE 9. Comparative quantitation curves for Lantus and Glulisine that were spiked into plasma at different levels as well as Humulin S which was spiked into each sample at a constant amount of 0.06 nM to replicate endogenous insulin. All AUC values were normalized to the porcine AUC response.



Conclusion

The targeted workflow successfully demonstrated selective and sensitive insulin variant extraction, LC separation, and quantitation using HR/AM MS for research. The pan insulin Ab was sensitive for all six insulin variants used in the study.

- The automated sample extraction utilized one step as opposed to multiple enrichment/extraction steps.
- Detection and quantitation ranges reached were at 0.015 nM in 0.5 L of plasma.
- The MSIA D.A.R.T. tips showed equivalent extraction and quantitative efficiency for singly or multiply spiked insulin variants at different concentration ranges.
- Decoupling data used for quantitative and qualitative analysis facilitates reprocessing for potential unknown insulin variants.
- The Pinpoint 1.3 software provides automated data extraction, verification, and quantification for all insulin variants.

References

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Development and Validation of Methods for Chemotherapy Drugs on the New Prelude SPLC LC-MS/MS System

Kerry Hassell, Dayana Argoti, Sarah J. Fair and Joseph Herman
Thermo Fisher Scientific, Franklin, MA, USA

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Overview

Purpose: To verify research methods to quantify a variety of chemotherapeutic drugs on the new Thermo Scientific™ Prelude™ sample preparation-liquid chromatography (SPLC) system, coupled to a Thermo Scientific TSQ mass spectrometer. The LC/MS/MS platform reduces solvent consumption, requires less maintenance, and is easier to use then traditional systems.

Methods: Prelude SPLC, Turbulent Flow Chromatography, LC/MS/MS, chemotherapeutics

Results: Methods for the chemotherapeutic drugs Busulfan, Docetaxel, Methotrexate and Imatinib, were validated using a Prelude SPLC system and TSQ mass spectrometer platform.

Introduction

Bioanalysis using LC-MS/MS can be difficult due to complex sample preparation and errors in sample handing, which can lead to variability. The use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify chemotherapeutic drugs (i.e., busulfan, methotrexate, imatinib, and docetaxel) is common practice. We demonstrate the application of Prelude SPLC system in developing a faster, more reproducible and lower solvent consuming methods for quantification of chemotherapeutic drugs.

Methods

Sample Preparation

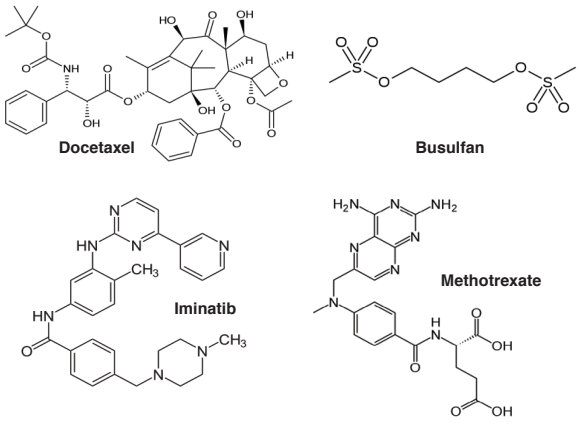
The samples in every method were prepared in human plasma . Sample preparation consisted of protein precipitation with organic solvents that contained internal standard, vortexed, followed by centrifugation. The supernatant was removed and injected into the Prelude SPLC system for analysis.

Liquid Chromatography

On-line sample clean-up was performed by 0.5x50 mm Thermo Scientific™ TurboFlow™ columns and analytical separation was performed with Thermo Scientific™ Accucore 50x2.1mm, 2.6 μm particle size columns. For iminatib, busulfan, and methotrexate, the TurboFlow HTLC-C18 XL column and Accucore PFP analytical column were used. For docetaxel, the TurboFlow HTLC-C8 XL column and Accucore C8 analytical column were used.

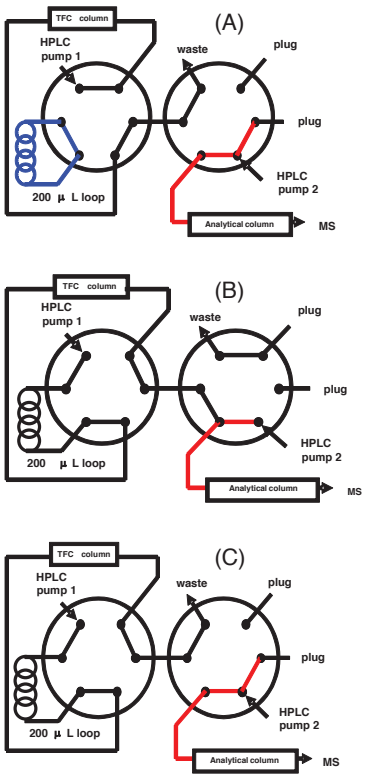
Mobile phases were (A) 10 mM ammonium formate, 0.05% formic acid in water; (B) 10 mM ammonium formate, 0.05% formic acid in methanol; and (C) 45/45/10 acetonitrile/isopropanol/acetone for iminatib, busulfan, and methotrexate. Docetaxol used acetonitrile for mobile phase B. All run times were 4 minutes or less and multiplexed to 2 minutes per sample. These methods consumed less than 3 mL of mobile phase per injection.

Structures of Chemotherapeutic Drugs



The Prelude SPLC system uses a two column method for on-line clean-up, as shown in Figure 1. The first step (Figure 1A – loading step) is to load the sample with aqueous mobile phase onto the TurboFlow column under turbulent flow conditions. Under turbulent flow conditions, large molecule (>15 kDa) cannot interact with the stationary phase and are washed to waste while the analytes of interest are retained on the column. Since the majority of matrix interferences are from the matrix proteins, the analyte of interest is removed from the matrix during step 1. Once the sample is removed from the matrix, the valves switch and the TurboFlow column is back-flushed with organic solvent stored in the loop of the first valve (filled from the previous injection), which elutes the analyte of interest from the TurboFlow column to the analytical column (Figure 1B – transfer step). In order to focus the analyte onto the analytical column, the flow from the TurboFlow column is teed to a valve with an aqueous flow from a second pump. The separation step on the analytical column provides gaussian chromatographic peaks and further separates any interferences. The elution step (Figure 1C) switches the second valve so that the sample is now eluted to the mass spectrometer and the loop can be filled with the correct percent of organic solvent for the next sample. Once these steps are complete, the valves are returned to the loading position where the columns can be cleaned and equilibrated for the next sample injection.

FIGURE 1. Valve positions for On-line Sample Clean up and Analytical Separation.



Mass Spectrometry

Detection of eluting analytes was done with a Thermo Scientific™ TSQ Vantage™ triple-stage quadrupole mass spectrometer, equipped with a heated electrospray ionization (HESI II) probe in positive ion mode using selected reaction monitoring (SRM).

Data Analysis

Quantitation was calculated with Thermo Scientific™ LCQUAN™ software.

Results

Accuracy and precision experiments were performed for system verification from three separate preparations of calibrators and controls on three different days. Interday and intra-day accuracy and precision results were obtained at concentration ranges of 20–2000 ng/mL for busulfan, 10–2000 ng/mL for imatinib, 5–1000 ng/mL for docetaxel, and 1–750 ng/mL for methotrexate. The method precision had RSD values less than 15% for all compounds tested. Additionally, accuracy was 15% of the theoretical value for all the methods. The correlation coefficient values for all the compounds ranged from 0.991 to 0.998, showing linearity throughout all concentrations and analytes. All the analytes passed carryover, bench top stability, autosampler stability, and specificity criterion. Recoveries, including matrix effects, were all ~>90%. Data are summarized in Tables 1–4. Figure 2 depicts representative standard curves for each compound tested. Representative chromatograms at the lower limit of quantitation (LLOQ) for each compound are shown in Figure 3.

The improvement in run times resulting from the lower void volumes of the Prelude SPLC system verses a conventional HPLC is illustrated in Figure 4 for Docetaxel. The same mobile phases and columns were used for the comparison. When using on-line clean-up, the duration of certain steps cannot be changed because they are dependent on the chromatographic separation needed. The duration of other steps in the process are related to how long it takes for solvent changes to reach the column. The sample clean-up and sample elution steps are dependent on the chromatography, and therefore, the time for those steps remain the same. However, the transfer, column cleaning and re-equilibration steps can be reduced. On a conventional HPLC the transfer step was 75 seconds vs. 60 seconds on the Prelude SPLC system. The column clean-up and equilibration steps were reduced from 150 to 60 seconds. The result is a reduction in run time of 29% (5:15 minutes to 3:45 minutes). A shorter run time also reduced solvent consumption by 33%.

TABLE 1. Method Range, Linearity, and Recovery.

Compound name	Method Range (ng/mL)	Linearity (r^2)	Recovery
Busulfan	20-2000	0.995-0.998	89.4-93.5
Docetaxel	5-1000		
Imatinib	10-2000	0.991-0.998	92.0-110.2
Methotrexate	10-750	0.992-0.998	102.0-110.2

TABLE 2. Intraday Accuracy and Precision.

Compound name	Intraday Accuracy (%Difference from Theoretical)			Intraday Precision (%RSD)		
	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
Busulfan	0.56-16.5	0.17-8.17	0.22-5.83	1.1-10.9	1.8-3.3	1.6-4.2
Docetaxel						
Imatinib	1.0-9.5	0.3-9.8	0.0-11.7	1.0-1.9	1.1-7.4	1.3-6.2
Methotrexate	0.13-18.5	0.12-9.74	0.10-10.5	3.3-7.5	0.6-5.9	2.8-7.8

TABLE 3. Interday Accuracy and Precision.

Compound name	Interday Accuracy (%Difference from Theoretical)			Interday Precision (%RSD)		
	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
Busulfan	4.76	0.35	3.85	5.6	5.4	3.9
Docetaxel						
Imatinib	11.00	1.33	3.74	4.0	2.0	5.9
Methotrexate	2.33	2.80	0.48	5.5	2.8	7.5

TABLE 4. Bench Top Stability, Autosampler Stability, and Selectivity.

Compound name	Bench Top Stability	Autosampler Stability	Selectivity (% of LOQ)
Busulfan	104.2-121.5	104.1-111.3	0.01-7.08
Docetaxel			
Imatinib	98.0-105.1	88.2-96.5	N/A
Methotrexate	102.4-102.9	101.4-102.5	2.14-5.50

FIGURE 2. Standard Curves for Each Compound Tested Using a Prelude SPLC System with TSQ MS

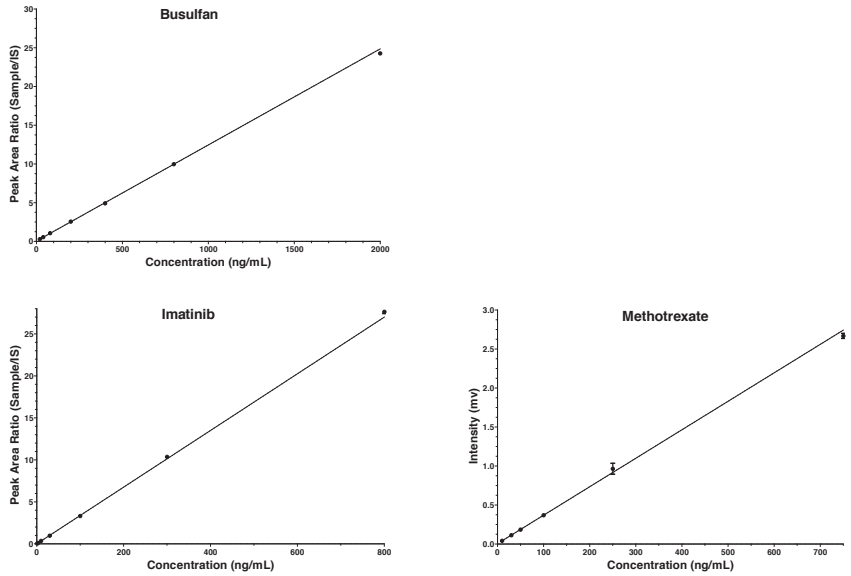


FIGURE 3. Representative Chromatograms at the LOQ for Each Compound Tested Using a Prelude SPLC System with TSQ MS

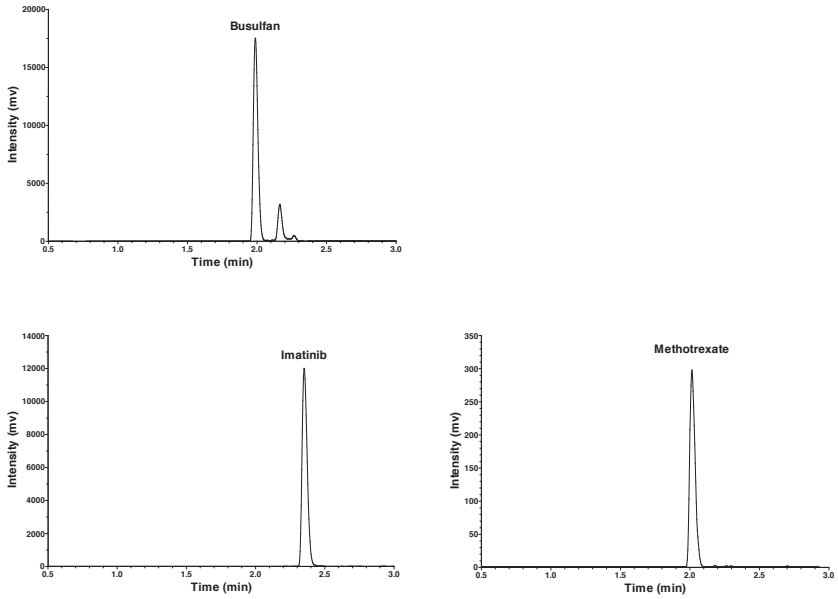
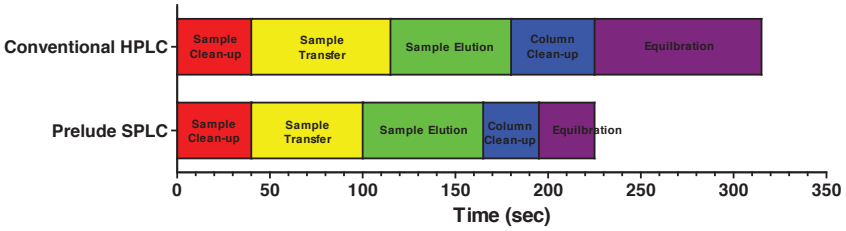


FIGURE 4. Comparison of the Method Run Time for Docetaxel on a Prelude SPLC System with TSQ MS to that of a Conventional HPLC System



Discussion

LC/MS methods for these chemotherapeutic drugs have been done previously with liquid-liquid extraction and longer HPLC methods (7-15 minutes)[1-3]. Off-line techniques such as liquid-liquid extraction are time consuming and costly from both a material and waste disposal point of view. On-line sample clean-up lowers the sample preparation duration and variability of the methods by and minimizing sample handling. Utilizing the new Prelude SPLC system reduced the run time to under 4 minutes. The shorter method time allows for higher sample throughput, which is increased further by the multiplexing capabilities of the Prelude SPLC system. The shorter run time also dramatically reduced the solvent consumption, leading to further reductions operating cost.

The Prelude SPLC system was specifically designed to reduce instrument maintenance, down time, and operating costs for high-throughput LC/MS/MS applications that require sample clean-up prior to HPLC analysis. The Prelude SPLC system utilizes syringe pumps designed to deliver the volume of mobile phase required for each sample analysis with a single push of the piston. This pump design greatly reduces the wear and tear on pump seals and check valves because the pistons in dual piston reciprocating pumps can move several hundred if not thousands of times per sample run. The majority of maintenance required on traditional HPLC pumps results from the wear of the seals and check valves; therefore, syringe pumps are more robust than traditional HPLC pumps. In addition, the Prelude SPLC system syringe pumps have no need for pulse dampers. The result is that the Prelude SPLC system has extremely low dead volumes, making rapid changes in mobile phases possible. The time required for many of the steps in a method to occur are reduced, resulting in shorter run times and lower solvent costs for equivalent methods

Prelude SPLC System & TSQ Vantage MS

Prelude SPLC System & TSQ Endura™ MS

Cross-validation of chemotherapeutics will be repeated on the Prelude SPLC system coupled to the new Thermo Scientific™ TSQ Endura™ triple-stage quadrupole mass spectrometry platform.

Conclusion

- Chemotherapeutic research methods were validated on the new Prelude SPLC with method durations of 4 minutes or less.
- The Prelude SPLC system has lower void volumes than conventional HPLC systems, resulting in sample run times that are 20-30% shorter. The reduced run time also results in reduced cost due to lower consumption of mobile phases.
- The Prelude SPLC system uses a single syringe fill per sample, which removes the need for pulse dampeners, reduces the mechanical wear and tear on the pumps, does not need proportioning valves, and removes the need for active check valves. The result: far less maintenance is required, further reducing cost and down time.

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The Utilization of Novel Platform in a LC-MS/MS Workflow for the Analysis of Vitamin D, Testosterone, Immunosuppressants, Chemotherapeutics and Cortisol

Dayana Argoti, Kerry Hassell, Sarah J. Fair, and Joseph Herman
Thermo Fisher Scientific, Franklin, MA, USA

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Overview

Purpose: To demonstrate the validity of the Prelude Sample Preparation Liquid Chromatography (SPLC) system, a new LC/MS/MS platform that reduces solvent consumption, requires less maintenance, and is easier to use then traditional systems.

Methods: Prelude SPLC™, Turbulent Flow Chromatography, LC/MS/MS, Multiplexing

Results: Methods for 25-hydroxy-vitamin D2 and D3, testosterone, the immunosuppressant drugs Sirolimus, Tacrolimus, Everolimus, and Cyclosporine A, the chemotherapeutic drugs Busulfan, Docetaxel, Methotrexate and Imatinib, and cortisol were validated using a Prelude SPLC™ LC/MS/MS platform.

Introduction

A new LC system was specifically designed to reduce instrument maintenance, down time, and operating costs for high-throughput, LC/MS/MS applications which require sample clean-up prior to HPLC analysis. The Prelude SPLC System utilizes syringe pumps designed to deliver the volume of mobile phase required for each sample analysis with a single push of the piston. This pump design greatly reduces the wear and tear on pump seals and check valves, because the pistons in dual piston reciprocating pumps can move several hundred if not thousands of times per sample run. The majority of maintenance required on traditional HPLC pumps results from the wear of the seals and check valves; therefore, syringe pumps are more robust than traditional HPLC pumps. The Prelude SPLC System's also have extremely low dead volumes making rapid changes in mobile phases possible. The time required for many of the steps in a method to occur are reduced resulting in shorter run times and lower solvent costs for equivalent methods.

In order to prove the utility of the Prelude SPLC platform, several LC/MS methods that are currently used by clinical researchers were validated. The successful validation of such a wide range of analytes using the new platform demonstrates that the Prelude SPLC offers a viable alternative to existing LC/MS systems. Reduced system void volumes resulted in methods that had run times 20-30% shorter then their equivalent methods run on a conventional HPLC and produce a corresponding reduction in mobile phase consumption.

Methods

All samples were vortexed, mixed with internal standard solution and centrifuged. Supernatant was removed and transferred into sampling containers for LC-MS/MS analysis. On-line sample clean-up using a 0.5x50 mm ThermoScientific HTLC-C18 XL TurboFlow column was followed by chromatographic separations of 25-OH-D_2, 25-OH-D_3, immunosuppressants, chemotherapeutics, cortisol and testosterone using a 50x2.1mm, 2.6 μm particle size ThermoScientific Accucore PFP analytical column. The detector was a TSQ Vantage triple quadrupole mass spectrometer with HESI-II ionization probe in positive mode. Mobile phases were (A) 10 mM ammonium formate in water, (B) 10 mM ammonium formate in Methanol, and (C) 45/45/10 acetonitrile/isopropanol/acetone. All run times were 4 minutes or less and when mutiplexed the effective analysis time was reduced to 2 minutes per sample. The Immunosuppresants were run in spiked human whole blood with cell lysis and protein precipitation occurring at the same time as the addition of the internal standard. Testosterone analysis was performed in spiked testosterone depleted human plasma. Chemotherapeutics were run in spiked human plasma. Cortisol was run using synthetic urine but was validated against human urine samples containing known levels of Cortisol

Results

Accuracy and precision experiments were performed for system verification from three separate preparations on calibrators and controls on three different days . The interday and intraday accuracy and precision results were obtained for 25-OH-D_2 and 25-OH-D_3 at a concentration range of range of 2-100 ng/mL. The range for testosterone was 0.02-10 ng/mL. Immunosuppressants and chemotherapeutics were analyzed in ranges from 1-2000 ng/mL. The method range for cortisol was 3.62 - 362 ng/mL (0.1-10 nM). The method precision had RSD values were less than 15.0% for all compounds tested. Additionally, accuracy was ±15% of the theoretical value for all the methods. The correlation coefficient values for all the compounds ranged from 0.991 to 0.999, showing linearity throughout all concentrations and analytes. All the analytes passed carryover, benchtop stability, autosampler stability, and

TABLE 1. Method Range, Linearity and Recovery

Compound Name	Method Range (ng/mL)	Linearity (r^2)	Recovery
Cyclosporin A	10 – 2000	0.992 – 0.998	87.3 – 93.9
Sirolimus	1 – 50	0.998 – 0.999	86.9 – 93.9
Everolimus	1 – 50	0.992 – 0.998	88.5 – 95.2
Tacrolimus	1 – 50	0.998 – 0.999	87.3 – 97.9
Testosterone	0.020 -10.0	0.994 – 0.999	99.9 – 103.5
Cortisol	3.62 - 362	0.997 – 0.999	88.3 – 114.1
Busulfan	20 - 2000	0.995 – 0.998	89.4 – 93.5
Docetaxel	10 - 1000	0.993 – 0.999	96.6 – 102.1
Imibitib	10 - 2000	0.991 – 0.998	92.0 – 110.2
Methotrexate	10 - 750	0.992 – 0.998	102 – 111.8
25-hydroxy Vit D2	2.0 - 100	0.992 – 0.998	92.2 – 94.5
25-hydroxy Vit D3	2.0 - 100	0.992 – 0.996	95.0 - 98.9

TABLE 2. Intraday Accuracy and Precision

Compound Name	Intraday Accuracy Range (% Difference from Theoretical)			Intraday Precession Range (%RSD)		
	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
Cyclosporin A	2.38 – 12.4	3.61 – 10.9	2.11 – 9.72	1.7 – 4.2	1.1 – 2.9	1.4 – 2.7
Sirolimus	1.78 – 16.5	2.33 – 14.9	0.11 – 13.6	7.5 -10.6	1.8 – 2.8	4.7 – 7.6
Everolimus	1.98 – 18.9	2.66 – 13.4	0.81 – 10.2	5.4 – 8.3	1.7 – 3.5	1.6 – 4.1
Tacrolimus	1.09 – 13.3	0.87 – 5.32	0.34 – 8.38	4.8 – 6.0	1.3 – 2.6	1.4 – 2.3
Testosterone	0.18 – 11.4	0.15 – 5.24	1.63 – 4.84	3.4 – 3.6	1.5 – 2.6	0.8 – 1.2
Cortisol	1.6 - 9.3	0.76 – 12.0	0.03 – 15.1	4.0 – 6.3	2.3 – 3.9	2.6 – 5.1
Busulfan	0.56 - 16.5	0.17 – 8.17	0.22 – 5.83	1.1 – 10.9	1.8 – 3.3	1.6 – 4.2
Docetaxel	0.37 – 11.9	0.14 – 5.61	0.26 – 6.98	1.6 – 9.4	1.1 – 3.7	0.9 – 3.4
Imatinib	1.0 - 9.5	0.3 – 9.8	0.0 – 11.7	1.0 – 1.9	1.1 – 7.4	1.3 – 6.2
Methotrexate	0.13 – 18.5	0.12 – 9.74	0.10 – 10.5	3.3 – 7.5	0.6 – 5.9	2.8 – 7.8
25-hydroxy Vit D2	0.5 – 14.8	0.09 – 12.5	0.3 – 11.2	5.0 – 11.5	2.9 – 6.6	1.9 – 5.1
25-hydroxy Vit D3	1.0 – 17.8	0.3 – 12.9	0.9 – 13.3	6.3 – 6.8	2.3 – 3.9	2.0 – 3.2

TABLE 3. Interday Accuracy and Precision

Compound Name	Interday Accuracy (% Difference from Theoretical)			Interday Precession (%RSD)		
	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
Cyclosporin A	2.00	0.75	3.06	12.2	9.7	12.2
Sirolimus	2.00	4.00	3.75	7.8	8.1	1.8
Everolimus	2.35	3.11	2.98	9.7	5.4	4.6
Tacrolimus	1.67	0.50	3.75	5.1	3.2	2.9
Testosterone	5.00	0.32	3.12	3.5	1.3	0.15
Cortisol	1.10	1.72	3.50	3.3	3.8	2.7
Busulfan	4.76	0.35	3.85	5.6	5.4	3.9
Docetaxel	2.66	1.51	1.28	4.2	4.4	3.1
Imatinib	11.0	1.33	3.74	4.0	2.0	5.9
Methotrexate	2.33	2.80	0.48	5.5	2.8	7.5
25-hydroxy Vit D2	4.83	2.52	2.87	3.9	4.0	4.8
25-hydroxy Vit D3	5.33	2.53	0.00	3.4	3.1	3.9

FIGURE 1. Standard Curves for Each Compound Tested Using a Prelude SLPC™ LC/MS/MS System

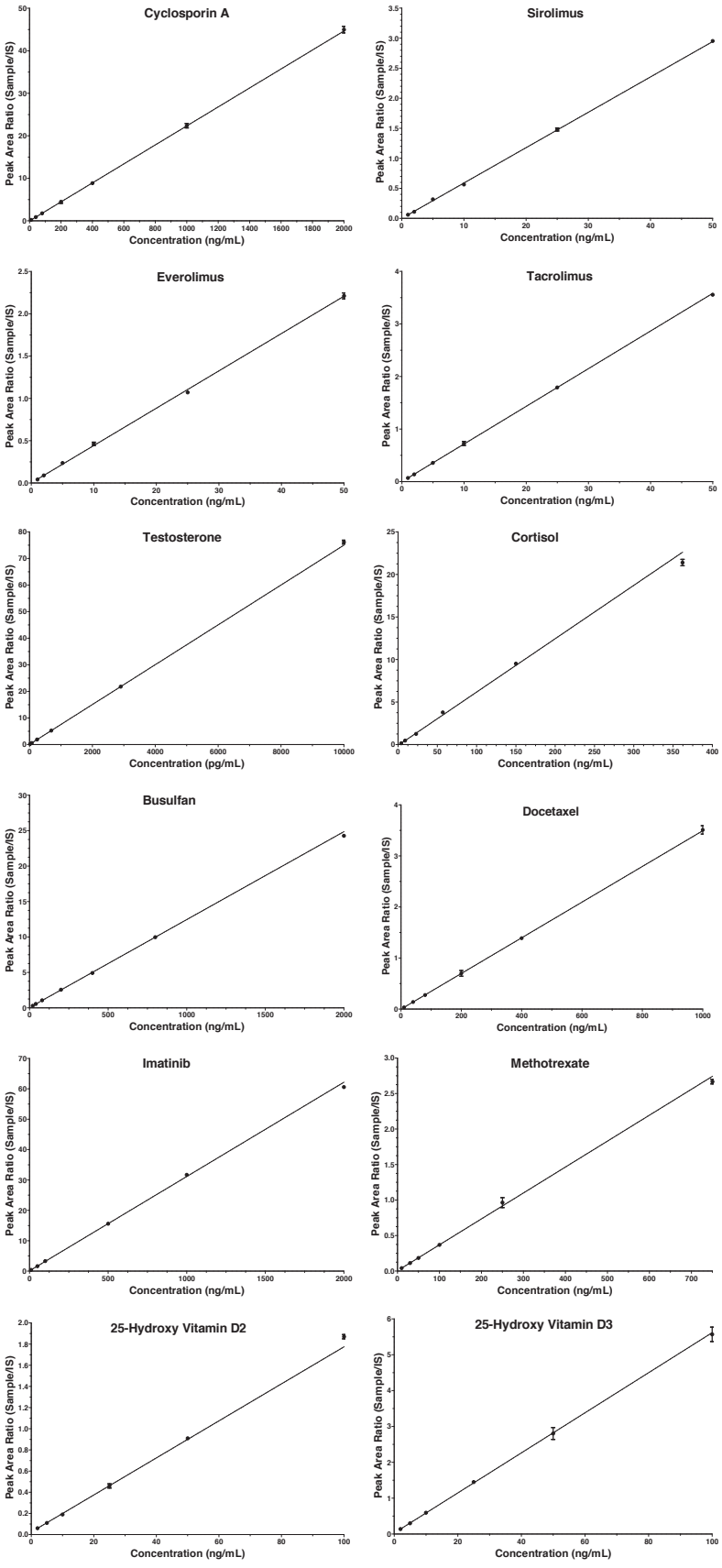


FIGURE 2. Representative Chromatograms at the LOQ for Each Compound Tested Using a Prelude SLPC™ LC/MS/MS System

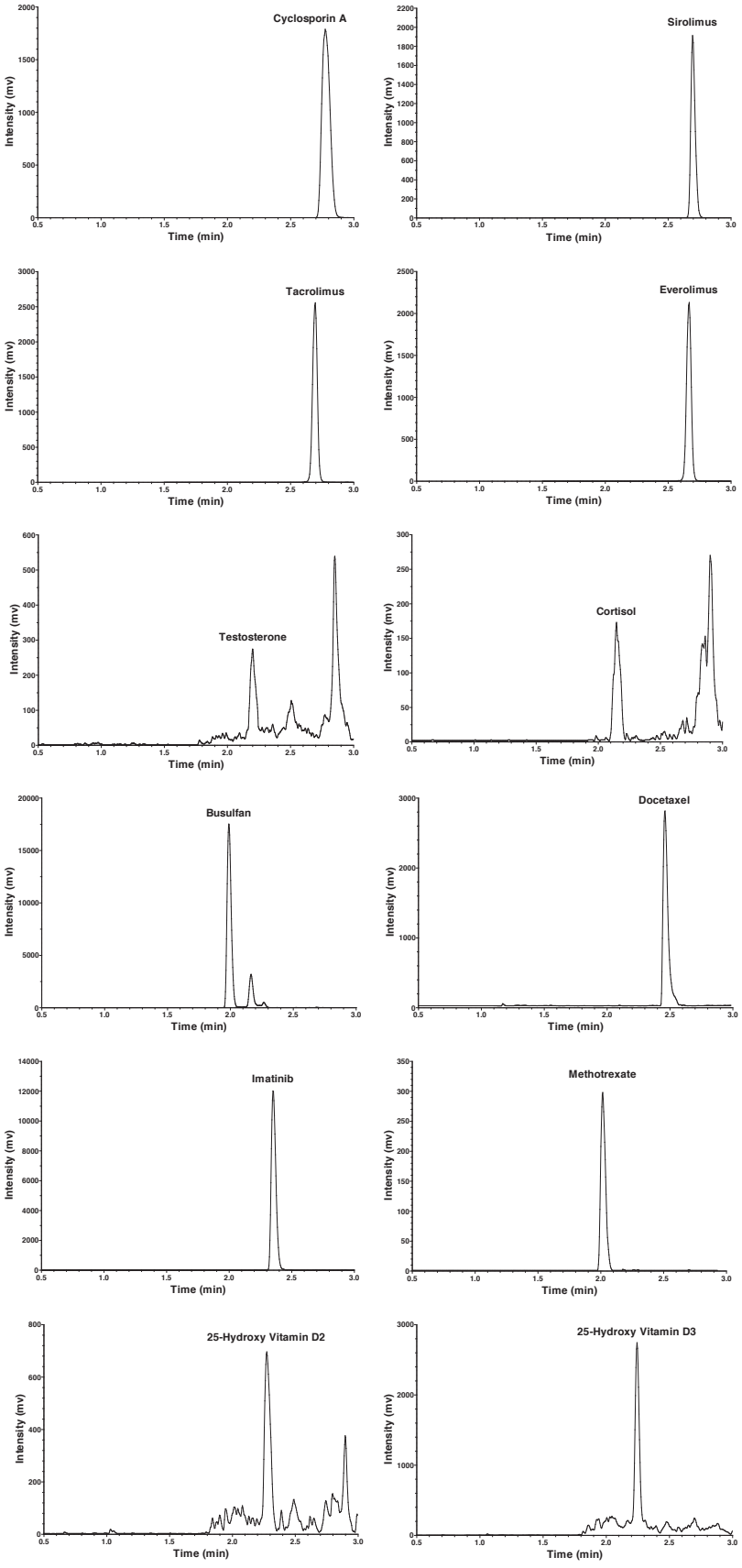
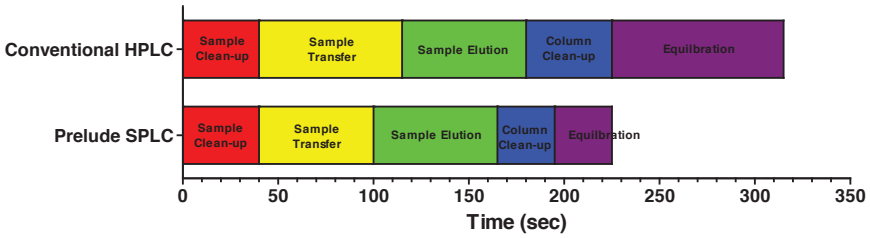


FIGURE 3. Comparison of the Method Run Time for Vitamin D on a Prelude SLPC LC/MS/MS System to that of a Conventional HPLC System



specificity criterion. Recoveries, including matrix effects, were all around 90% or higher. All the data is summarized in Tables 1 to 3. Figure 1 depicts representative standard curves for each compound tested. Representative chromatograms at the lower limit of quantitation (LLOQ) for each compound are shown in Figure 2.

The improvement in run times resulting from the lower void volumes of the Prelude SPLC System verses a conventional HPLC is illustrated in Figure 3 for vitamin D. The same mobile phases and columns were used for the comparison. When using on-line clean-up the duration of certain steps cannot be changed because they are dependent on the chromatographic separation needed. The duration of others steps in the process are related to how long it takes for solvent changes to reach the column. The sample clean-up and sample elution steps are dependent on the chromatography and; therefore, the time for those steps remain the same. However, the transfer, column cleaning and re-equilibration steps can be reduced. On a conventional HPLC the transfer step was 75 sec vs. 60 seconds on the Prelude SPLC. The column clean-up and equilibration steps were reduced from 150 to 60 seconds. The result is a reduction in run time of 29% (5:15 minutes to 3:45 minutes). A shorter run time also reduced solvent consumption by 33%.

Conclusion

- A large number of compounds, with logP values ranging from -1 to 5, have been validated on a new LC/MS/MS platform demonstrating the viability of the Prelude SPLC System for compounds of interest to clinical researchers..
- The Prelude SPLC System's lower void volume results in sample run times that are 20-30% shorter. The reduced run time results in reduced cost due to lower consumption of mobile phases and less waste disposal.
- The Prelude SPLC uses a single syringe fill per sample, which removes the need for pulse dampeners, reduces the mechanical wear and tear on pump parts such as pump seal and active check valves, and does not need proportioning valves. The result is far less required maintenance, reducing operating cost and down time.

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Therapeutic Drug Monitoring of 9 New Anticancer Agents by High-Performance Liquid Chromatography-Tandem Mass Spectrometry

V. Thibert, B. Duretz Thermo Fisher Scientific, Courtaboeuf, France
I. Gana, I. Andriamanana, A. Hulin GH Henri Mondor, Créteil, France

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Introduction

The treatment of some cancers has shifted from conventional chemotherapy drugs to chronic treatment with molecular targeted therapies. Targeted therapies include drugs such as Tyrosine kinase inhibitors (eg: Imatinib, Dasatinib, Nilotinib, Sunitinib, Sorafenib, Vandetanib, Lapatinib, Vatalanib and Erlotinib) that present better efficiency and lower side effects than conventional anti cancer drugs.

Goal

The goal was to develop and validate a fast, specific and sensitive method for the quantitation of Tyrosine kinase inhibitors (eg: Imatinib, Dasatinib, Nilotinib, Sunitinib, Sorafenib, Vandetanib, Lapatinib, Vatalanib and Erlotinib) in plasma samples using liquid chromatography coupled to mass spectrometry.

Method

Equipment

The liquid chromatography consisted of a Thermo Scientific (Courtaboeuf, France) Accela® autosampler and a quaternary pump. Separation was performed on an Hypersil Gold® PFP (2.1x100 mm; pore size 1.9 µm) analytical column placed in a thermostated column heater at 50°C. The chromatographic system was coupled to a triple quadrupole (TSQ) Quantum Ultra mass spectrometer (MS) from Thermo Fisher Scientific, Inc. equipped with an Ion Max electrospray ionization (ESI) interface and operated with XCalibur 2.07 software (Thermo Fisher Scientific, Courtaboeuf France).

LC conditions

The mobile phase used for chromatography was 10 mM ammonium formate buffer containing 0.1% (v/v) formic acid (solution A), and acetonitrile with 0.1 % (v/v) formic acid (solution B). The mobile phase was delivered using the following stepwise gradient elution program: initial conditions of 95:5 (A:B) maintained for 0.5 minutes, run from 95:5 (A:B) at 0.5 minutes to obtain 5:95 (A:B) at 2 minutes, conditions 5:95 (A:B) maintained from 2 to 4 minutes, wash using 100% of phase C from 4 to 7 minutes, run from 5:95 (A:B) at 7.01 minutes to 95:5 (A:B) at 7.5 minutes, conditions 95:5 (A:B) maintained to 10 minutes for equilibration. The flow was 300 µl/min. The thermostated column heater was set at 50°C and the autosampler was maintained at 4°C.

MS conditions

The MS conditions were as follows: ESI in positive mode, capillary temperature: 325 °C: 10V, tube lens voltages range: reported in Table 1; spray voltage: 3500 V; sheath and auxiliary gas (nitrogen) flow-rate: 45 and 25 (arbitrary units), respectively. The Q2 collision gas (argon) pressure was 1.5 mTorr. Data are acquired in selected reaction monitoring (SRM) mode.
The SRM transitions, the collision energy and ions ratio for each analyte are reported in Table 1.

Sample preparation

Calibrators and QCs preparation

For each drug, two primary stock solutions were prepared at 1 mg/ml by dissolving 10-mg base equivalent aliquots of each drug in 10 mL of methanol. Stock solutions were mixed together in order to get 2 methanolic working solutions containing all drugs at 100 µg/mL, 10 µg/mL and 1 µg/mL.
The first set was used for the preparation of the calibration standards ranging from 2 to 250 ng/mL for BORT, DASA and SUNI and from 50 to 3 500 ng/mL for the others drugs. The second set was used for the preparation of the 5 quality controls (QCs): 7, 75, 150, 750 and 1 500 ng/mL for each drug.

Only QCs at 7, 75 and 150 ng/mL were used for BORT, DASA and SUNI while QCs at 75, 150, 750 and 1 500 ng/mL were used for the other TKIs. A 0.5 mg/mL d8-imatinib, internal standard (IS) stock solution was prepared by dissolving 1 mg of the chemical in 2 ml of methanol. Plasma calibration samples and three plasma quality control (QC) samples were prepared by adding the appropriate volume of each working solution to blank plasma.

Analyte	Retention time	Precursor Ion	Product ion	TL/CE	Product ion	CE	Ion Ratio
Bortezomib	3.11	367.1	226.0	192/-18	208.0	-28	60
Dasatinib	3.01	488.2	401.0	184/-29	231.9	-38	40
Erlotinib	3.12	394 .2	277.9	136/-21	336.0	-22	40
Imatinib	2.96	494.3	394.1	170/-25	222.0	-27	20
D8-Imatinib	2.96	502.3	394.1	170/-25			
Lapatinib	3.28	581.1	349.9	185/-36	364.9	-38	75
Nilotinib	3.26	530.1	288.9	199/-29	261.0	-42	45
Sorafenib	3.59	465.1	251.9	176/-31	270.0	-21	75
Sunitinib	3.06	399.2	282.9	134/-28	326.0	-20	60
Vandetanib	2.99	475.1	83.1	142/-32	111.9	-64	15

Table 1: Retention time, precursor molecular ion/product ion for quantification, precursor molecular ion/product ion for confirmation and detection parameters (tube lens voltage (TL)/collision energy(CE)) for each analyte

Plasma sample extraction procedure

Aliquots of 50 µl of the plasma unknowns, blank, calibration standards and QCs were placed in appropriate labeled 1.5 mL microcentrifuge tubes and mixed with 200 µl of acetonitrile containing 20 ng/mL IS. After automatic vortexing for 10 minutes, each sample was centrifuged at 6 000g at 4°C for 15 minutes. Hundred microliters of supernatant were diluted two-fold using the mobile phases A and B in a 50/50 (v/v) ratio. After capping and vortexing, the vials were transferred into the autosampler tray that was maintained at +4°C. Twenty-five microliters aliquots of the extract were injected into the HPLC system.

Results

Chromatograms

The proposed method enables the simultaneous quantification of commonly used TKIs in 50µL-plasma aliquots by liquid chromatography coupled with tandem MS. Typical chromatographic profiles of the highest calibrator sample containing all are shown in Fig. 2.

Internal standard, calibration curve and lower limit of quantification

Imatinib-D8 was used as IS with a satisfactory chromatographic profile and a negligible memory effect. Calibration curves over the entire ranges of concentrations were best described by 1/x weighted linear regression of the peak-area ratio of each TKI to IS *versus* the concentrations of the respective TKI in each standard sample.

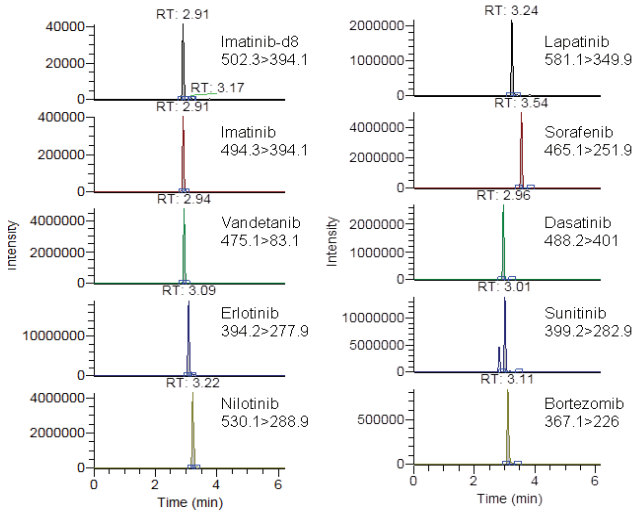

Fig. 1: Chromatogram of the highest calibrator sample containing each TKI.

This model was optimal for the 9 TKIs. Standard curves, prepared from different biological plasmas (EDTA), were performed in plasma on twenty consecutive days. The assay proved to be linear and acceptable, as the regression coefficients were >0.99 for each of the twenty standard curves excepted for sorafenib (mean r^2 0.9894) (Table 2).

A linearity test has been performed to compare theorical values, mean and standard deviations of the back-calculated values to each nominal concentration used in the low and the high standard curves. Then the accuracies were calculated for each analyte. In all cases, slopes of these linear curves were ranging between 0.9987 to 1.019 and statistics showed slopes significantly different from 1 ($p<0.0001$). The LLOQ was established at 2 ng/mL for BORT, DASA and SUNI and 50 ng/mL for the others drugs in human plasma.

Analyte		Slope	Intercept	R^2
	Mean	0.000179	0.0000483	0.9935
BORT	CV	14.8	142.9	0.48
	Mean	0.000989	-0.0004033	0.9967
DASA	CV	9.3	181.7	0.26
	Mean	0.00820	0.2222	0.9913
ERLO	CV	7.1	40.8	0.46
	Mean	0.0198	-0.009083	0.9980
IMAT	CV	5.4	137.8	0.10
	Mean	0.000286	-0.0004005	0.9964
LAPA	CV	11.5	164	0.22
	Mean	0.002519	-0.02377	0.9911
NILO	CV	3.88	91.8	1.44
	Mean	0.000657	-0.020596	0.9894
SORA	CV	10.8	24.0	0.69
	Mean	0.00514	0.00121	0.9919
SUNI	CV	6.9	183.9	0.46
	Mean	0.0000199	-0.002118	0.9943
VAND	CV	12.2	163.0	0.32

Table 2: Data detailing the slopes, intercepts, coefficient correlations (r^2) for 9TKIs (n=20).

Accuracy and precision

Precision and accuracy determined with 3 and 4 controls samples are given in Table 3. The levels of control samples were selected to reflect low, medium and high range of the two sets of calibration curves. They were chosen to encompass the clinically range of concentrations found in patients plasma. The mean intra-assay precision was similar over the entire concentration range and lower than 8.2 %. Overall, the mean inter-day precision was good with CVs within 5.3 and 13.8%. The intra-assay and **inter-assay** bias from the nominal concentrations of QCs for each considered TKI were contained between and 86.8 and 113.5 %. Ratios of ion transitions were reproducible for all TKIs and standard deviation for all of them below 25%.

Concentration	BORT		DASA		SUNI	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
2	98.4	19.8	106.9	16.2	119.8	20.0
5	93.2	19.5	101.5	8.2	99.7	6.3
10	93.9	8.9	98.3	11.9	97.6	7.0
20	108.2	13.1	97.8	6.0	93.9	11.0
50	104.0	10.1	97.2	7.7	90.7	5.1
100	98.2	5.8	98.4	5.6	91.3	4.0
250	99.5	3.9	102.2	3.0	105.8	2.1

Concentration	ERLO		IMAT		LAPA	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
50	91.8	13.8	93.4	12.8	105.5	7.7
100	94.3	10.5	98.1	9.9	96.8	6.6
200	113.9	7.1	107.6	7.8	109.2	6.1
500	109.0	5.7	98.3	5.9	90.5	7.8
1000	103.5	5.1	100.2	5.1	96.8	6.1
2000	101.4	4.4	99.2	3.8	99.6	4.8
3500	94.9	3.6	100.8	2.3	101.9	2.7

Concentration	NILO		SORA		VAND	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
50	111.5	7.8	113.1	5.4	86.5	16.2
100	99.7	4.6	98.9	3.3	91.7	11.0
200	111.1	4.5	108.2	5.8	111.2	17.4
500	98.5	5.3	91.8	5.7	103.8	10.7
1000	93.0	7.0	91.2	8.4	108.5	5.0
2000	97.5	3.3	98.3	2.7	101.3	2.4
3500	103.2	3.0	105.3	3.3	96.0	3.3

Table 3: Assay performance data of the low calibration samples for BORT, DASA, SUNI and of the high calibration samples for ERLO, IMAT, LAPA, NILO, SORA, VAND in human plasma (n=20)

Selectivity and specificity

No peaks from endogeneous compounds were observed at the drugs retention time in any of the 10 blank plasma extracts evaluated. The endogeneous responses in blank plasma were always below 6.5 % of the signal at the LLOQ of 2 ng/mL for BORT, DASA, SUNI and at 50 ng/mL for the others. The endogeneous responses in plasma provided from polymedicated patients were always less than 7.1% of the signal at each LLOQ. There were no effects of others concomitant treatments (40 mg/l of amikacin, 20 mg/l of gentamycin, 25 mg/l of vancomycin, ceftazidime, imipenem and cisplatin, 0.5 mg/l of morphine, 3 mg/l of docetaxel, 5 mg/l of voriconazole, posaconazole, itraconazole and fluconazole).

Extraction recovery and matrix effect

The assessment of matrix effects and extraction recoveries is reported in Table 5. A value above or below 100% for the matrix effects indicates an ionization enhancement or suppression, respectively. Matrix effects and extraction yields were ranged from 84.6 to 109 % and 84.0 to 101.2% respectively. Overall recoveries were ranged from 77.8 to 93.3 % for lower concentrations, 78.6 to 98.4% for medium concentrations and from 79.8 to 105.6 % for higher concentrations. The extraction recovery of D8-imatinib was 93.7%. There was no effect of hyperbilirubinemia, hyperlipemia and haemolysis on matrix effect as evaluated in medium CQs.

Stability

The stability of TKIs in human plasma samples was studied with low and high QC samples left at room temperature up to 48h. The variations are contained within ± 15% of starting concentrations indicating that TKIs can be considered stable at RT excepted for lapatinib which decreases of -36% at RT after 24h and of -76% after 48h. It has been demonstrated that lapatinib was stable at RT for 6 hours. Sunitinib is sensitive to light and decreases by -15% after 48h even light protection. By contrast, all TKIs in plasma samples left during the same period of time at +4°C were found stable.

QC samples prepared in human plasma undergoing three freeze-thaw cycles showed no significant degradation (variation < 8.2 %) for all analytes.

Long-term stability studies indicated that all analytes were stable in human plasma when stored at -70°C for 150 days (ratios between 96.0 to 100.5%, degradation < 7.9%).

The stability of stock solutions held at -70°C and left in the dark for 10 months showed decrease less than 6% for each analyte.

In neutral extracts, all analytes were stable up to 7h when left in the autosampler without any degradation allowing more than 40 samples to be analyzed simultaneously within a single chromatographic batch.

External quality controls

The external quality controls (low and high concentrations) for imatinib (18 laboratories), nilotinib and dasatinib (9 laboratories) showed a good accuracy (97.2 to 101.4%) in comparison to data obtained from others laboratories.

Application to biological samples

We applied the assay to the analysis of samples obtained from patients receiving imatinib, nilotinib, dasatinib, sunitinib or sorafenib.

DASA, IMAT and NILO were frequently detected in patients with chronic myeloid leukemia (n=75). In 71 patients treated with 400 (84%) or 600 mg imatinib daily, detected though concentrations were around 871 ng/mL (median: 789 ng/mL). Among these 71 patients, 45 % of them presented a major molecular response associated with a trough concentration higher than 1,000 ng/mL such as recommended [50].

We applied the assay to samples provided from an obese patient treated with 50 mg sunitinib for a renal carcinoma. The profile of SUNI concentrations measured in this obese woman showed no difference with AUC (1592 ± 41 ngh/ml) observed in patients without obesity.

Conclusion

In overall, the method that has been developed is precise, accurate and sensitive. It concerns nine inhibitors of tyrosine kinase acquired in a single run Confirmation is performed using confirmation/quantification ion ratios criteria. The method is very simple and therefore used in a routine environment for clinical studies; it is also possible to add new TKIs that could potentially have an interest in clinical practices and performed a partial analytical validation. The dynamic range of the concentrations allow to carry out some pharmacokinetics studies.

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LC-MS Quantitative Screening Method for 18 Anabolic Steroids in Oral Fluid Using MS2 Spectra Data Collected with Q Exactive Orbitrap Mass Spectrometer

Marta Kozak, Thermo Fisher Scientific, San Jose, CA, USA

Thermo SCIENTIFIC

Overview

Purpose: To develop a sensitive method for quantitative screening of 18 anabolic steroids in oral fluid.

Methods: Samples were processed with LLE, analyzed with a 15 min. LC gradient, and compounds were identified with ion ratio calculated for fragments in MS2 spectrum

Results: The LLOQ was 1ng/mL for all analytes except for 6β-Hydroxyfluoxymesterone (6 ng/mL). The UPLQ was between 60-1500 ng/mL, and it was lower for compounds producing high signal in mass spectrometer detector. Matrix effects were not observed: percent recovery in spiked blank oral fluid and analyzed with calibration standards prepared in solvent was in range 78.5-118%.

Introduction

Androgenic-anabolic steroids (AAS) are drugs which mimic effects of testosterone and dihydrotestosterone in the human body. They increase protein synthesis within cells which results in buildup of cellular tissue, especially in muscles. Use of anabolic steroids by athletes to increase body weight is referred to as doping and is banned by major sporting bodies.

In this work we implemented Thermo Scientific™ Q Exactive™ ultra high resolution mass spectrometer to ensure high method specificity and sensitivity.

Methods

Sample Preparation - LLE

1. To 200 µL of oral fluid (in preservation buffer), add 40 µL of internal standard solution (10 µg/mL Testosterone $^{13}C_3$ in MeOH) and 1 mL MTBE
2. Vortex, let samples rest for 5 min. at room temperature
3. Store samples for 30 min. at -20 °C
4. Transfer solvent upper layer to glass tube
5. Evaporate at 37 °C
6. Reconstitute in 50% MeOH
7. Inject 30 µL of the sample onto LC-MS

Liquid Chromatography

Column: Thermo Acucore C18, 100x3 mm, 2.6 µm

Mobile phase:

A: 0.2% Formic Acid in DIW
B: 0.1% Formic Acid in MeOH
C: ACN/IPA/Acetone=45/45/10 v/v/v

LC gradient:

	Time	A%	B%	C%	D%	µl/min
0	0.00	95.0	5.0	0.0	0.0	1000.0
1	0.49	95.0	5.0	0.0	0.0	1000.0
2	0.50	95.0	5.0	0.0	0.0	500.0
3	2.00	50.0	50.0	0.0	0.0	500.0
4	10.00	0.0	100.0	0.0	0.0	500.0
5	10.01	0.0	100.0	0.0	0.0	1000.0
6	11.00	0.0	100.0	0.0	0.0	1000.0
7	11.01	0.0	0.0	100.0	0.0	1000.0
8	12.00	0.0	0.0	100.0	0.0	1000.0
9	12.01	95.0	5.0	0.0	0.0	700.0
10	13.00	95.0	5.0	0.0	0.0	1000.0
11	15.00	95.0	5.0	0.0	0.0	1000.0

Mass Spectrometer

Ionization source: APCI
Resolution: 35K
Isolations width: 2 mu
AGC target: 2e5
Maximum IT = 250 ms
Acquisition mode: t-MS2

MS2 spectra are collected with optimized collision energies specified in method inclusion list (Figure 1) together with acquisition time windows.

Figure 1. MS method inclusion list

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	Mass [m/z]	Polarity	Start [min]	End [min]	nCE	CS [z]	Comment
1	259.07630	Positive	2.75	3.75	40 %		Clenbuterol
2	259.07630	Positive	7.25	8.25	40 %		19-Norandosterone
3	275.20060	Positive	6.00	7.00	50 %		Nandrolone
4	283.20560	Positive	6.12	7.12	40 %		Methandrosterone
5	285.18490	Positive	3.70	4.70	40 %		6b-Hydroxyboldenone
6	287.20060	Positive	5.70	6.70	35 %		Boldenone
7	287.20060	Positive	6.70	7.70	50 %		DHEA
8	289.21620	Positive	5.90	7.90	40 %		Oxandrolone/Testosterone/Epitestosterone
9	303.19550	Positive	3.70	4.70	45 %		Formestane
10	311.24820	Positive	7.10	8.10	90 %		Stanozolol
11	313.21620	Positive	7.44	8.44	50 %		THG
12	319.22680	Positive	6.70	8.70	50 %		Oxymesterone
13	323.17720	Positive	7.00	8.00	40 %		Clostebol
14	292.22630	Positive	6.40	7.40	40 %		Testosterone_3C13
15	337.21740	Positive	5.90	6.90	50 %		Fluoxymesterone
16	345.25360	Positive	5.80	6.80	80 %		3-Hydroxystanozolol
17	353.21230	Positive	4.20	5.20	45 %		6b-Hydroxyfluoxymesterone
18							

Data Processing

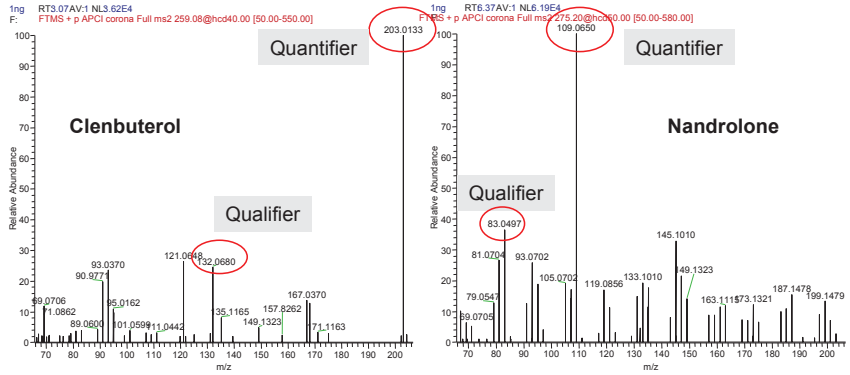
Two most abundant fragments (Table 1) in MS2 spectra (Figure 2) were selected for quantification and confirmation. Ion ratio was calculated and EU guidelines[1] for maximum permitted tolerance were applied.

Table 1. List of analytes, m/z values for parent ion and fragments in MS2 spectrum

Analyte	Formula	m/z	m/z in MS source	Ret Time (min)	Fragment 1	Fragment 2
Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	277.0869	259.0763	3.2	203.0129	132.0679
19-Norandrosterone	$C_{18}H_{28}O_2$	277.2162	259.2056	7.7	241.1942	145.1007
Nandrolone	$C_{18}H_{26}O_2$	275.2006	275.2006	6.5	109.0647	83.0494
Methandrosterone	$C_{20}H_{28}O_2$	301.2161	283.2056	6.6	173.0956	147.0800
6β-Hydroxyboldenone	$C_{29}H_{26}O_3$	303.1955	285.1849	4.3	121.0645	147.0798
Boldenone	$C_{19}H_{26}O_2$	287.2006	287.2006	6.2	121.0648	135.1166
DHEA	$C_{19}H_{28}O_2$	289.2162	287.2006	7.2	97.0653	109.0651
Oxandrolone	$C_{19}H_{30}O_3$	307.2268	289.2162	6.4	135.1165	121.1012
Testosterone	$C_{19}H_{28}O_2$	289.2162	289.2162	6.9	97.0651	109.0650
Epitestosterone	$C_{19}H_{28}O_2$	289.2162	289.2162	7.4	97.0651	109.0650
Formestane	$C_{19}H_{26}O_3$	303.1955	303.1955	4.2	121.0649	171.0802
Stanozolol	$C_{21}H_{32}N_2O$	329.2587	311.2482	7.6	81.0542	107.0857
THG	$C_{21}H_{28}O_2$	313.2162	313.2162	7.9	241.1576	159.0798
Oxymesterone	$C_{20}H_{30}O_3$	319.2268	319.2268	7.2	113.0595	125.0593
Clostebol	$C_{19}H_{27}ClO_2$	323.1772	323.1772	7.5	143.0254	131.0254
Fluoxymesterone	$C_{20}H_{29}FO_3$	337.2173	337.2173	6.4	241.1576	131.0851
3-Hydroxystanozolol	$C_{21}H_{32}N_2O_2$	345.2536	345.2536	6.3	97.0400	107.0855
6β-Hydroxyfluoxymesterone	$C_{20}H_{29}FO_4$	353.2122	353.2123	4.7	95.0857	239.1419
Testosterone-$^{13}C_3$	$C_{16}{}^{13}C_3H_{28}O_2$	292.2263	292.2263	6.9	100.0753	112.0751

Results

FIGURE 2. MS2 spectra for selected analytes collected for 1 ng/mL calibration standard



Linearity Range, LOQ, LOD

Figure 3. Chromatographic peaks reconstructed with m/z accuracy of 5 ppm at LOQ of 1 ng/mL (*3 ng/mL, **6 ng/mL)

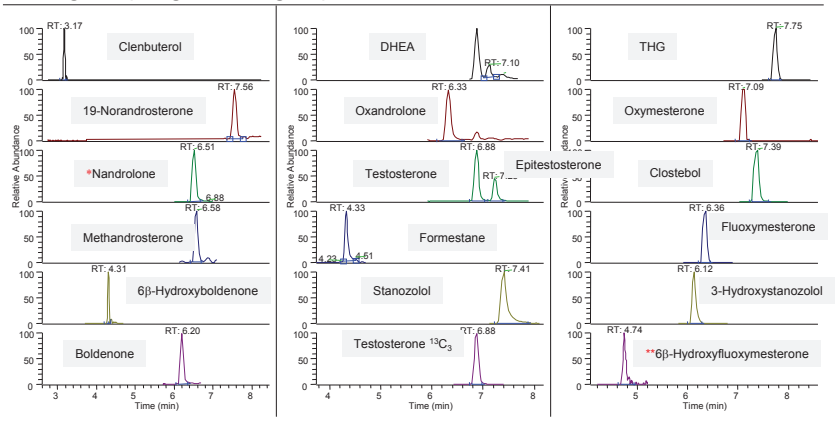
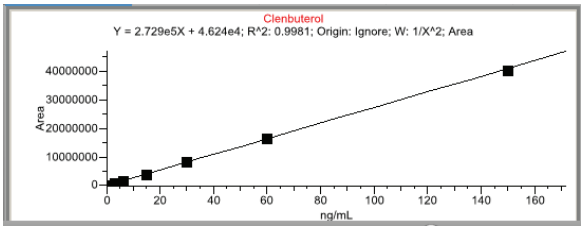
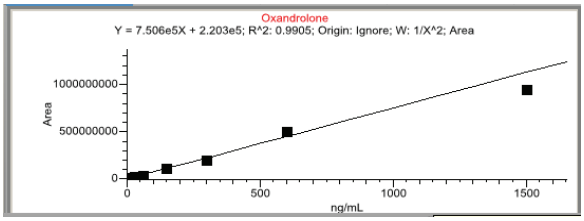


Figure 4. Calibration curves for selected analytes



Theo Amt	Calc Amt	Excluded	% Diff
1.000	0.986		-1.40
3.000	3.119		3.97
6.000	6.187		3.12
15.000	13.899		-7.34
30.000	30.735		2.45
60.000	60.561		0.94
150.000	147.492		-1.67



Theo Amt	Calc Amt	Excluded	% Diff
1.000	0.974		-2.60
3.000	3.058		1.93
6.000	6.557		9.28
15.000	15.531		3.54
30.000	32.688		8.96
60.000	57.033		-4.95
150.000	147.968		-1.35
300.000	267.250		-10.92
600.000	672.905		12.15
1500.000	1259.275		-16.05

Table 2. Linearity ranges, LOQ, LOD

Analyte	Linearity range	R^2	LOQ	LOD
Clenbuterol	1-150 ng/mL	0.9981	1 ng/mL	<1 ng/mL
19-Norandrosterone	1-1500 ng/mL	0.9937	1 ng/mL	< 1 ng/mL
Nandrolone	3-150 ng/mL	0.9926	3 ng/mL	<1 ng/mL
Methandrosterone	1-600 ng/mL	0.9931	1 ng/mL	<1 ng/mL
6β-Hydroxyboldenone	1-600 ng/mL	0.9852	1 ng/mL	<1 ng/mL
Boldenone	1-600 ng/mL	0.9939	1 ng/mL	<1 ng/mL
DHEA	1-600 ng/mL	0.9898	1 ng/mL	<1 ng/mL
Oxandrolone	1-1500 ng/mL	0.9905	1 ng/mL	<1 ng/mL
Testosterone	1-300 ng/mL	0.9896	1 ng/mL	<1 ng/mL
Epitestosterone	1-600 ng/mL	0.9889	1 ng/mL	<1 ng/mL
Formestane	1-600 ng/mL	0.9882	1 ng/mL	<1 ng/mL
Stanozolol	1-300 ng/mL	0.9911	1 ng/mL	<1 ng/mL
THG	1-600 ng/mL	0.9914	1 ng/mL	<1 ng/mL
Oxymesterone	1-300 ng/mL	0.9923	1 ng/mL	<1ng/mL
Clostebol	1-150 ng/mL	0.9961	1 ng/mL	<1 ng/mL
Fluoxymesterone	1-300 ng/mL	0.9916	1 ng/mL	<1 ng/mL
3-Hydroxystanozolol	1-60 ng/mL	0.9952	1 ng/mL	<1 ng/mL
6β-Hydroxyfluoxymesterone	6-150 ng/mL	0.9838	6 ng/mL	3 ng/mL

Method Precision
QC samples with concentrations across calibration range (2 ng/mL, 15 ng/mL, 90 ng/mL, 450 ng/mL) were prepared in blank oral fluid. QC samples were analyzed in 5 replicates in 3 separate batches to obtain intra- and inter- assay precision (Table 3).

Table 3. Intra-assay and inter-assay results

Analyte	Intra assay				Inter assay			
	2 ng/mL	15 ng/mL	90 ng/mL	450 ng/mL	2 ng/mL	15 ng/mL	90 ng/mL	450 ng/mL
Clenbuterol	<10.5	<3.3	<6.2	<15.1	12.6	8.4	5.8	11.0
19-Norandrosterone	<12.4	<11.6	<12.5	17.9	16.3	9.4	12.0	14.1
Nandrolone	NA	<14.2	<12.3	<13.0	NA	12.7	10.0	10.4
Methandrosterone	<13.1	<11.9	<13.9	<18.3	11.5	12.7	13.9	17.3
6β-Hydroxyboldenone	<7.9	<13.3	<11.5	<20.0	14.5	10.8	9.5	13.6
Boldenone	<15.1	<9.4	<11.1	<12.9	12.6	11.3	16.1	18.2
DHEA	<16.6	<13.4	<10.5	<9.7	14.2	10.2	10.2	8.9
Oxandrolone	<11.0	<14.4	<12.9	<19.9	10.6	10.4	10.2	13.7
Testosterone	<14.6	<9.0	<11.9	<19.1	11.5	7.6	9.5	16.7
Epitestosterone	<16.4	<14.4	<10.8	<13.2	14.3	9.8	7.7	8.3
Formestane	<10.4	<10.6	<10.0	<18.1	18.7	13.5	14.3	19.9
Stanozolol	<20.9	<10.9	<10.5	<15.2	19.9	10.9	8.2	13.1
THG	<19.5	<10.1	<11.0	<16.9	16.3	11.1	7.5	13.4
Oxymesterone	<25.0	<12.3	<6.0	<15.0	24.5	9.0	4.9	12.6
Clostebol	<14.8	<12.4	<10.3	<12.8	14.1	11.0	6.6	9.7
Fluoxymesterone	<18.0	<9.6	<11.6	<19.2	24.0	9.0	7.5	14.0
3-Hydroxystanozolol	<15.1	<5.0	<5.3	<12.5	24.8	8.0	5.8	11.0
6β-Hydroxyfluoxymesterone	NA	<12.8	<6.5	<13.7	NA	9.1	9.4	14.2

Matrix Effect
Matrix effects (Table 4) were evaluated by spiking blank oral fluid with all analytes at concentrations of 2 ng/mL, 10 ng/mL, 100 ng/mL and analyzing these samples with calibration standards prepared in solvent.

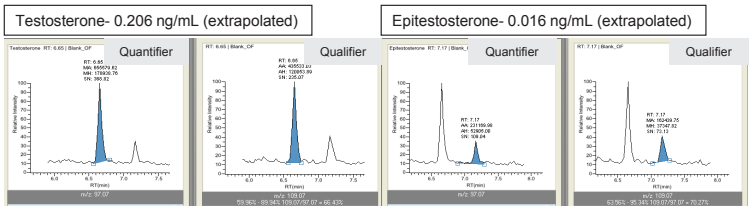
Table 4. Percent recovery in spiked blank oral fluid

Analyte	2 ng/mL	10 ng/mL	100 ng/mL
Clenbuterol	121	131	107
19-Norandrosterone	101	123	101
Nandrolone	ND	97.7	93.5
Methandrosterone	95.0	104	103
6β-Hydroxyboldenone	102	92.4	94.3
Boldenone	101	103	99.6
DHEA	100	127	115
Oxandrolone	93.5	124	109
Testosterone	90.5	105	96.8
Epitestosterone	78.5	99.8	102
Formestane	90.5	92.6	95.3
Stanozolol	80.0	81.5	92.8
THG	94.0	100	95.9
Oxymesterone	89.0	109	113
Clostebol	99.7	110	118
Fluoxymesterone	96.5	101	104
3-Hydroxystanozolol	93.5	92.0	105
6β-Hydroxufluoxymesterone	80.5*	102	104

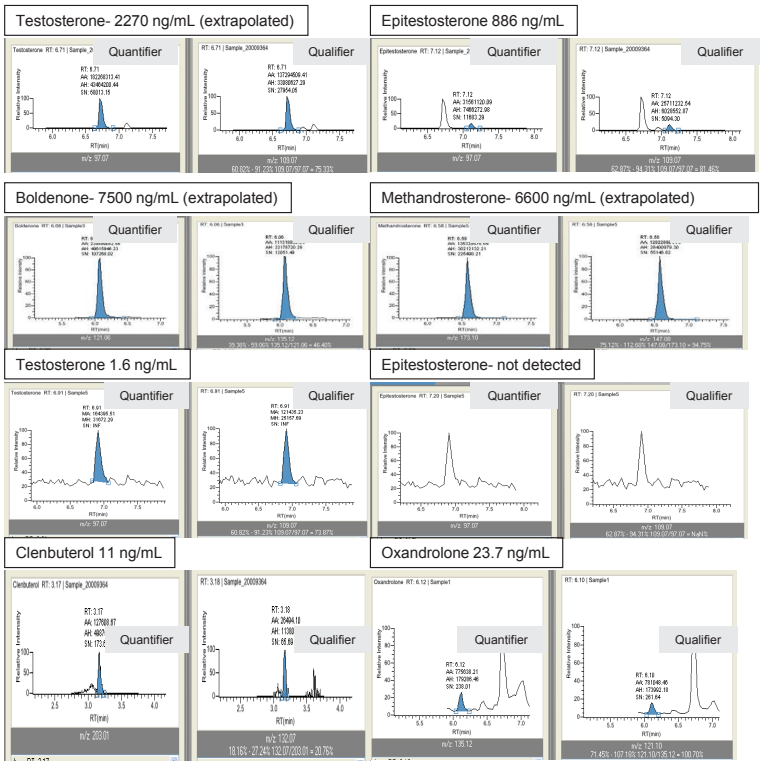
ND: not detected; *concentration (1.61 ng/mL) below LOQ

Donor Samples

Testosterone and Epitestosterone in negative tested oral fluid processed with LLE.



Compounds detected in selected positive tested samples prepared in collaborator lab with protein precipitation method.



Conclusion

We developed sensitive and robust quantitative screening method to analyze anabolic steroids in human oral fluid.

- Implementation of the ultra high resolution Q Exactive mass spectrometer to collect MS2 spectra and ion ratio confirmation results in high confidence in compound identification.
- Method was validated using LLE for sample preparation, but we also detected all analytes in positive tested samples processed with protein precipitation and provided by collaborator laboratory.

Acknowledgement

We would like to thank Erica Guice, Research Director, Western Slope Laboratory, for scientific advice and for providing samples for method testing.

References

1. Draft SANCO 1805/2000 Rev.1 [Revised Commission Decision 93/256 of 14 April 1993] laying down performance criteria for analytical methods to be used for certain substances and residues there of in live animals and animals products according to Council Directive 96/23/EC

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A Novel On-Line Sample Cleanup and Liquid Chromatography Platform for LC/MS Analysis in the Clinical Research Laboratory

Christopher Esposito, Joseph Di Bussolo and François Espourteille
Thermo Fisher Scientific, Franklin, MA, USA

Thermo SCIENTIFIC

Overview

Purpose: Describe a reliable and rugged sample preparation liquid chromatography system - Prelude SPLC™ - which utilizes novel pumps and fluidics configuration to multiplex two channels, for high-throughput LC-MS applications.

Methods: TurboFlow™ on-line extraction coupled to high efficiency HPLC utilizing core enhanced technology prior to tandem mass spectrometry were optimized for measuring immunosuppressant drugs, drugs of abuse and steroidal compounds.

Results: Typical throughput was 20 samples per hour while conserving consumables and minimizing user intervention. Quality-control (QC) sample results from three different Prelude SPLC systems operated at three different locations typically varied by less than ten percent coefficient of variation (%CV).

Introduction

Clinical research and forensic toxicology laboratories have a need for rapid and reproducible methods automated by systems that are easy-to-use and maintain. We describe a new system, which encompasses a novel HPLC pump design and fluidics configuration, enabling the user to perform on-line sample cleanup using TurboFlow technology and high-performance liquid chromatography (HPLC) on two channels multiplexed to a mass spectrometer (MS). Reproducibility, linearity, and other performance data are discussed. Several applications (immunosuppressant drugs (ISDs), pain management drugs (PMDs), 25-OH-vitamin D and various steroids in blood) have been satisfactorily tested. They displayed significantly reduced solvent consumption and shortened run times with reproducible results.

Methods

Sample Preparation & Liquid Chromatography

A Prelude SPLC system (Thermo Scientific) processed 20 uL injections of supernatants from protein-precipitated samples using a Cyclone-P™ TurboFlow column, transferred extracted analytes to an Accucore™ PFP HPLC column (2.1 x 50 mm) in which the analytes were separated, and then eluted to the MS system.

Mass Spectrometry

A TSQ Vantage™ tandem mass spectrometer (MS/MS) with heated electrospray ion (HESI-II) source (Thermo Scientific) was used for selective reaction monitoring (SRM) of analytes.

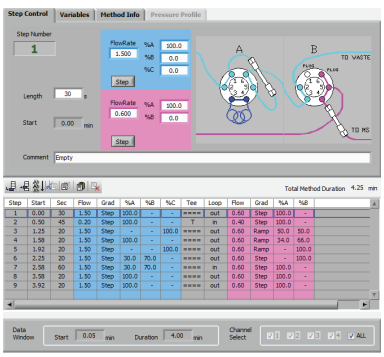
System Control & Data Analysis

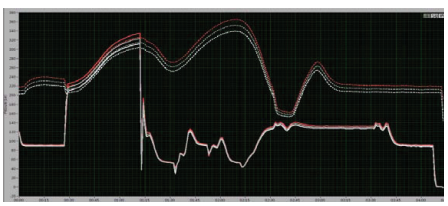
TraceFinder™ 2.1 software was used to control the SPLC-MS system and to collect and process the MS/MS data.

System Suitability

A rigorous LC-MS/MS testing protocol was designed to determine inter- and intra-system precision and ruggedness of the system (Figure 1). Using a test mix of four compounds - Atenolol, Warfarin, Lidocaine and Imipramine, in both aqueous and plasma matrices, both channels on multiple Prelude SPLC systems were tested. %RSD values were generated for peak areas as well as retention times across channels and across systems.

FIGURE 1. SPLC System Suitability Method and Representative Pressure Trace.





Solvents:

A: Water + 10 mM ammonium formate + 0.05% formic acid

B: Methanol + 10 mM ammonium formate + 0.05% formic acid

C: 45% acetonitrile + 45% isopropanol + 10% acetone

Results

Whole-System Testing Verified Performance

To simulate a typical bio-analytical application, plasma spiked with our test mix was mixed with a 3-fold volume of acetonitrile and centrifuged. To test the reproducibility and ruggedness of the SPLC-MS/MS system, we ran a batch of 500 injections of the supernatant, which had a duration of 34 hours. The peak retention times and areas for each compound were reproducible as illustrated in Figure 2. Without the benefit of smoothing or internal-standard compensation, peak area RSDs were below 9% (Figure 3).

FIGURE 2. 500 Matrix Injections - *over 34 hrs of run time!*

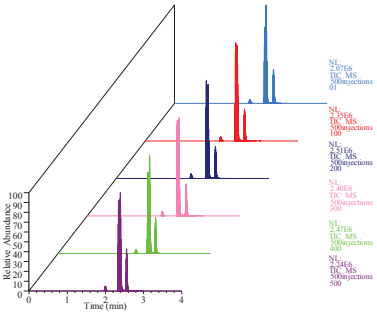
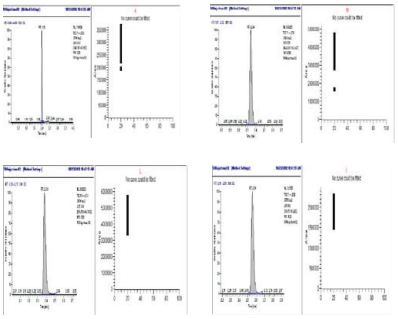


FIGURE 3. Peak Area %RSDs



Pressure and Retention Times were Reproducible

While the reproducibility of raw area counts speaks to the removal of matrix effect and its impact on data, the burden of 500 matrix injections and its impact on the aging of the SPLC system and its columns can be significant. For that reason retention time drift, pressure trace drift and peak shape changes were evaluated for the same data set. As shown by Figures 4, 5 & 6, retention times, pressure traces and peak shapes were remarkably stable throughout the 500-injection 34-hour batch.

FIGURE 4. Retention Time Drift for four compounds over 34 hrs of run time

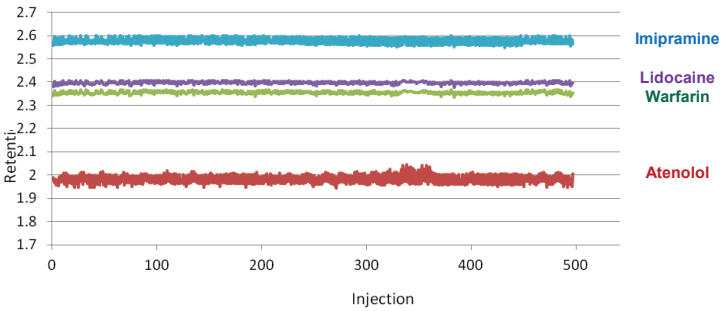
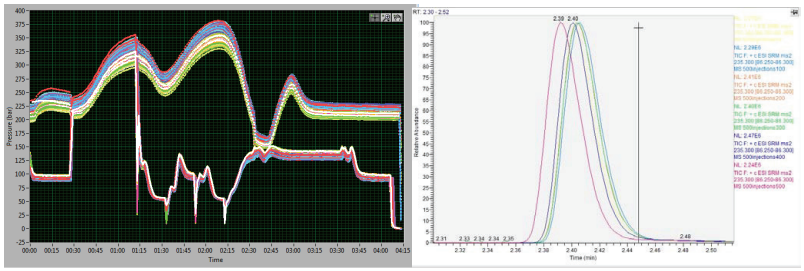


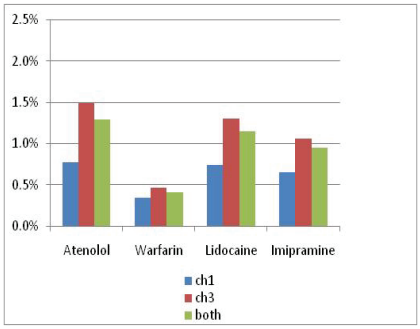
FIGURE 5. Pressure Trace Overlay across 34 hrs & Peak Overlay at injections 1,100, 200, 3 and 500



Inter-System Testing was Acceptable

While performance and ruggedness of any single LC-MS system is essential, inter-system performance is equally important. The typical workflow from Development to Production of a new method relies on inter-system ruggedness and reproducibility. For this reason, data from three prototype SPLC systems were gathered over the course of 5 months of testing and retention time performance across the three systems were analyzed. Reproducibility of retention times for each of the four test compounds generated from both channels of the three systems is summarized in Figure 7. The percent coefficient of variation (%CV) values were calculated from A random selection of 9 data points for each compound.

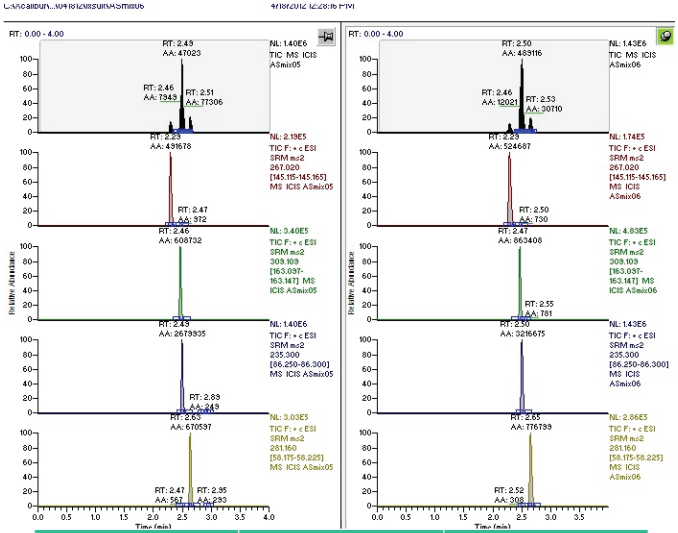
FIGURE 7. Retention Time %CVs from 3 different prototype systems.



A Purpose-Built Method clearly showed System Performance

Knowing that a rigorous LC-MS method with stringent data criteria would be the best test of the Prelude SPLC system, a method that tests for common problems with chromatographic separations was devised. The Suitability test has four compounds the first, Atenolol, the earliest eluter, is used to help elucidate problems that might exist with the refocusing of analytes on the analytical column. Retention time and peak shape differences in Warfarin and Lidocaine peaks will help detect any problems that may exist with gradient formation and/or column deterioration, as their RT shifts with compositional mobile phase differences. Imipramine is highly susceptible to degradation in peak shape if the mobile phases are not fresh or made precisely as prescribed by the method. In concert, the test mix serves as powerful diagnostic tool. The installation protocol for Prelude SPLC systems requires all four compounds to pass 20 injections (10 per channel) with RSD or CV of 10% or less with no internal standard correction for retention times and peak areas. Figure 8 shows typical performance.

FIGURE 8. System Suitability run of both channels of the Prelude SPLC System

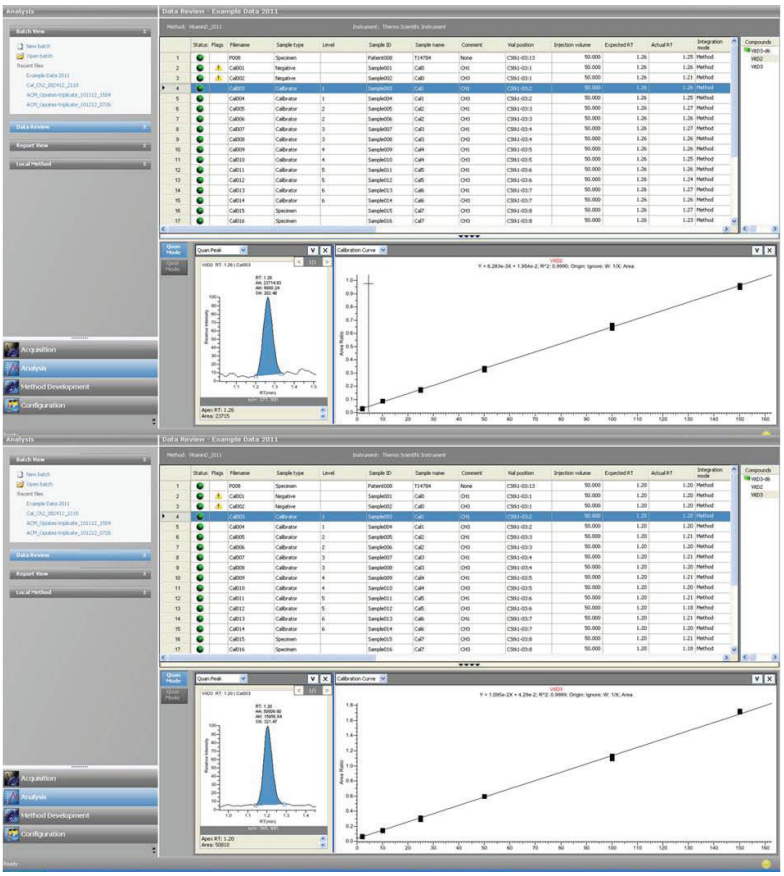


Compound	Area Count RSD%	RT CV%
Atenolol	1.98%	0.31%
Warfarin	3.64%	0.18%
Lidocaine	4.69%	0.15%
Imipramine	3.81%	0.10%

System was Suitable for well-known Clinical Research Methods

In order to asses the scope of applications for the Prelude SPLC system, popular LC-MS methods used in clinical research were considered. Methods for steroids, pain management drugs, immunosuppressant drugs and 25-OH-Vitamin D2 and D3, were developed and evaluated. We monitored linearity within the experimental range, inter- and intra-day reproducibility, long-term system stability, solvent consumption as compared to other platforms, and other relevant parameters. Please see other posters for more details on some of these methods. Figure 9 shows typical quantitative results for the Vitamin D compounds - excellent reproducibility for peak areas and retention times while achieving the desired sensitivity and linearity.

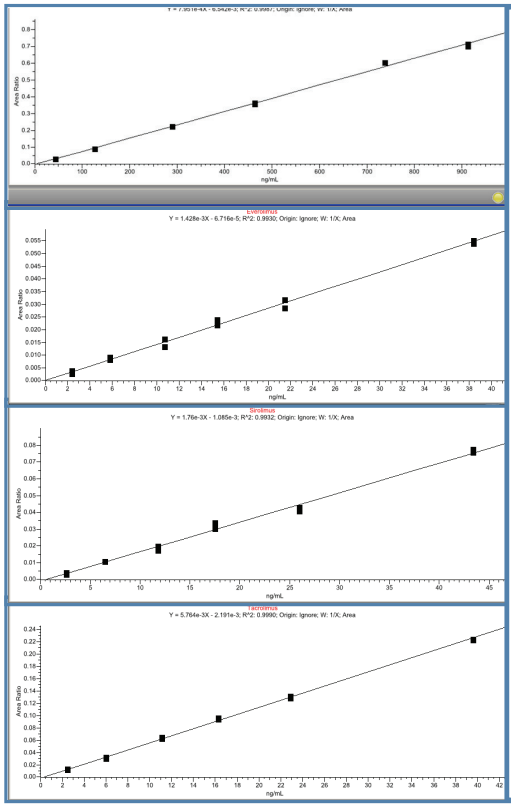
FIGURE 9. Quantitative Results – 25-OH-Vitamin D2 and D3



Even for Immunosuppressant Drug applications

Measuring Everolimus, Sirolimus, Tacrolimus and Cyclosporin A in whole-blood samples presents many challenges, from sample preparation to detection of each analyte and internal standard by the MS/MS system. To evaluate the Prelude SPLC system's ability to handle such an application, ChromSystems® multilevel calibrators and MassCheck® whole blood controls were processed using D_{12}-Cyclosporin A (Alsachim, Illkirch-Graffenstaden, France) as the internal standard (IS) for Cyclosporin A and Tacrolimus-$^{13}CD_2$ (Toronto Research Chemicals, Canada) as the IS for Everolimus, Sirolimus and Tacrolimus. Typical RSDs of peak areas for the two IS compounds were less than 12%.Typical quantitative results, collected over a span of 30 days from three systems in different locations - Cleveland, Baltimore and Boston, are shown in Figure 9.

FIGURE 10. Quantitative Results Immunosuppressant Drugs



Cyclosporin A QCs

Level	Expected	Average	RSD
I	53	53	4.6%
II	276	260	3.5
III	514	515	2.1
IV	1111	1172	6.4

Everolimus QCs

Level	Expected	Average	RSD
I	2.3	2.3	11.7%
II	4.4	4.4	11.0
III	8.5	8.8	8.4
IV	28.8	28.6	6.1

Sirolimus QCs

Level	Expected	Average	RSD
I	2.9	2.9	8.5%
II	10.1	10.0	4.6
III	20.4	20.6	5.2
IV	38.5	38.6	6.2

Tacrolimus QCs

Level	Expected	Average	RSD
I	2.6	2.8	5.3%
II	7.3	7.1	6.1
III	16.7	16.4	4.1
IV	34.2	33.8	4.1

n=15 from 3 systems within 30 days

Conclusion

Far too often LC/MS methods and instruments fall short of the rigorous performance criteria Clinical Research Labs require for everyday testing. The complex nature of the samples being injected on the system and the number of samples which need to be processed tax the instrumentation and columns. The system suitability method we developed proved a valuable whole-system testing procedure and demonstrated consistent performance of the Prelude SPLC systems in three different locations. This purpose-designed testing facilitates the implementation of rigorous evaluation standards for LC-MS systems used for clinical research. Availability of a standard system suitability test allows vendors and scientists to verify LC/MS system performance under controlled conditions which are similar to actual operating circumstances and has proven to be a valuable tool which is utilized from manufacture to installation of Prelude SPLC Systems. System performance was also verified by calibration and QC results for ISDs that matched expected values under typical operating conditions at two different clinical research facilities.

Acknowledgements

The authors thank the following who hosted our testing program at their laboratories:

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Dr. Mark Kellogg & Dr Roy Peake of Boston Children's Hospital and

Dr. Sihe Wang & Jessica Gabler of the Cleveland Clinic.

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Quantitation of Immunosuppressant Drugs in Blood Using a Second-Generation High-Resolution, Accurate-Mass Mass Spectrometer

Kristine Van Natta and Marta Kozak
Thermo Fisher Scientific, San Jose, CA

Application Note 577

Key Words
Immunosuppressant, tacrolimus, sirolimus, everolimus, cyclosporine A, Exactive Plus, TraceFinder, ClinSpec, clinical research, FK-506

Goal
Evaluate the combined use of a second-generation Thermo Scientific™ Orbitrap™-based high-resolution mass spectrometer and an immunosuppressants test kit for clinical research analysis of immunosuppressant drugs in whole blood. Apply the method to analysis of samples previously analyzed using a validated method on a triple-stage quadrupole mass spectrometer and compare the results.

Introduction
The Thermo Scientific™ ClinSpec™ Immunosuppressant Test kit was developed for clinical research liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis of tacrolimus, sirolimus, everolimus, and cyclosporine A in whole blood specimens. The kit consists of six different calibrator levels, up to five quality control levels, internal standard and extraction reagent. Here, the kit is used with a second-generation high-resolution, accurate-mass (HR/AM) mass spectrometer to analyze for these compounds. The results are compared to results previously obtained using a validated method and a triple-stage quadrupole mass spectrometer.

Experimental

Sample Preparation
Samples were prepared per the package insert in the ClinSpec kit.[1] Briefly, whole blood samples were processed by precipitation with $ZnSO_4$/methanol solution containing internal standards ascomycin and cyclosporine D. The samples were shaken for 30 minutes at room temperature before being centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to an autosampler vial, capped, and 50 µL was injected onto the HPLC system.

Liquid Chromatography
Chromatographic separation was performed using a Thermo Scientific™ Accela™ 600 HPLC pump and Thermo Scientific™ Hypersil GOLD™ Javelin™ guard column, (10 x 2.1 mm, 5 µm particle size), maintained at 80 °C. Mobile phases A and B consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol, respectively. Mobile phase C was acetonitrile/1-propanol/acetone (45:45:10). The total run time was 2 minutes.

Mass Spectrometry
Samples were analyzed with a Thermo Scientific™ Exactive™ Plus high-performance benchtop mass spectrometer equipped with an Orbitrap mass analyzer. An atmospheric pressure chemical ionization (APCI) probe was used as an ion source. The instrument was operating in positive full-scan mode at a resolution of 70,000 (FWHM) at *m/z* 200. Relevant scan and source parameters are shown in Tables 1 and 2.

Table 1. Scan parameters for Exactive Plus mass spectrometer

Scan Parameter	Value
Mass range:	*m/z* 800–4000
Resolution:	70,000
Polarity:	Positive
Microscans:	1
Lock mass:	Off
AGC target:	1×10^6
Max inject time:	200 msec

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Table 2. Source parameters for APCI probe

Source Parameter	Value
Sheath gas:	15
Aux gas:	17
Sweep gas:	1
Discharge Current:	4.6 kV
Capillary temperature:	275 °C
S-Lens voltage:	75 V
Vaporizer temperature:	300 °C

Validation

Validation consisted of analyzing replicates of quality controls along with a calibration curve on multiple days. We also analyzed donor samples previously analyzed using a validated method on a Thermo Scientific™ TSQ Access MAX™ triple-stage quadrupole mass spectrometer and compared the results.

Data Analysis

Data was acquired and processed using Thermo Scientific™ TraceFinder™ software. Ascomycin was used as internal standard for tacrolimus, sirolimus, and everolimus. Cyclosporine D was used as internal standard for cyclosporine A. All of the compounds form ammoniated adducts (Table 3). Extracted ion chromatograms (XIC) for individual compounds were reconstructed from the full-scan data with a mass tolerance of 5 ppm. Figure 1 shows representative chromatograms for analytes at their respective LOQs and internal standards.

Table 3. Exact masses of the ammoniated adducts of the immunosuppressant drugs and internal standards

Compound	*m/z*	Compound	*m/z*
Ascomycin	809.5158	Everolimus	975.6152
Tacrolimus	821.5158	Cyclosporine A	1219.8752
Sirolimus	931.5890	Cyclosporine D	1233.8908

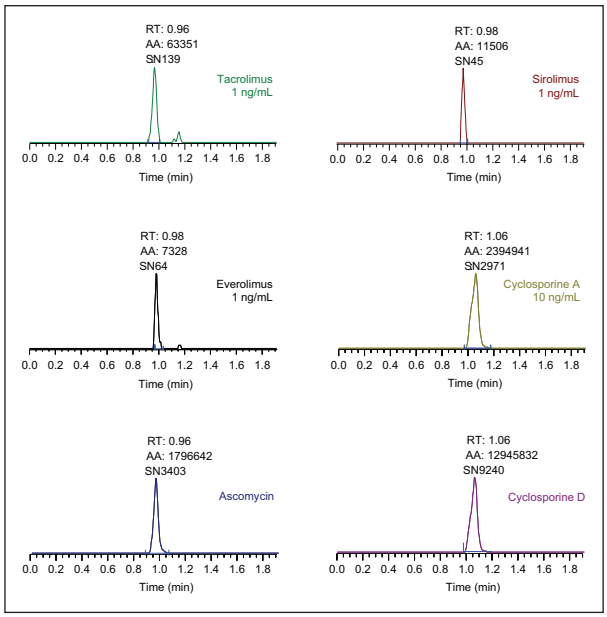

Figure 1. Extracted ion chromatograms with 5 ppm mass windows for analytes tacrolimus, sirolimus, everolimus (each at 1 ng/mL), and cyclosporine A (10 ng/mL), and internal standards ascomycin and cyclosporine D

Results and Discussion

Linearity

All compounds were linear within the test kit calibrator ranges of 1–30 ng/mL for tacrolimus, sirolimus, and everolimus; and 10–1500 ng/mL for cyclosporine A. Figure 2 shows representative calibration curves for all compounds. Standards back-calculated to within 6.3% for tacrolimus, 10.9% for sirolimus, 14.7% for everolimus, and 7.5% for cyclosporine A.

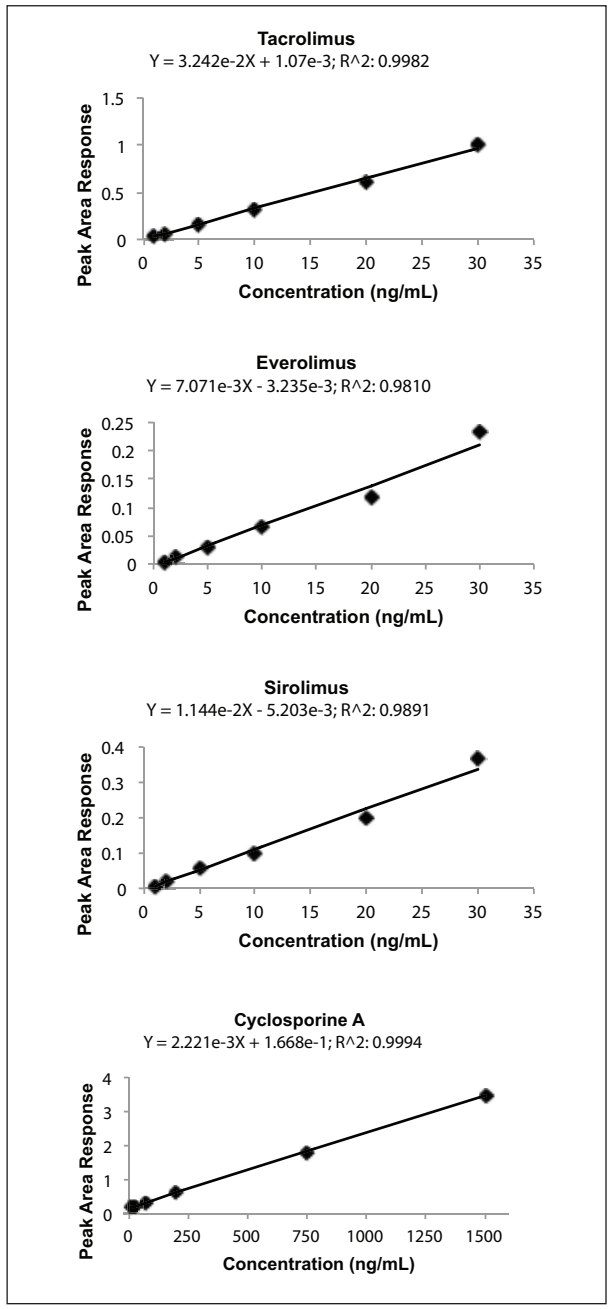

Figure 2. Representative calibration curves for immunosuppressant drugs

Quality Controls

Quality control samples analyzed in this study showed good recovery and reproducibility. Table 4 shows validation statistics for quality controls analyzed in this study. Imprecisions, as given by %CV, were also better than those given in the package insert for all compounds and levels tested except for those for everolimus, which still compared favorably to the test kit (data not shown).

Table 4. Mean %bias and %CV of quality controls

Analyte	Control 1 (3/30)*	Control 2 (12/125)*	Control 3 (25/375)*	Control 4 (0/700)*
Tacrolimus	-3.20/6.60	-4.60/2.22	-1.85/4.17	NA
Sirolimus	0.691/12.9	-10.1/7.56	-14.7/4.66	NA
Everolimus	-9.17/18.6	-10.9/9.49	-5.18/8.12	NA
Cyclosporine A	5.12/7.07	-3.20/4.94	1.71/3.98	8.10/3.78

**Concentration of (tacrolimus, sirolimus, everolimus)/cyclosporine A in ng/mL*

Method Comparison Samples

Donor samples previously analyzed with a validated method utilizing a triple-stage quadrupole mass spectrometer were reanalyzed on the Exactive Plus MS. A total of 114 samples containing tacrolimus, 34 containing sirolimus, and 32 containing cyclosporine A were analyzed. No donor sample values were available for everolimus. Figure 3 shows the correlation between the two methods. All slopes were greater than 0.9, indicating good agreement between the two methods. R-squared values were also greater than 0.99 for tacrolimus and cyclosporine A and greater than 0.94 for sirolimus.

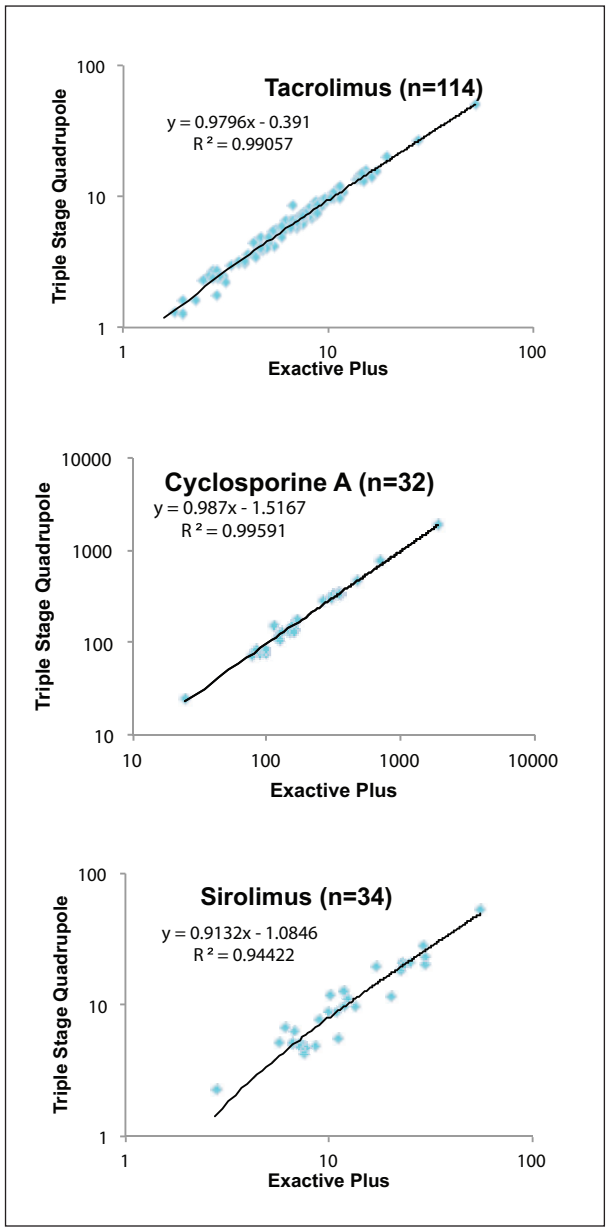

Figure 3. Correlation of results from Exactive Plus and triple-stage quadrupole instruments

Conclusion

- The method was easy to set up. No compound tuning was required.
- The method showed good linearity across the calibration ranges.
- Controls indicated good method precision and robustness.
- The Exactive Plus MS produced results comparable to a triple-stage quadrupole method, showing the suitability of Orbitrap technology for routine quantitation of whole blood samples by clinical research laboratories.

Acknowledgement

The authors would like to thank Dr. William Clarke of Johns Hopkins Medical Institute for his valuable insights and discussions.

Reference

1. Thermo Scientific ClinSpec Immunosuppressants Test product insert.

AN63667_E 01/13S

Application Note: 518

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Simultaneous Quantitative Analysis of Four Immunosuppressive Drugs Using High Resolution Accurate Mass LC-MS

Neil Leaver[1], Bevean Chiboho[1], Helen Welchman[2], Sarah Robinson[2]
[1]Royal Brompton & Harefield NHS Foundation Trust, Harefield Hospital, Harefield, UK;
[2]Thermo Fisher Scientific, Hemel Hempstead, UK

Key Words

- Exactive
- Accela U-HPLC
- Therapeutic Drug Monitoring
- Clinical Research

Introduction

Immunosuppressive drugs have been quantitatively analyzed by selected reaction monitoring (SRM) analysis using tandem mass spectrometry for over 10 years in the clinical research setting. High resolution accurate mass (HRAM) mass spectrometry offers the same quantitative performance characteristics with the added benefit of significantly faster method development. The HRAM method development time depends only on the sample preparation and chromatography conditions. In addition, mass analysis methods can be established rapidly because there is no requirement to tune SRM transitions, collision energies, or transfer lens voltages.

Goal

In this preliminary evaluation a set of calibrators, clinical samples, and QCs are investigated with the analysis of multiple replicates over the course of 7 days. The current in-house validated liquid chromatography – tandem mass spectrometry (LC-MS/MS) method data is directly compared against the use of HRAM LC-MS data.

Experimental Conditions

Sample Preparation

Commercial calibration standards in frozen stabilized whole blood were sourced from Chromsystems (München, Germany). Commercial quality control material in stabilized whole blood was sourced from More Diagnostics (Los Osos, CA, USA). All calibrators, QCs, and whole blood samples were extracted using a plate-based solid phase extraction (SPE) procedure.

HPLC

Chromatographic separation was accomplished using a Thermo Scientific Accela U-HPLC system. A Thermo Scientific AQUASIL C18 column (150 x 2.1 mm, 5 µm) heated to 50 °C, was used with an isocratic gradient of 90% MeCN + ammonium acetate (2 mM). For each sample, 20 µL was injected.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific Exactive high performance benchtop mass spectrometer powered by Orbitrap™ technology. Atmospheric pressure chemical ionization (APCI) was used to generate the $[M+NH_3]^+$ ions for tacrolimus, sirolimus, and everolimus, and the $[M+H]^+$ ions for cyclosporin, as well as two internal standards: ascomycin (for cyclosporin and tacrolimus) and desmethoxyrapamycin (for sirolimus and everolimus).

The Exactive™ mass spectrometer was set to scan at 50 K resolution over the range *m/z* 700 – 1300 and was calibrated once at the start of the 7-day analysis. Data acquisition and analysis were carried out with Thermo Scientific LCQUAN software.

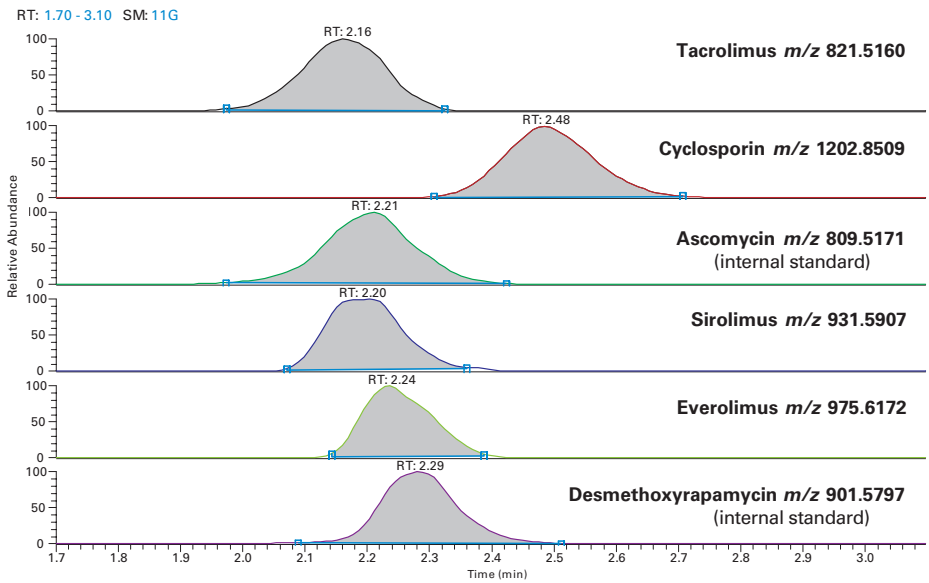

Figure 1. XIC of lowest calibration standard

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Results and Discussion

An accurate mass extracted ion chromatogram of the lowest calibration standard for each compound is presented in Figure 1. An example calibration line for each of the analytes is presented in Figure 2 A, B, C and D.

Inter-assay variability was determined by processing 30 replicates of each quality control over multiple batches. The precision data for inter-assay validation are presented in Table 1. The limit of quantitation (LOQ) has been set at 1 ng/mL for each analyte, and the highest CVs obtained at this concentration were 10.2%. The lower limit of quantitation (LLOQ) has not yet been fully investigated. Although cyclosporin, which also has the largest concentration range, achieved CVs of 12.5% at 0.3 ng/mL.

A total of 360 clinical research samples were analyzed by the HRAM method. The results were compared to the current LC-MS/MS method. Analysis of the clinical specimens by both HRAM LC-MS and LC-MS/MS demonstrate good correlation for cyclosporin, tacrolimus, and sirolimus across the required therapeutic range. No clinical research specimens were available for the method comparison of everolimus.

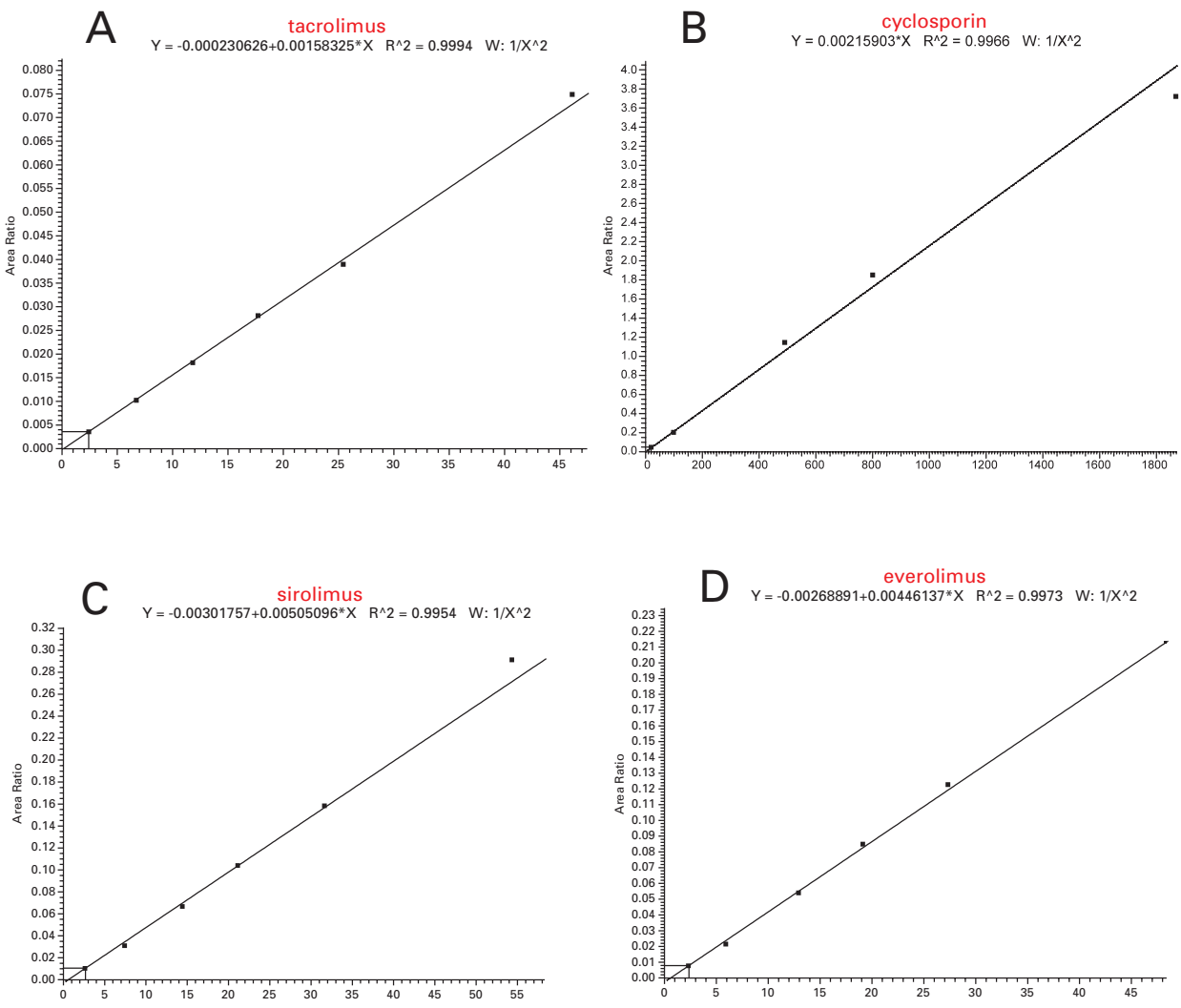

Figure 2. Calibration curves for (A) tacrolimus, (B) cyclosporin, (C) sirolimus, and (D) everolimus

Table 1. Method variability for each analyte

Analyte		Control 1	Control 2	Control 3	Control 4
Tacrolimus	Mean (ng/mL)	1.6	7.4	10.8	20.2
	%CV	12.8	4.2	4.2	2.9
Sirolimus	Mean (ng/mL)	3.3	14.4	25.3	41.6
	%CV	12.1	5.6	6.5	6.6
Everolimus	Mean (ng/mL)	2.9	13.9	24.2	41.8
	%CV	9.4	3.7	4.4	5.5
Cyclosporin	Mean (ng/mL)	83	176	362	787
	%CV	8.1	11.1	7.2	4.7

Conclusion

The HRAM analysis using the Exactive mass spectrometer demonstrates SRM comparable specificity, dynamic range, LOQ and precision in whole blood matrix. There is good correlation between SRM and HRAM results for the immunosuppressant drugs monitored.

The precision of HRAM LC-MS analysis meets current consensus guidelines and has acceptable performance to be used as a candidate clinical research method following further evaluation. All the method development time for this application was associated with the sample preparation and chromatography conditions. The mass analysis method was established in less than 5 minutes since there is no requirement to tune SRM transitions, collision energies or transfer lens voltages.

Acknowledgement

We would like to thank Dr. Mark Harrison for advice during the method set up.

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Validated LC-MS/MS Method for the Analysis of Immunosuppressant Drugs in Whole Blood Using the RECIPE ClinMass® Complete Kit

Samuele Scurati[1], Anke Trebstein[2], Lea Bonnington[2], Norbert Dirsch[2], Glenn Damkroeger[3], Miriam Drayss[3]; [1]Thermo Fisher Scientific, Rodano, Italy; [2]RECIPE Chemicals + Instruments GmbH, Munich, Germany; [3]Thermo Fisher Scientific, Dreieich, Germany

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Key Words

- TSQ Vantage
- Clinical Research
- Therapeutic Drugs

Introduction

Immunosuppressant drugs inhibit the immune system and are used in organ transplant patients to prevent organ rejection. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a widely accepted technique for the determination of immunosuppressant drugs in whole blood by clinical research laboratories. Tools providing reagents for sample extraction, calibrators, and QCs for analysis of these molecules are useful in facilitating analysis and increasing throughput.

Goal

To set up and validate an LC-MS/MS method for the analysis of Tacrolimus, Sirolimus, Everolimus, and Cyclosporin A in whole blood for clinical research laboratories by using the RECIPE ClinMass® Complete Kit with the Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer.

Experimental

This method has been developed using the RECIPE ClinMass® Complete Kit for the determination of immunosuppressants in whole blood according to the instruction manual.

Sample preparation

In a sample preparation vial, 200 µL of precipitation reagent, 20 µL of internal standard, and 100 µL of whole blood sample were combined. The sample was mixed for 30 seconds and incubated at ambient temperature for 5 minutes. The sample was mixed again for 10 seconds and centrifuged. Then, 50 µL of the supernatant was injected into the LC-MS/MS system.

HPLC

High performance liquid chromatography (HPLC) analysis was performed online by use of a 6-port, 3-channel, automatic switching valve and two Thermo Scientific Accela HPLC pumps working in isocratic mode. The sample was injected onto the solid phase extraction (SPE) column (with the switching valve in the "load" position), which extracted the analytes selectively from the sample matrix. The matrix components passed the SPE column widely unhindered and were eluted to waste. Meanwhile, the analytical column was re-equilibrated from the previous injection cycle. When the automatic switching valve switched to the "inject" position, the extracted analytes were eluted from the SPE column in backflush mode and transferred to the analytical column. After elution of the analytes, the automatic switching valve returned to the "load" position. Both columns (SPE and analytical) were re-equilibrated for the next injection. The effective run time was two minutes.

MS

Mass spectrometry analysis was performed using a TSQ Vantage™ triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization source (H-ESI II). The parameters are summarized in Table 1. MS analysis was performed in positive selected reaction monitoring (SRM) data acquisition mode. SRM parameters for all of the analytes and internal standards are shown in Table 2.

Table 1. Optimized ion source parameters

Ion Source	H-ESI II, positive
Resolution Q1 and Q3	0.7 amu
Spray Voltage	3500 V
Vaporizer Temp	300 °C
Sheath Gas Pressure	40
Ion Sweep Gas Pressure	2.0
Aux Gas Pressure	15
Capillary Temp	200 °C
Declustering Voltage	-2 V
Collision Pressure	1.5 mTorr

Table 2. SRM parameters used for the analysis

Compound	Precursor Ion	Product Ion	Scan Time [msec]	Collision Energy
Tacrolimus	821.6	768.4	50	18
Ascomycin	809.5	756.6	50	18
Sirolimus	931.7	864.6	75	15
Everolimus	975.7	908.8	75	16
d_4-Everolimus	979.7	912.6	75	16
Cyclosporin A	1220.0	1203.3	50	17
Cyclosporin D	1234.0	1217.0	50	17

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Results and Discussion

Figure 1 displays the representative lower limit of quantification (LLOQ) chromatograms for Tacrolimus, Sirolimus, Everolimus, Cyclosporin A, and the internal standards.

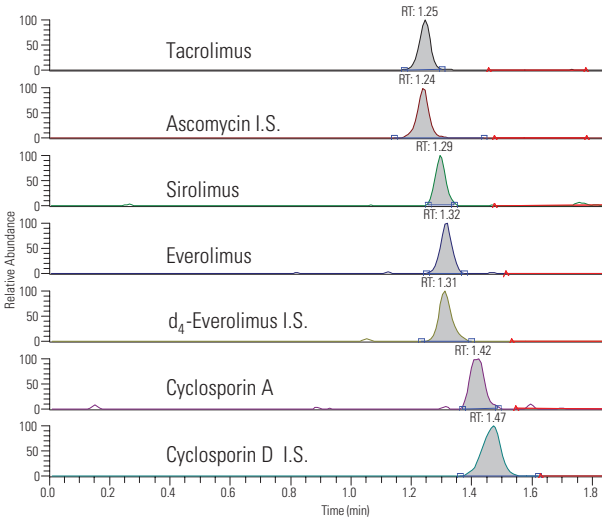

Figure 1: Chromatograms of the lowest calibration standard

In Table 3, the LLOQ and the linearity range for each analyte are reported and compared to the therapeutic range.

As shown in Tables 4 and 5, the intra- and inter-day variabilities were excellent as well as accurate. For each analyte, intra-day variability and accuracy were determined by performing two different extractions of each QC sample and analyzing them two times. Inter-day variability and accuracy were determined by repeating the intra-day procedure on three different days. Sample extractions were performed by different people.

Conclusion

A fast and reliable LC-MS/MS method for the quantification of Tacrolimus, Sirolimus, Everolimus, and Cyclosporin A in whole blood was validated using the RECIPE ClinMass® Complete Kit.

This method fulfills accuracy, precision, and dynamic range requirements of a routine method for clinical research.

Table 3. Summary of assay performance and therapeutic range

	Therapeutic Range [ng/mL]	LOQ [ng/mL]	Linearity Range [ng/mL]	I.S.
Tacrolimus	2 - 15	0.13	1.3 - 46.7	Ascomycin
Sirolimus	5 - 15	0.13	1.3 - 46.9	d_4-Everolimus
Everolimus	6 - 8	0.13	1.3 - 47.4	d_4-Everolimus
Cyclosporin A	100 - 350	24.90	24.90 - 1264.0	Cyclosporin D

Table 4. Intra-day variability (%RSD) and accuracy

	QC 1			QC 2			QC 3		
	Value	%RSD	%Accuracy	Value	%RSD	%Accuracy	Value	%RSD	%Accuracy
Tacrolimus	3.28	6.7	90.1	6.67	2.9	96.3	13.3	5.5	99.4
Sirolimus	3.64	2.7	81.7	11.20	3.8	93.6	18.9	5.2	101.8
Everolimus	3.34	7.2	90.1	10.60	7.1	97.4	18.2	7.2	101.5
Cyclosporin A	62.50	11.4	101.7	258.00	6.2	102.9	1341.0	2.8	94.6

Table 5. Inter-day variability (%RSD) and accuracy

	QC 1			QC 2			QC 3		
	Value	%RSD	%Accuracy	Value	%RSD	%Accuracy	Value	%RSD	%Accuracy
Tacrolimus	3.28	4.7	92.5	6.67	2.1	97.4	13.3	3.3	99.4
Sirolimus	3.64	8.4	89.6	11.20	4.6	95.7	18.9	5.1	102.8
Everolimus	3.34	7.6	96.7	10.60	5.1	96.5	18.2	4.7	100.9
Cyclosporin A	62.50	15.6	103.4	258.00	6.7	99.0	1341.0	12.0	102.9

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Quantitative Analysis of Mevalonate in Plasma Using LC-MS/MS

Flavio Giavarini[1], Omar Maschi[1], Samuele Scurati[2], Donatella Caruso[1]
[1]Department of Pharmacological Sciences, Università degli Studi di Milano, Milano, Italy;
[2]Thermo Fisher Scientific, Rodano, Italy

Key Words

- LTQ Ion Trap
- Clinical Research
- Cholesterol Synthesis

Introduction

Cholesterol is synthesized *in vivo* through a multiple step pathway. Because mevalonate is the key intermediate of this process, its plasmatic levels are an indirect measure of *in vivo* cholesterol synthesis and, therefore, facilitate clinical research into pharmacological activity of anti-hypercholesterolemic drugs such as statins.

Goal

To develop a reliable and fast analytical method for the quantitative determination of mevalonate in plasma using a Thermo Scientific LTQ linear ion trap mass spectrometer.

Experimental

Sample Preparation

The plasma sample (500 µL) was spiked with 20 ng of Mevalonate-D_7. Samples were acidified with hydrochloric acid allowing the conversion of mevalonate to mevalono-lactone (Figure 1). After purification through solid phase extraction (SPE), samples were dried and dissolved in 400 µL of 0.2% ammonium hydroxide to restore the non-lactonic form. Then 10 µL were injected.

Quantitative analysis was performed on the basis of calibration curves, ranging from 2.5 to 250 ng/mL.

HPLC Conditions

High performance liquid chromatography (HPLC) analysis was performed using a Thermo Scientific Surveyor autosampler and pump. The 10 µL sample was injected directly on a Thermo Scientific BioBasic AX column (150 × 2.1 mm, 5 µm). A gradient LC method used mobile phases A (10 mM ammonium formate, pH 8) and B (acetonitrile) at a flow rate of 200 µL/min.

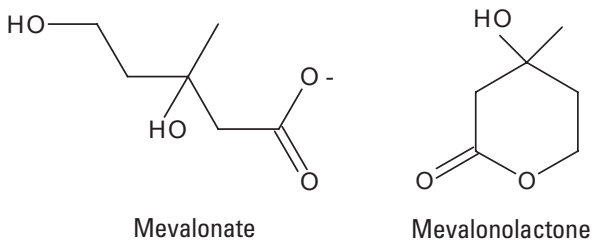

Figure 1. Structure of mevalonate and mevalonolactone

Mass Spectrometry

MS analysis was carried out on a LTQ™ linear ion trap mass spectrometer equipped with a Thermo Scientific Ion Max source with an electrospray ionization (ESI) probe.

Ion polarity:	Negative
Spray voltage:	2 kV
Sheath/Auxiliary gas:	Nitrogen
Sheath gas pressure:	40 (arbitrary units)
Auxiliary gas pressure:	10 (arbitrary units)
Sweep gas pressure:	5 (arbitrary units)
Ion transfer tube temperature:	300 °C
Scan type:	Full Scan MS/MS
Collision gas:	Helium
Collision energy:	30%
Divert valve:	3.0-6.5 min to source
Selected ions for quantification:	*m/z* 147→ 59 for mevalonate *m/z* 154→ 59 for mevalonate-D_7

Results and Discussion

Figure 2 shows the ion chromatograms of a lower sample of the calibration curve. Excellent linearity (r^2 = 0.999) fits for the calibration curve were observed over the range of 2.5 - 250 ng/mL plasma (Figures 3 and 4). The intraday CV% (n=3) was in the range 0.5% - 4%. The limit of detection (LOD) was 2 pg, and the limit of quantification (LOQ) was 2.5 ng/mL.

Figure 5 reports an ion chromatogram of a plasma sample of a healthy volunteer (24 ng/mL plasma), extracted and analyzed as described.

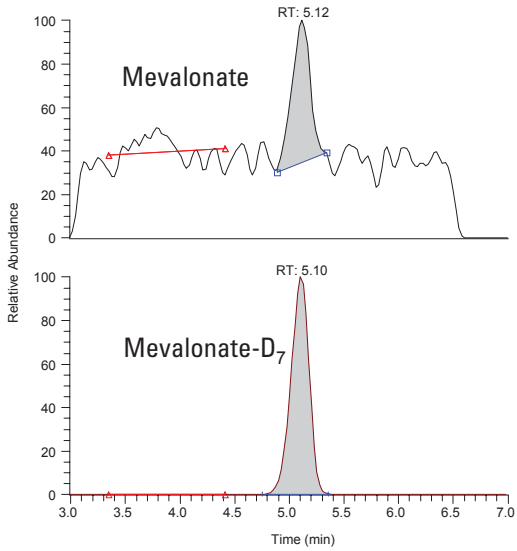

Figure 2. Ion chromatograms of 2.5 ng/mL calibration standard

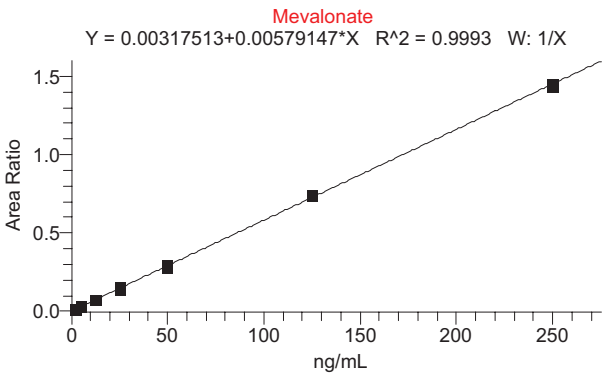

Figure 3. Calibration curve of mevalonate

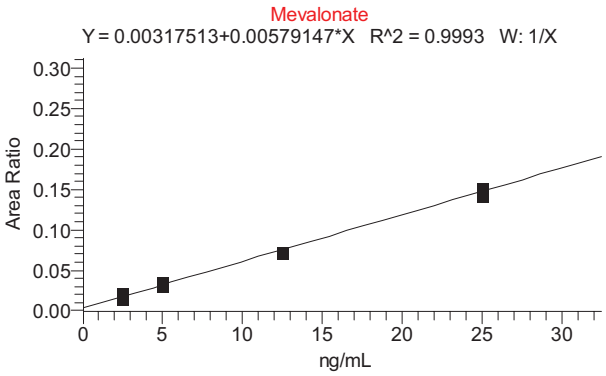

Figure 4. Zoom on low calibration points

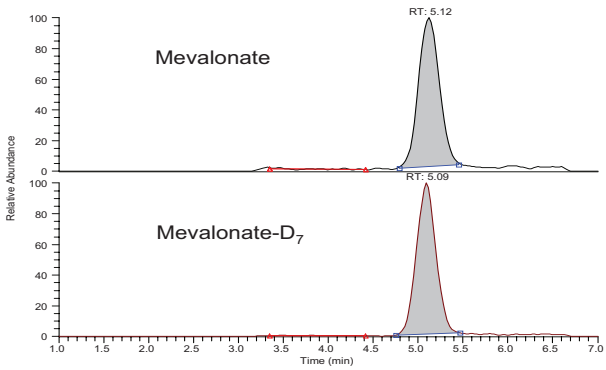

Figure 5. Ion chromatograms of plasma sample containing 24 ng/mL

Conclusion

A robust 10-minute method for the quantification of mevalonate with a dynamic range of 2.5 - 250 ng/mL plasma has been developed for clinical research using fast SPE purification and the LTQ linear ion trap mass spectrometer.

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Quantitative Analysis of Immunosuppressant Drugs in Whole Blood Using High Throughput LC-MS/MS

Marta Kozak[1], Raimund Ruzicka[2], Vani Bodepudi[2], Jeff Zonderman[3], [1]Thermo Fisher Scientific, San Jose, CA, USA, [2]Thermo Fisher Scientific, Fremont, CA, USA, [3]Thermo Fisher Scientific, Franklin, MA, USA

Key Words

- TSQ Quantum Ultra
- Transcend LX-2 System
- Clinical Research
- Multiplexing

Introduction

Immunosuppressant drugs inhibit the body's immune system and are used in organ transplant patients to prevent organ rejection. Liquid chromatography-mass spectrometry (LC-MS/MS) is a widely-accepted technique for the determination of immunosuppressant drugs in whole blood by research laboratories.

This application note describes a fast, sensitive, reliable, and accurate LC-MS/MS quantitative method for use by research laboratories for the simultaneous analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in whole blood.

Experimental Conditions

Sample Preparation

A protein precipitation solution was prepared by mixing MeOH containing internal standards (Ascomycin and Cyclosporin D) with $ZnSO_4$ solution. Blood samples were processed by adding precipitation solution. The mixture was vortexed and centrifuged. Supernatant was injected into the LC-MS/MS system.

HPLC

HPLC analysis was performed using Thermo Scientific Transcend LX-2 advanced multiplexing system. Samples were injected into a Thermo Scientific Javelin C18 guard column at 80 °C and analyzed with a 2-minute gradient method.

Mass Spectrometry

MS analysis was performed using a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source in selective reaction monitoring (SRM) data acquisition mode. Optimized SRM parameters for all of the analytes and internal standards are shown in Table 1.

Table 1: Optimized SRM parameters

Compound	Parent Ion	Fragment Ion	Collision Energy	Tube Lens Offset
Tacrolimus	821.4	768.3	18	190
Sirolimus	931.6	864.5	15	190
Everolimus	975.7	908.4	16	190
Ascomycin	809.4	756.4	18	190
Cyclosporin A	1219.9	1202.9	17	190
Cyclosporin D	1234.0	1216.9	17	190

Results and Discussion

Figure 1 displays the representative limits of quantitation (LOQ) chromatograms for tacrolimus, sirolimus, everolimus, cyclosporin A, and the internal standards. As shown in Tables 2 and 3, the intra- and inter-day variability were excellent. For each analyte, intra-day variability was determined by processing and analyzing 5 replicates of each QC sample. Inter-day variability was determined with 5 replicates of each QC sample in 3 different batches. The method tested negatively for all interferences and cross-reactivity. No ion suppression or enhancement was observed.

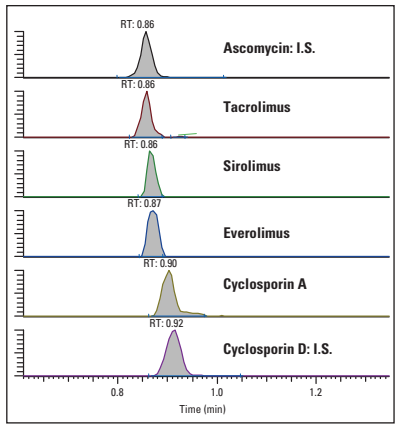

Figure 1: Chromatogram of lowest calibration standard

Assay performance summary

Target Analytes	Tacrolimus, Sirolimus, Everolimus and Cyclosporin A
Matrix	Whole blood
LOQ	1 ng/mL (Tacrolimus, Sirolimus, Everolimus) 10 ng/mL (Cyclosporin A)
Assay Linearity	1-50 ng/mL (Tacrolimus, Sirolimus, Everolimus) 10-2000 ng/mL (Cyclosporin A)
Analysis Time	2.0 min; 1.0 min with column multiplexing

Table 2: Intra-day variability (%RSD)

Analyte	QC1	QC2	QC3	QC4	QC5
Tacrolimus	6.8	4.6	4.9	-	-
Sirolimus	6.7	6.1	3.9	-	-
Everolimus	8.6	5.1	4.5	-	-
Cyclosporin A	5.5	4.6	3.4	3.4	3.5

Table 3: Inter-day variability (%RSD)

Analyte	QC1	QC2	QC3	QC4	QC5
Tacrolimus	4.2	4.1	1.7	-	-
Sirolimus	4.4	7.0	7.5	-	-
Everolimus	7.5	2.3	6.8	-	-
Cyclosporin A	1.8	2.0	2.4	1.7	4.7

Conclusion

A fast, sensitive, reliable and accurate method was developed for the quantification of tacrolimus, sirolimus, everolimus and cyclosporin A in whole blood by research laboratories. The use of column multiplexing technology allows for a 1 min analytical method, which enhances sample throughput.

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Research Analysis of Clozapine and Norclozapine in Plasma Using Automated Sample Preparation and LC-MS/MS

Phillip Morgan, Kings College Hospital NHS Foundation Trust, London, UK
Shane McDonnell, Sarah Robinson, Thermo Fisher Scientific, Hemel Hempstead, UK

Key Words

- TurboFlow Technology
- TSQ Quantum Ultra
- Clinical Research

Introduction

Clozapine (Figure 1) is a tricyclic dibenzodiazepine drug used in the treatment of schizophrenia. It is uniquely effective in patients resistant to therapy with other antipsychotics. In addition to mandatory hematological monitoring to minimize the risk of agranulocytosis, there are large variations (50-fold) among patients' clozapine dose requirements. Moreover, changes in smoking habits can have a large effect on the clozapine dose requirement (on average, the clozapine dose for non-smokers is half that required for smokers) due to the induction of cytochrome P450 (CYP) enzymes in smokers.[1] Studies have indicated that accurate quantification of clozapine levels may help researchers better understand, and conduct analysis of, issues related to dose optimization and adherence.[2]

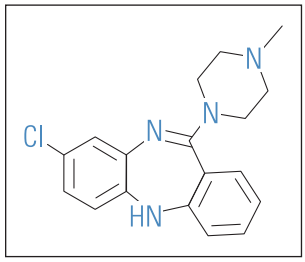

Figure 1: Structure of clozapine

Clozapine is metabolized via *N*-demethylation, *N*-oxidation, and aromatic hydroxylation, amongst other pathways. A few drugs, notably fluvoxamine, block all four CYP enzymes that can metabolise clozapine. Measurement of *N*-desmethylclozapine (norclozapine), which accumulates in plasma to concentrations similar to that of clozapine, can give useful information regarding adherence with medication, sample timing in relation to the last dose of clozapine and drug-drug interactions, such as that with fluvoxamine.

Current research methodology in our laboratory for clozapine and norclozapine involves off-line liquid-liquid extraction with manual transfer to a high pressure liquid chromatography-ultra violet (HPLC-UV) system. The Thermo Scientific Aria TLX-1 System powered by TurboFlow™ automated sample preparation technology is being investigated to simplify sample preparation, reduce the risk of operator error, improve sample throughput, and gain further selectivity by utilizing tandem mass spectrometry.

Goal

To assess Thermo Scientific TurboFlow automated sample preparation technology with tandem mass spectrometry for the research analysis of clozapine and norclozapine levels in plasma samples.

Experimental

Sample Preparation

Calibration standards (n=6) were prepared in the range 0.05 mg/L to 2 mg/L by addition of clozapine and norclozapine to newborn calf serum. Similarly, both analytes were added to drug-free human plasma to give internal quality control (IQC) solutions at 0.15, 0.40, and 1.20 mg/L. After centrifugation at 11,000 *g* for 2 min, 10 µL plasma was injected directly onto the Aria™ TLX-1 system.

The eluent gradients for both pumps are displayed in Table 1.

TurboFlow LC

Column:	TurboFlow Cyclone 50 x 0.5 mm
Mobile phase A:	0.05% (v/v) aqueous formic acid
Mobile phase B:	0.05% (v/v) formic acid in methanol
Mobile phase D:	45/45/10 Propan-2-ol/acetonitrile/acetone

Analytical LC

Column:	Thermo Scientific Hypersil GOLD C18 50 x 2.1 mm, 3 µm
Mobile phase A:	0.05% (v/v) aqueous formic acid
Mobile phase B:	0.05% (v/v) formic acid in methanol

			TurboFlow Method								Analytical			
Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	00:00	30	1.50	Step	100	-	-	-	====	out	0.50	Step	100	0
2	00:30	60	0.25	Step	100	-	-	-	T	in	0.25	Step	100	0
3	01:30	60	1.50	Step	-	-	-	100	====	in	0.50	Ramp	5	95
4	02:30	60	1.50	Step	70	30	-	-	====	in	0.50	Step	5	95
5	03:30	60	1.50	Step	100	-	-	-	====	out	0.50	Step	100	0

Table 1: Gradient programs for both TurboFlow and analytical methods (flow rate is mL/min)

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Mass Spectrometry

Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer	
Ion Source & Polarity:	APCI, positive ion mode
Discharge Current:	4.0 µA
Vaporizer Temperature:	325 °C
Sheath Gas:	60 units
Ion Sweep Gas:	0 units
Auxillary Gas:	10 units
Capillary Temperature:	275 °C
Collision Gas Pressure:	1.5 mTorr

The selective reaction monitoring (SRM) transitions used are presented in Table 2.

Analyte	Parent	Product	Scan Time	Collision Energy	Tube Lens
Clozapine	327.20	192	25 ms	60	47
		270	25 ms	21	47
Norclozapine	313.20	164	25 ms	67	113
		192	25 ms	41	113

Table 2: SRM transitions monitored in the experiment

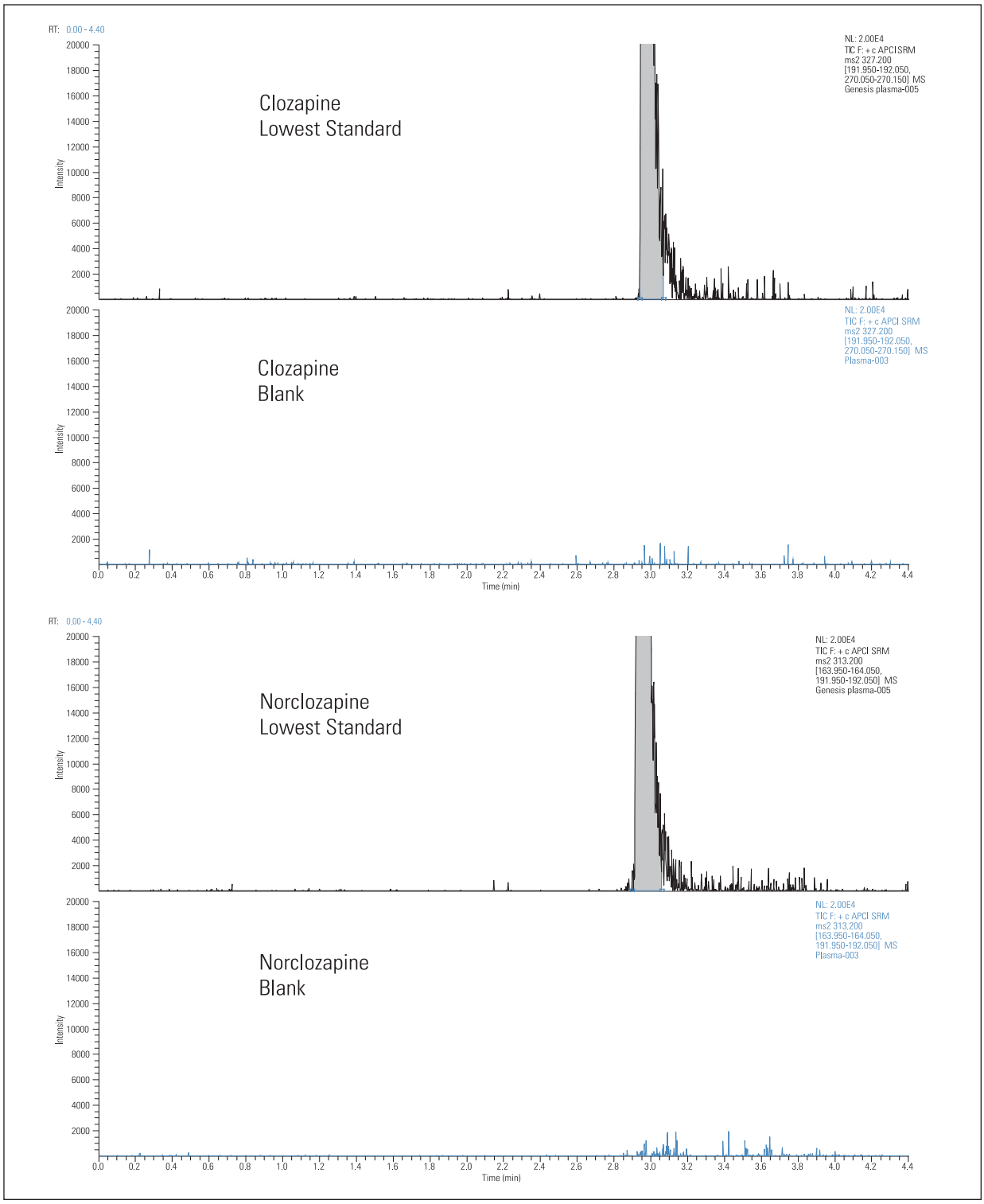

Figure 2: Extracted ion chromatogram of the plasma blank

Results and Discussion

Plasma was centrifuged prior to analysis. Calibration standards were analyzed from low to high concentration followed by IQCs. An injection of solvent after the highest concentration IQC was used for evaluation of carry-over. The volume of plasma injected was 10 µL, and all plasma analyses were in triplicate.

The extracted ion chromatograms of the plasma blank and lowest and highest concentration calibrators are presented in Figures 2, 3, and 4, respectively. The calibration curves for clozapine and norclozapine covered the range 0.05 - 2.00 mg/L (Figure 5 and 6). No internal standard was used, and thus, this work demonstrates the reproducibility of the system using external standard calibration.

Reproducibility and variance of the calibrators are shown in Figure 7.

Carry-over was calculated by comparing the response for clozapine and for norclozapine with that of a solvent blank injected immediately after a 1.2 mg/L IQC sample. This was shown to be ~0.1% for both clozapine and norclozapine. Additional clozapine metabolites were not investigated as part of this evaluation.

Conclusion

The research use of TurboFlow technology for automated sample preparation and tandem MS detection allowed the selective analysis of clozapine and norclozapine in plasma. The only sample preparation was the centrifugation of plasma. The sample volume required was one-tenth that used by the existing method – liquid-liquid extraction (LLE) followed by HPLC-UV – and provided lower limits of detection and quantitation. The calibration curves for all analytes were linear over the concentration range and carry-over was minimal. Use of the automated TurboFlow method has effectively eliminated two hours of sample preparation time for a 100-sample batch.

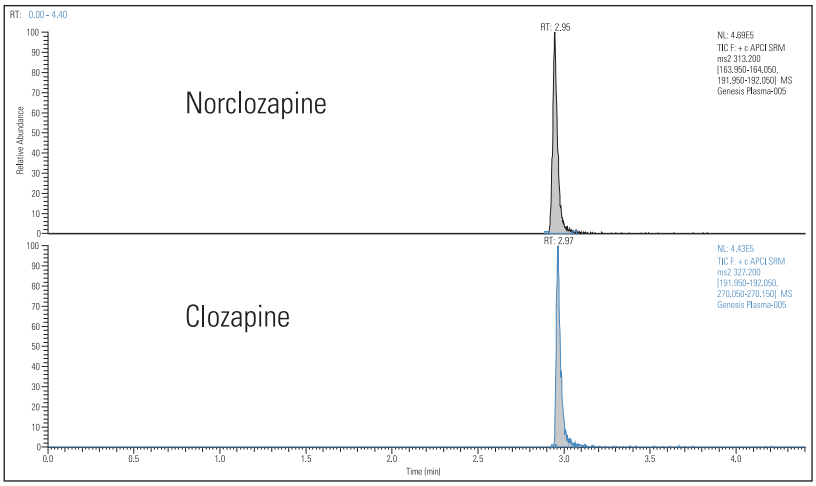

Figure 3: Clozapine and Norcloxapine lowest calibration from plasma, 0.05 mg/L

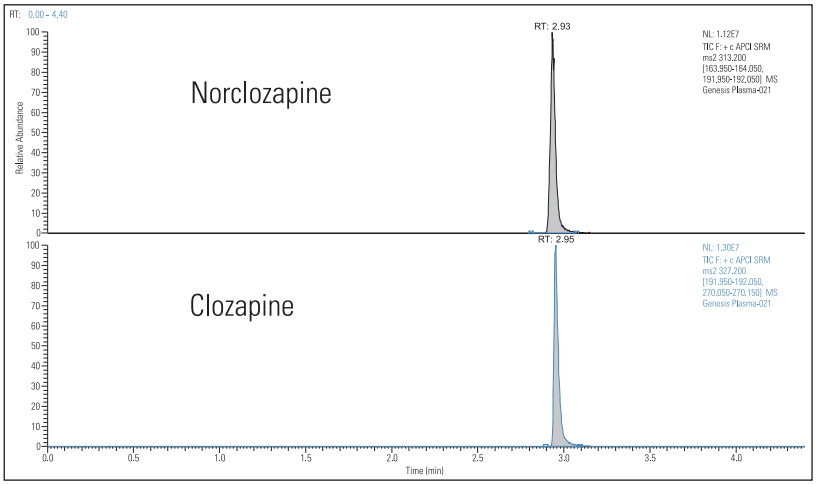

Figure 4: Clozapine and norcloxapine lowest calibration from plasma, 2 mg/L

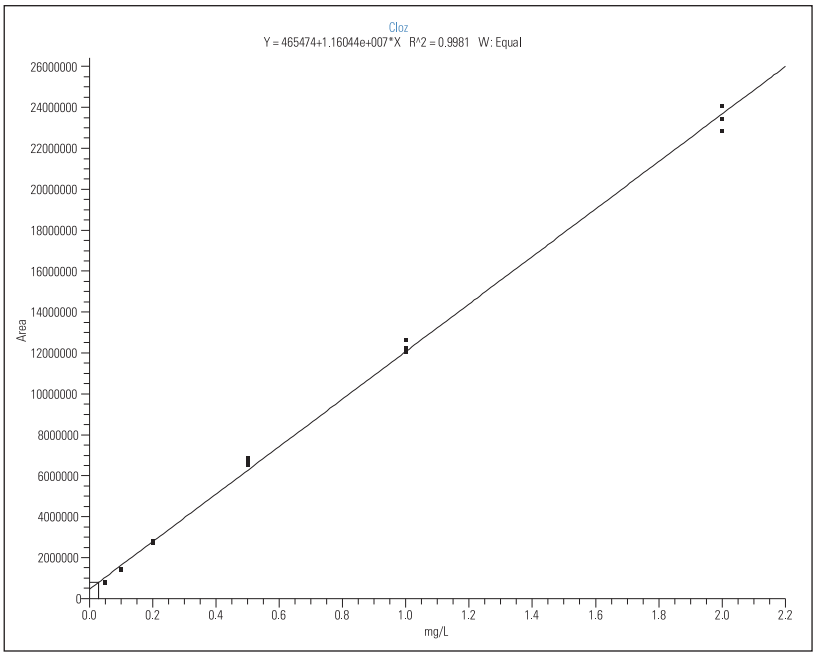

Figure 5: Clozapine calibration curve, 0.05 – 2 mg/L

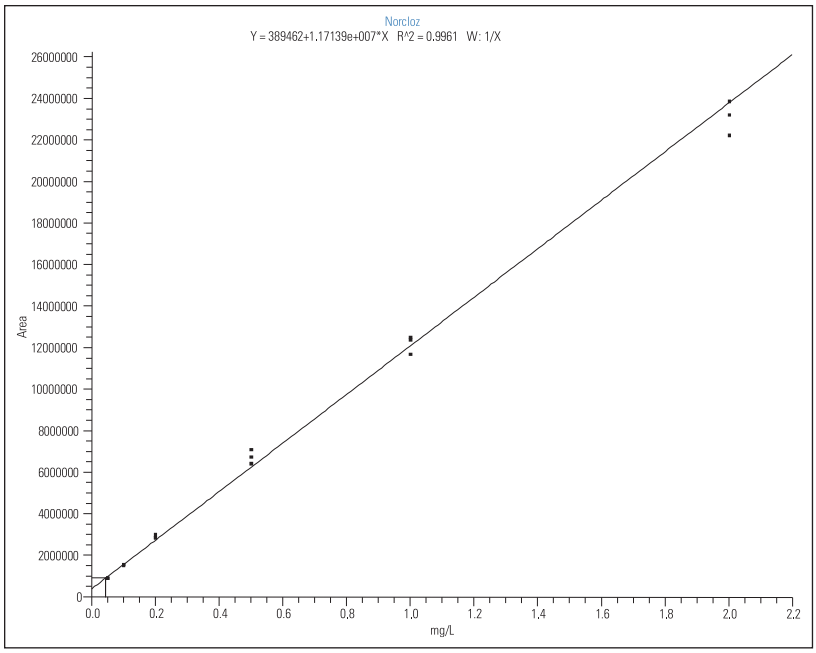

Figure 6: Norclozapine calibration curve, 0.05 – 2 mg/L

Clozapine					Norclozapine				
Concentration	Response	Specified Conc	Calculated Conc	% CV	Concentration	Response	Specified Conc	Calculated Conc	% CV
0.05 mg/L	784733	0.05	0.03	1.2	0.05 mg/L	905801	0.05	0.04	0.4
0.05 mg/L	797712	0.05	0.03	1.2	0.05 mg/L	897792	0.05	0.04	0.4
0.05 mg/L	780137	0.05	0.03	1.2	0.05 mg/L	900825	0.05	0.04	0.4
0.10 mg/L	1415271	0.10	0.08	1.7	0.10 mg/L	1555897	0.10	0.10	1.1
0.10 mg/L	1456027	0.10	0.09	1.7	0.10 mg/L	1554377	0.10	0.10	1.1
0.10 mg/L	1411624	0.10	0.08	1.7	0.10 mg/L	1525338	0.10	0.10	1.1
0.20 mg/L	2745962	0.20	0.20	1.8	0.20 mg/L	2847998	0.20	0.21	3.1
0.20 mg/L	2743289	0.20	0.20	1.8	0.20 mg/L	2859029	0.20	0.21	3.1
0.20 mg/L	2832044	0.20	0.20	1.8	0.20 mg/L	3006773	0.20	0.22	3.1
0.50 mg/L	6889405	0.50	0.55	2.6	0.50 mg/L	7099512	0.50	0.57	5.0
0.50 mg/L	6682781	0.50	0.54	2.6	0.50 mg/L	6741516	0.50	0.54	5.0
0.50 mg/L	6549395	0.50	0.52	2.6	0.50 mg/L	6420812	0.50	0.51	5.0
1.00 mg/L	12624439	1.00	1.05	2.3	1.00 mg/L	12521697	1.00	1.04	3.6
1.00 mg/L	12261014	1.00	1.02	2.3	1.00 mg/L	12383684	1.00	1.02	3.6
1.00 mg/L	12054848	1.00	1.00	2.3	1.00 mg/L	11695815	1.00	0.97	3.6
2.00 mg/L	24055429	2.00	2.03	2.5	2.00 mg/L	23888229	2.00	2.01	3.5
2.00 mg/L	22868295	2.00	1.93	2.5	2.00 mg/L	22259134	2.00	1.87	3.5
2.00 mg/L	23457123	2.00	1.98	2.5	2.00 mg/L	23241437	2.00	1.95	3.5

Figure 7: Clozapine/Norclozapine reproducibility and variance

References

1. Rostami-Hodjegan A, Amin AM, Spencer EP, Lennard MS, Tucker GT, Flanagan RJ, *J Clin Psychopharmacol* 2004; 24 (1): 1-9
2. Flanagan RJ, *CPD Clin Biochem* 2006; 7 (1): 3-18

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Bioanalytical Assay for Neurotransmitters in Whole Blood by LC-MS/MS

Yang Shi, Catherine Lafontaine, Francois A. Espourteille, Thermo Fisher Scientific, Franklin, MA, USA

Key Words

- Aria TLX-1
- TSQ Quantum Ultra
- TurboFlow Technology
- Parkinson's Disease

Introduction

Taken orally in conjunction with Levodopa (L-DOPA), Carbidopa (C-DOPA) inhibits the metabolism of L-DOPA before it reaches the brain so that more is available to be converted into dopamine in the brain. 3-methoxy-L-tyrosine (3-OMD) is an important metabolite produced after L-DOPA administration. The following LC-MS/MS method using TurboFlow™ technology for on-line sample extraction using a Thermo Scientific Aria™ TLX-1 system coupled with Thermo Scientific TSQ Quantum Ultra™ triple quadrupole mass spectrometer demonstrates its suitability as a research method for these compounds in human whole blood.

Goal

To develop a quantitative, fast, automated LC-MS/MS method for analysis of neurotransmitters in human whole blood.

Method Information

These analytes were extracted on-line from crashed human whole blood. Calibration curves were analyzed using an Aria TLX-1 LC system coupled with a TSQ Quantum Ultra with heated electrospray ionization (H-ESI) source. Internal standards used were 4-chloro-L-phenylalanine and L-DOPA-d_3.

Experimental Conditions

Sample Preparation

A standard stock solution of 50 µg/mL L-Dopa, C-Dopa and 3-OMD in methanol was prepared. Methanol-quenched human whole blood (K_2 EDTA) was centrifuged at 10,000 RPM for 10 minutes. Calibrators were prepared in the supernatant. Analyte concentration ratio of spiking solution was 4 to 1 of L-DOPA and 3-OMD to C-DOPA. Final internal standard concentrations were 90 ng/mL for 4-chloro-L-phenylalanine and 225 ng/mL for L-DOPA-d_3, respectively. Injection volumes were 0.010 mL.

Aria TLX-1 System Parameters

Two 0.5 x 50 mm Thermo Scientific Cyclone™ MAX TurboFlow columns with a C18 HPLC column (4.6 x 150 mm, 5 µm particle size).

LC Method Mobile Phases

Loading Pump

Mobile Phase A:	10 mM Ammonium Acetate with 0.2% Ammonium Hydroxide (aq)
Mobile Phase B:	0.1% Formic Acid (aq)
Mobile Phase C:	50 mM Ammonium Acetate with 10% Formic Acid (aq)
Mobile Phase D:	50 mM Ammonium Acetate with 10% Formic Acid in Methanol

Elution Pump

Mobile Phase A:	0.1% Formic Acid (aq)
Mobile Phase B:	0.1% Formic Acid in Acetonitrile

Mass Spectrometer Parameters

Ion Polarity:	Positive ion mode
Vaporizer Temperature:	400 °C
Capillary Temperature:	300 °C
Sheath Gas Pressure (N_2):	60 units
Auxiliary Gas Pressure (N_2):	55 units
Scan Type:	Highly-selective reaction monitoring (H-SRM)
Scan Time:	0.050 s
Q1 (FWHM):	0.7
Q3 (FWHM):	0.7

Positive single reaction mode (+SRM) transitions and other MS parameters for test compounds are shown in Table 1. The whole experiment was controlled by Aria software.

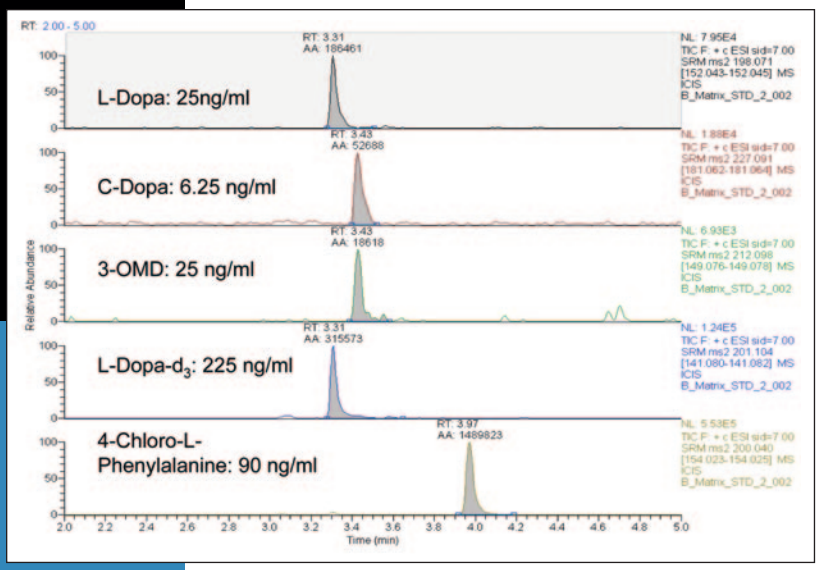

Figure 1: The representative chromatogram for the assay at the low end of the calibration curve

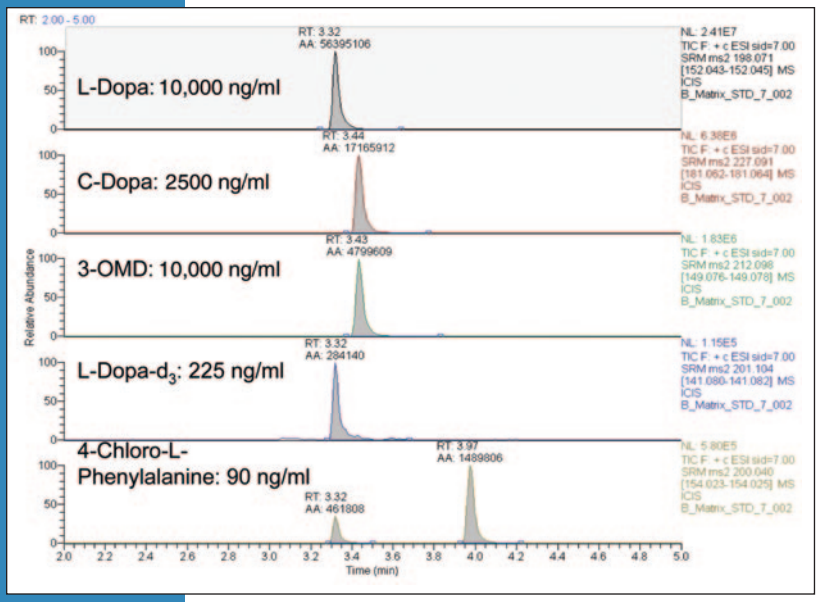

Figure 2: The representative chromatogram for the assay at the high end of the calibration curve

Results

Figures 1 shows a representative chromatogram for the assay at the low end of the curve. Figure 2 shows a representative chromatogram for the assay at the high end of the curve. Linearity of the calibration curves (N=3) ranged from 0.9942 to 0.9989 (with 1/x weighting). Figure 3 shows the representative linear calibration curves for all three test compounds. The excellent linear fits were over the range of 100-10000 ng/mL for L-Dopa and 3-OMD and 25-2500 ng/mL for C-Dopa. The limit of detection (LOD) levels were five-times lower for all compounds. The % CV values were less than 20% deviation for LLOQ and less than 15% deviation for all the other points on the calibration curve. Carryover was determined to be much less than 20% of lower limit of quantitation (LLOQ). A minimum of 85% recovery was achieved. The variability was determined by processing and analyzing five replicates of each of four QC samples. The test was repeated in three batches, Table 2. The results show that the %RSDs were well below the validation guideline of 15%.[1]

Table 1: Positive single reaction mode (+SRM) transitions and other MS parameters for test compounds

Compound	Parent Ion	Fragment Ion	Collision Energy (eV)	Tube Lens Offset
L-DOPA	198.071	152.044	14	72
C-DOPA	227.091	181.063	12	77
3-OMD	212.098	149.077	15	75
L-DOPA-d_3	201.104	141.081	16	87
4-Chloro-L-Phenyl-Alanine	200.040	154.024	14	61

Table 2: Low internal standard variability demonstrated the reliability of the method

L-Dopa-d_3 in QC Samples

	Batch #1	Batch #2	Batch #3
Number of Samples	20	20	20
RSD (%)	6.2	6.6	4.7

4-Chloro-L-Phenylalanine in QC Samples

	Batch #1	Batch #2	Batch #3
Number of Samples	20	20	20
RSD (%)	2.0	1.6	2.3

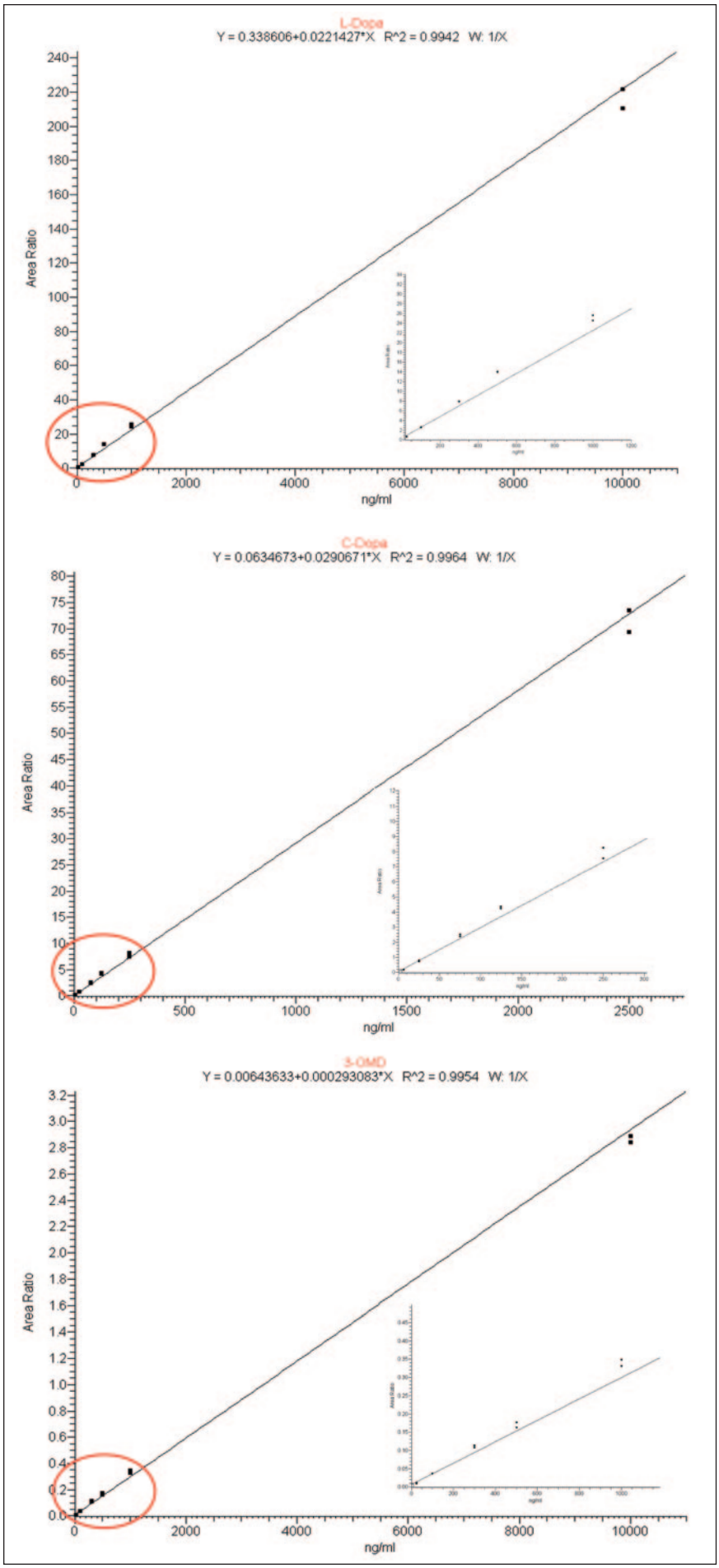

Figure 3: Representative linear calibration curves for all three test compounds

Conclusion

TurboFlow technology is a powerful technique for the direct analysis of drugs in biological fluids without the need for an extensive number of sample preparation steps. In this study, the use of an Aria TLX-1 LC system in front of a TSQ Quantum Ultra allows for low levels of detection (6.25 ng/mL for C-Dopa; 25 ng/mL for L-Dopa and 3-OMD) of each of these neurotransmitter compounds in human whole blood extract and yields results in less than 10 minutes per sample. With the Aria TLX-4 multiplexed system, the results will be available about every 2.5 minutes using only one mass spectrometer. The low variability of the results demonstrates the reliability of this research method.

Reference

1. Guidance for Industry Bioanalytical Method Validation, Food and Drug Administration, May 2001.

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Determination of Digoxin in Serum by Liquid Chromatography–Tandem Mass Spectrometry

François-Ludovic Sauvage[1], Pierre Marquet[1,2]

[1]*Department of Pharmacology-Toxicology, University Hospital, Limoges, France.*
[2]*Laboratory of Pharmacology, Faculty of Medicine, University of Limoges, France.*

Key Words

- TSQ Quantum
- Clinical Research
- Toxicology

Introduction

Digoxin is a cardiac glycoside that can be used at very low concentrations. Identification and quantitation of this compound necessitate a sensitive and specific method. This study aims to describe a method using liquid chromatography/ tandem mass spectrometry and permitting to quantify digoxin at low concentrations for research applications.

Goal

The goal of this study was to identify and quantify digoxin in serum. This report demonstrates the use of the TSQ Quantum for this application.

Experimental Conditions/Methods

Chemicals and Reagents

Digoxin and 3-aminophenylsulfone (internal standard) were purchased from Sigma. Ammonium formate and formic acid (>99 % pure) were also purchased from Sigma. All reagents and solvents used in the extraction procedures were of analytical grade.

Sample preparation

To 1 mL of serum were added 50 µL of a 2.5 µg/mL aqueous solution of 3-aminophenylsulfone (Internal Standard), 1 mL of a solution of pH 9.50 carbonate buffer and 8 mL of Ether-Dichloromethane-Isopropanol (30:40:30 by volume). The tubes were vortex-mixed and shaken on an oscillatory mixer. After centrifugation at 3,400 g for 5 min, the organic phase was poured in a conical glass tube and evaporated under a stream of nitrogen at 37°C. The dried extracts were reconstituted in 50 µL of acetonitrile : pH 3.0, 2 mmol/L ammonium formate (30:70 by volume) and 10 µL were injected into the chromatographic system.

Instrumentation Methods

HPLC Conditions

The chromatographic system consisted of a CTC HTS PAL Autosampler kept at 6°C and a binary high-pressure pump. A C18, 5 µm (50×2.1 mm) column, maintained at 25°C, was used with a linear gradient of mobile phase A (pH 3.0, 2 mmol/L ammonium formate) and mobile phase B (acetonitrile:pH 3.0, 2 mmol/L ammonium formate (90:10; v/v)), flow rate of 200 µL/min, programmed as follows: 0-1.2 min, 20% B; 1.2–8.2 min, 20 to 80% B; 8.2–10.2 min, 80% B; 10.2–10.7 min, decrease from 80 to 20% B; 10.7–13 min, equilibration with 20% B.

MS Conditions

Mass Spectrometer: Thermo Scientific TSQ Quantum
Source: ESI mode
Ion Polarity: Positive
Spray Voltage: 3800 V
Sheath/Auxiliary gas: Nitrogen
Sheath gas pressure: 30 (arbitrary units)
Auxiliary gas pressure: 30 (arbitrary units)
Ion transfer tube temperature: 250°C
Scan type: SRM
Collision gas: Argon
Collision gas pressure: 1.5 mTorr

SRM Conditions

Settings were optimized by infusing at 5 µL/min a 1 µg/L solution containing the studied compound in acetonitrile: pH 3.0, 2 mmol/L ammonium formate (30:70, by volume). The structure of these compounds is shown in Figure 1.

Compounds	Quantification transition	Collision energy	Confirmation transition	Tube lens voltage
Digoxin	798.5/651.4	20	798.5/781.5	84
3-aminophenylsulfone	249.1/93.2	24		126

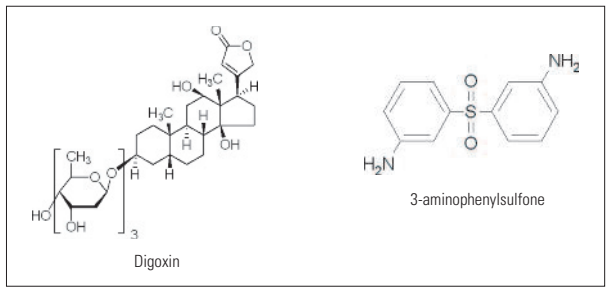

Figure 1: Structures of the investigated compounds

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Results and Discussion

The LC-ESI/SRM chromatograms for 3-aminophenylsulfone and digoxin for a blank serum sample and a blank serum sample spiked at 0.5 ng/mL are shown in Figures 2A and 2B respectively. Identification of digoxin was achieved with two characteristic SRM transitions and their relative retention time.

Linearity

Calibration curve obtained for digoxin spiked in serum samples is presented in Figure 3. Concentration range was comprised between 0.5 ng/mL and 100 ng/mL.

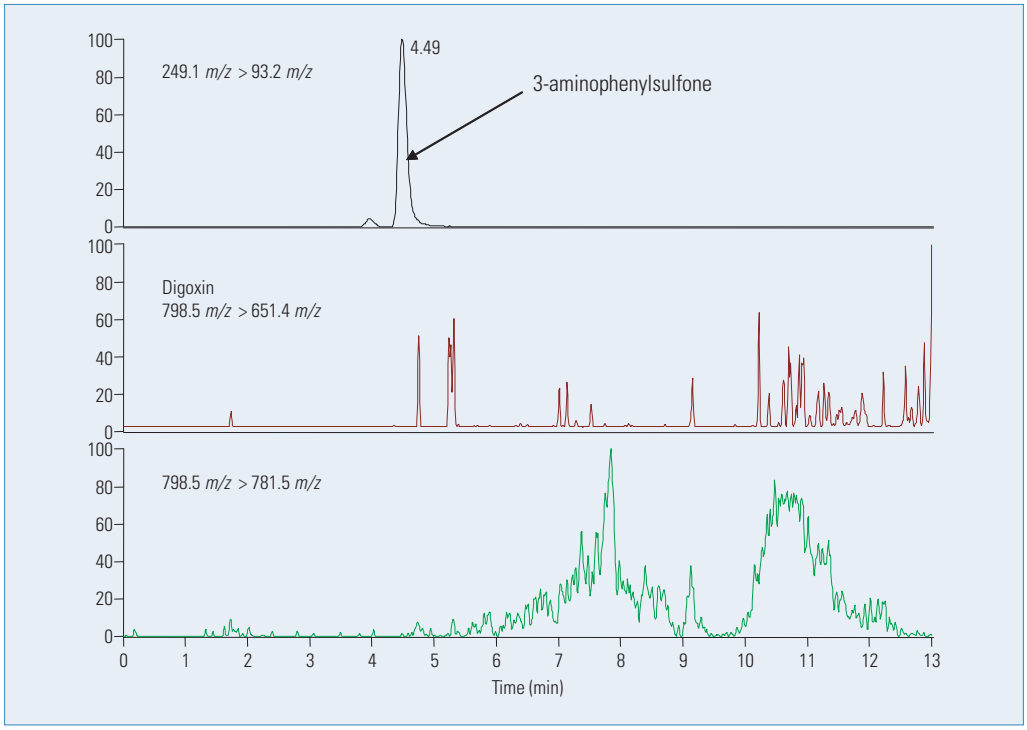

Figure 2A: Chromatogram of a blank serum

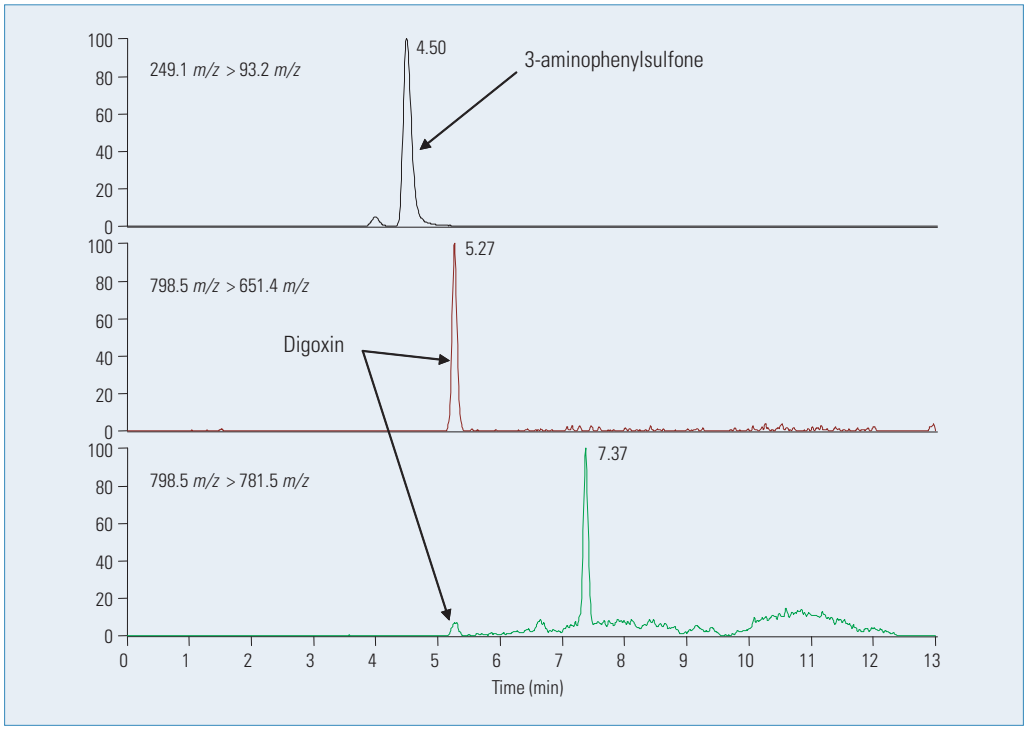

Figure 2B: Chromatogram of a blank serum spiked at 0.5 ng/mL

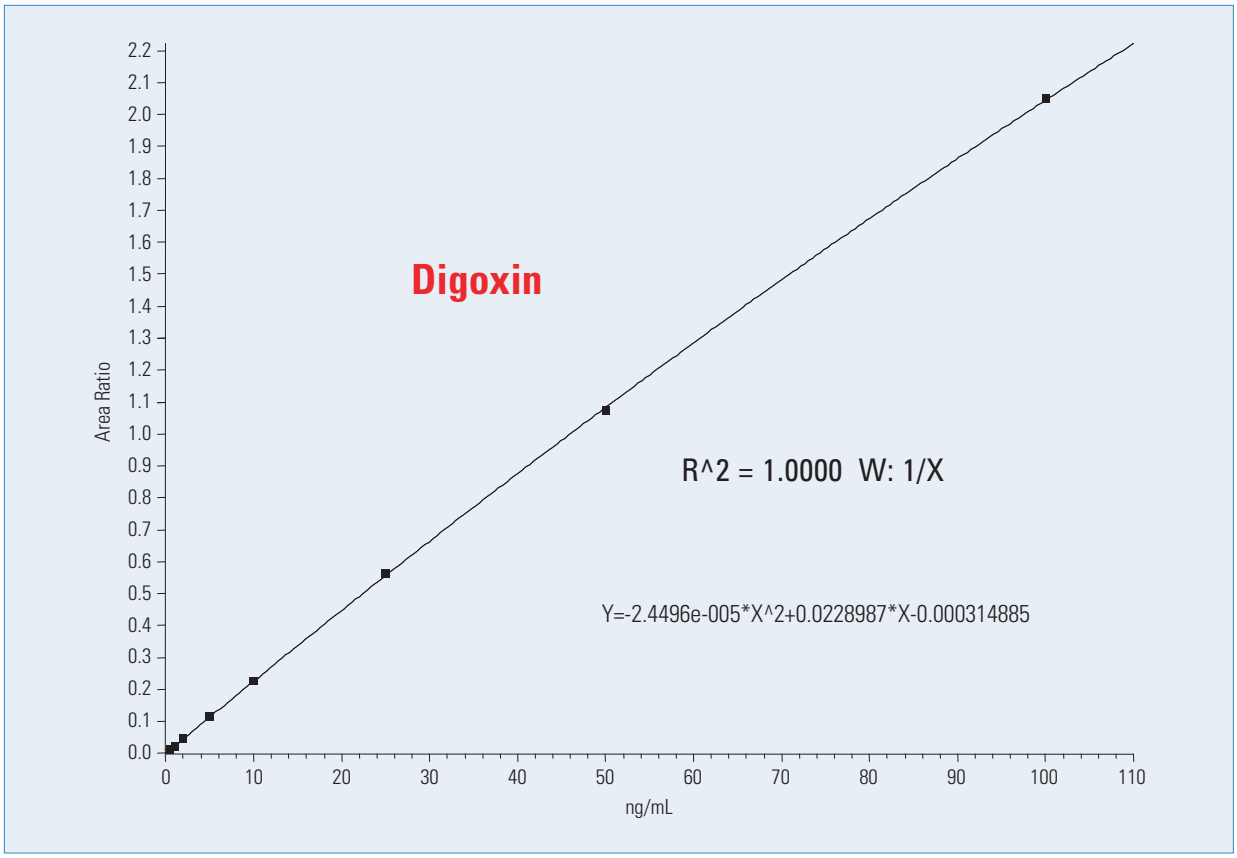

Figure 3: Representative calibration curve from standards spiked in serum

Corresponding Results of Calibration Standards

	Quadratic 1/x	
Specified Concentration (ng/mL)	Calculated Amount (ng/mL)	% Diff
0.5	0.503	0.53
1	.0972	-2.79
2	2.041	2.07
5	5.001	0.02
10	9.985	-0.15
25	25.239	0.95
50	49.614	-0.77
100	100.149	0.15

Intra-assay Accuracy and Precision (n=6)

	Quadratic 1/x	
Specified Concentration (ng/mL)	Calculated Amount (ng/mL)	% Diff
0.5	0.514	2.76
0.5	0.496	-0.75
0.5	0.546	9.10
0.5	0.558	11.62
0.5	0.510	1.97
0.5	0.563	12.62

Accuracy and precision

Intra-assay accuracy and precision (n=6) have been studied at the lowest concentration (0.5 ng/mL). Relative Standard Deviation was equal to 5.28% and Mean Relative Error to 6.23%.

Conclusion

This application note describes a sensitive and specific method developed for the quantitation of digoxin in serum for research applications.

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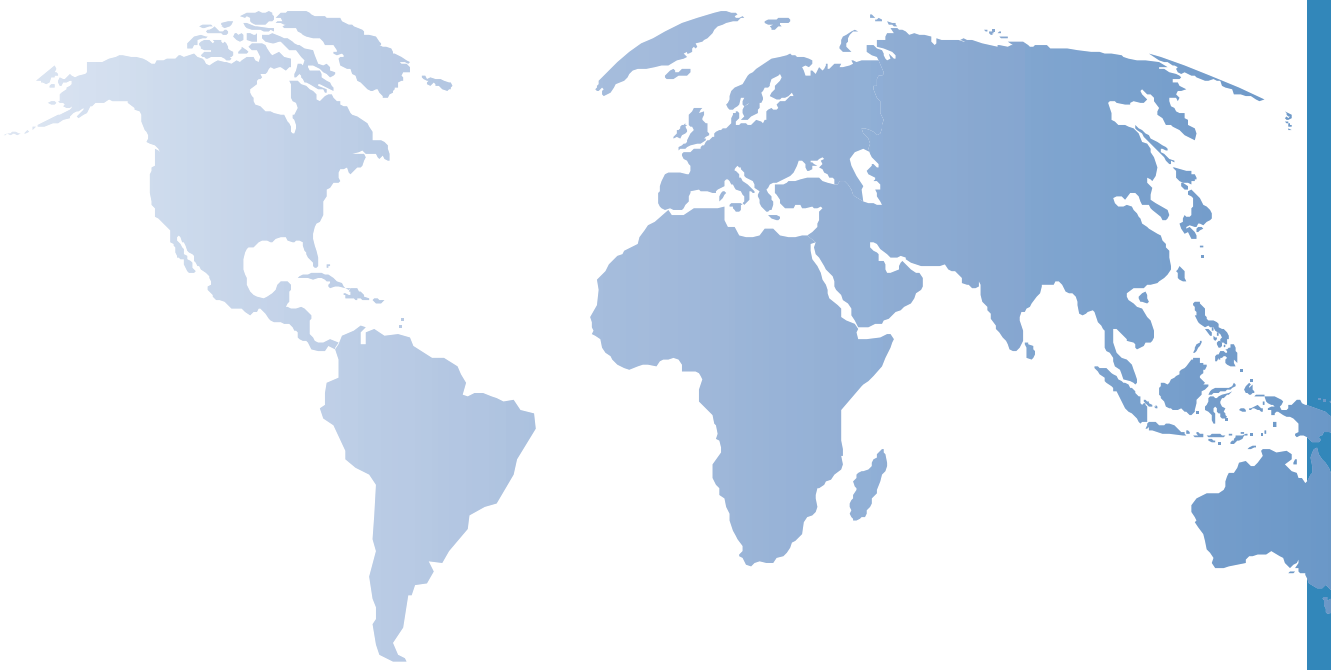

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Improved Quantitative Selectivity of Clenbuterol in Human Urine Using High Resolution on the TSQ Quantum Mass Spectrometer

Mark Churchill,[1] Mark Harrison,[1] John Henninge,[2] and Hanne Lund[2]

[1]*Thermo Fisher Scientific, Stafford House, 1 Boundary Park, Boundary Way, Hemel Hempstead, HP2 7GE, UK;*
[2]*Aker University Hospital, Hormone Laboratory, Section for Doping Analysis, Trondheimsveien, N-0514, Oslo, Norway*

Key Words

- TSQ Quantum™
- High Resolution Analysis
- Improved Sensitivity
- Quantitation

The data presented here was acquired on a TSQ Quantum mass spectrometer.

Introduction

Clenbuterol (Figure 1) is a beta-2-adrenergic agonist, an effective bronchodilator drug used for the treatment of human asthma. It relieves bronchial airway smooth muscle contractions caused by Chronic Obstructive Pulmonary Disease (COPD) and allergy-induced respiratory distress.

Clenbuterol has significant anabolic effects and could be used as a drug of abuse in athletes and livestock for its muscle growth stimulant properties. It raises the body temperature and hence facilitates fat tissue catabolism. Due to Clenbuterol having these anabolic properties, it must be routinely monitored in biological samples by veterinary and human doping control laboratories.

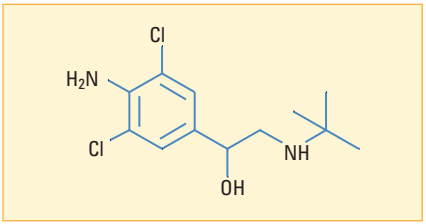

Figure 1: Chemical structure of Clenbuterol

Goal

One of the limitations to quantitation is the unequivocal identification of analytes in biological samples due to endogenous matrix interferents.

This report describes the use of high resolution on the Thermo Scientific TSQ Quantum to exploit the negative mass defect of a compound containing Chlorine, such as Clenbuterol, and hence improve the selectivity of the quantitative assay.

Clenbuterol ($C_{12}H_{18}Cl_2N_2O$, molecular weight 276.08 amu) was infused, 0.1 ng/µL, into the ESI source and the four most abundant product ions for the MS/MS breakdown were determined using the automated compound optimization procedure on the TSQ Quantum (Figure 2).

The transition yielding the most abundant product ion (*m/z* 203.0) was selected for the analysis of Clenbuterol.

Experimental Conditions

Sample Preparation: Human urine extracts were prepared using a C18 Solid Phase Extraction media. The extracted urine was spiked with Clenbuterol in the concentration range 0.1, 0.5, 1, 5, 10, 50 and 100 pg/µL for the calibration standards. No internal standard was used in this study.

Sample Analysis: The spiked urine extracts were chromatographed using a Thermo Scientific Surveyor™ LC on a C18 100 mm × 2.1 mm column at a flow rate of

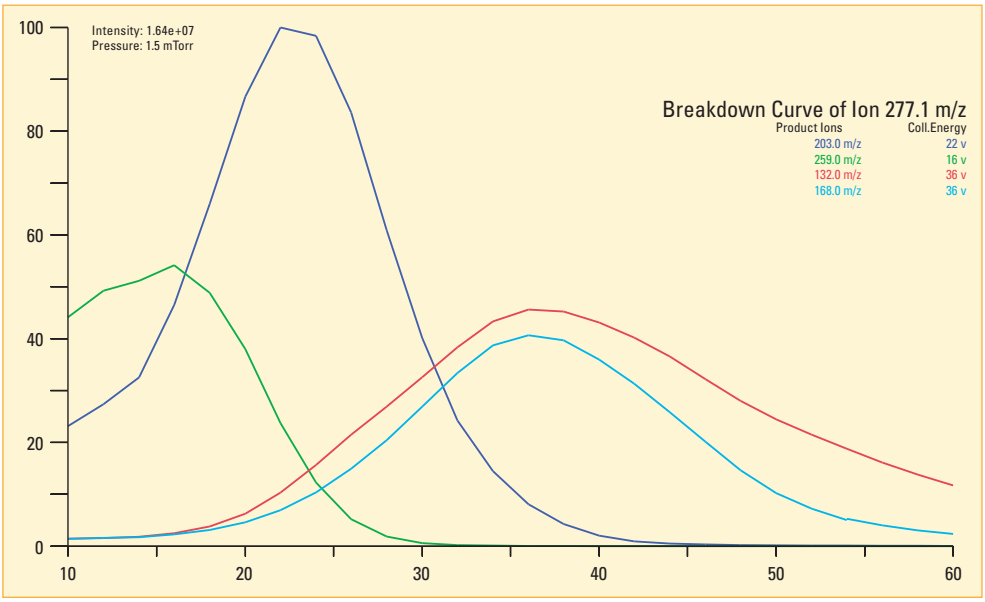

Figure 2: Automated optimization of MS/MS parameters for Clenbuterol

Thermo SCIENTIFIC

300 µL/min with a linear gradient of 10% solvent B (Methanol/Ammonium acetate [10 mM] 90/10 v/v) to 100% B over 5 minutes. Solvent A was Ammonium acetate (10 mM). The calibration standards were injected in duplicate at volumes of 10 µL.

MS Conditions

Mass spectrometer: TSQ Quantum

Ionization mode: Electrospray (ESI), positive ion

SRM: Clenbuterol 277.1 → 203.0 ± 0.3 Da, 22 eV
Collision energy Resolution

Experiment 1: 0.7 Da FWHM on Q1 and Q3

Experiment 2: 0.1 Da FWHM on Q1, 0.7 Da FWHM on Q3

Two separate quantitative analyses were performed at peak widths of 0.1 Da and 0.7 Da Full Width Half Maximum (FWHM) on Q1 in SRM mode. A peak width of 0.7 Da FWHM was used on Q3 for all analyses.

Results

The chromatogram of a pure standard of Clenbuterol in aqueous solvent demonstrates the retention time at 5.8 minutes (Figure 3).

Experiment 1: Quantitative Analysis Performed at 0.7 Da FWHM

The data below shows the quantitative analysis of Clenbuterol in Human urine at peak width settings of 0.7 Da FWHM on Q1 and Q3. Chromatograms are shown for blank urine (Figure 4) and urine containing Clenbuterol at 0.1 pg/µL (Figure 5).

A calibration curve of Clenbuterol analyzed at 0.7 Da FWHM was constructed using linear fit of peak area plotted against concentration, weighted 1/x (Figure 6). A correlation coefficient of r^2=0.9990 with an equation of Y=8496.82+266143*X was obtained for the curve.

The peak area, back-calculated values and precision of all calibration standards are shown in Table 1.

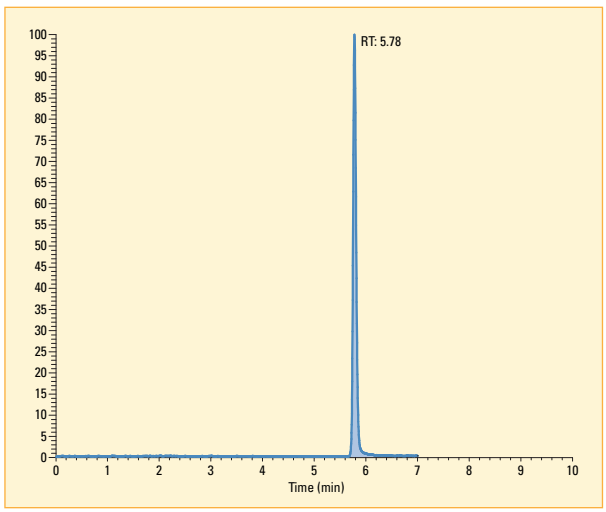

Figure 3: Determination of Clenbuterol retention time

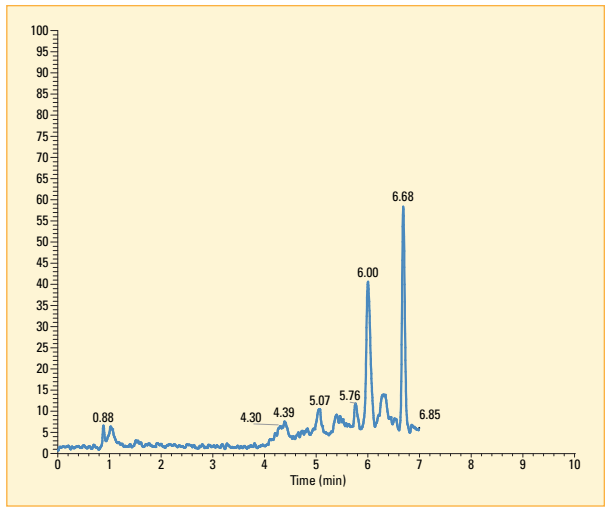

Figure 4: Urine blank, 0.7 Da FWHM

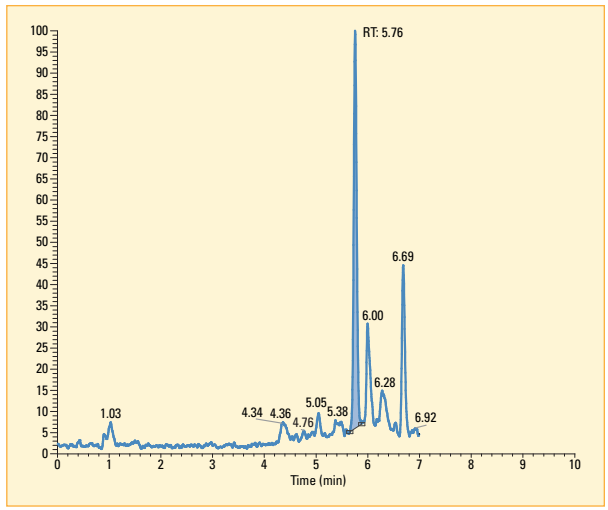

Figure 5: Clenbuterol, 0.1 pg/µL in urine, 0.7 Da FWHM

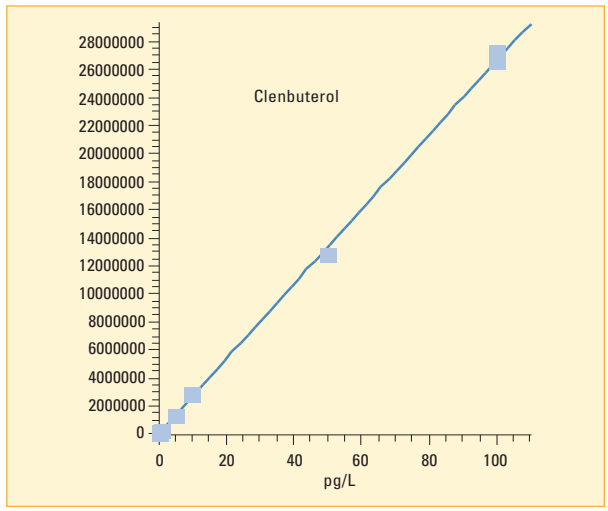

Figure 6: Clenbuterol curve at 0.7 Da FWHM

Experiment 2: Quantitative Analysis Performed at 0.1 Da FWHM

The data below shows the quantitative analysis of Clenbuterol in Human urine at peak width settings of 0.1 Da FWHM on Q1 and 0.7 Da FWHM on Q3. Chromatograms are shown for blank urine (Figure 7) and urine containing Clenbuterol at 0.1 pg/µL (Figure 8).

A calibration curve of Clenbuterol analyzed at 0.1 Da FWHM was constructed using linear fit of peak area plotted against concentration, weighted 1/x (Figure 9). A correlation coefficient of r^2=0.9994 with an equation of Y=2661.76+85951.1*X was obtained for the curve.

The peak area, back-calculated values and precision of all calibration standards are shown in Table 2.

SAMPLE NAME	AREA	CALC AMT	UNITS	%RSD
Urine blank	0.00	0.00	pg/L	
Urine blank	0.00	0.00	pg/L	
Cal 0.1 pg/L	33516.83	0.09	pg/L	4.5%
Cal 0.1 pg/L	31977.14	0.09	pg/L	4.5%
Cal 0.5 pg/L	136967.28	0.48	pg/L	0.6%
Cal 0.5 pg/L	137996.57	0.49	pg/L	0.6%
Cal 1 pg/L	289917.16	1.05	pg/L	1.3%
Cal 1 pg/L	295117.95	1.07	pg/L	1.3%
Cal 5 pg/L	1353210.91	5.05	pg/L	0.8%
Cal 5 pg/L	1338935.79	4.99	pg/L	0.8%
Cal 10 pg/L	2856289.00	10.70	pg/L	0.5%
Cal 10 pg/L	2877525.09	10.78	pg/L	0.5%
Cal 50 pg/L	12837781.41	48.20	pg/L	0.2%
Cal 50 pg/L	12797548.82	48.05	pg/L	0.2%
Cal 100 pg/L	27232776.65	102.29	pg/L	1.7%
Cal 100 pg/L	26578332.48	99.83	pg/L	1.7%

Table 1: Calculated standards at 0.7 Da FWHM

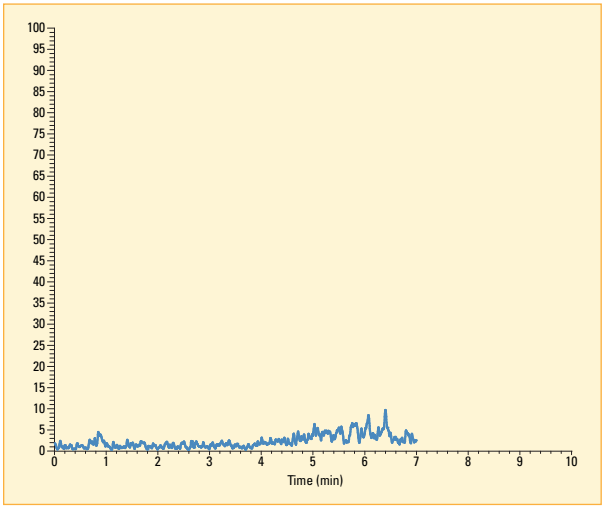

Figure 7: Urine blank, 0.1 Da FWHM

Discussion

Analysis, in SRM mode, of the spiked urine samples at a resolution setting of 0.7 Da FWHM resulted in a Clenbuterol peak eluting from the column upon a broad chemical noise background signal containing interferent peaks from the urine.

The same urine samples analyzed at a peak resolution setting of 0.1 Da FWHM resulted in elimination of the interfering isobaric mass peaks and the broad background chemical noise previously seen in the analysis at a peak width setting of 0.7 Da FWHM. The selected reaction monitoring analysis performed at a higher resolution setting of 0.1 Da FWHM resulted in increased selectivity of the assay and hence an increase in the precision that could be achieved.

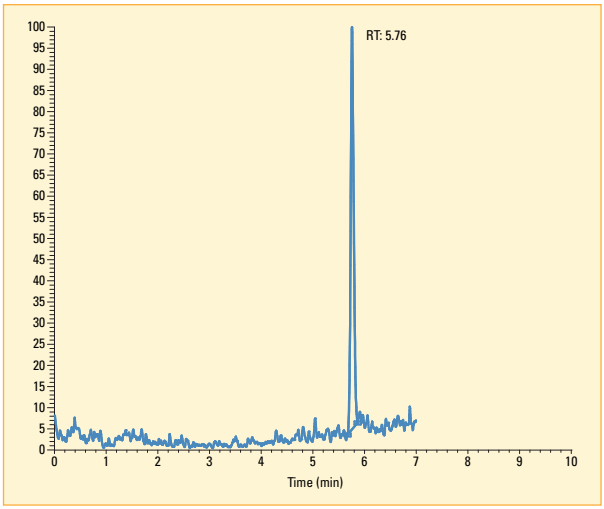

Figure 8: Clenbuterol, 0.1 pg/µL in urine, 0.1 Da FWHM

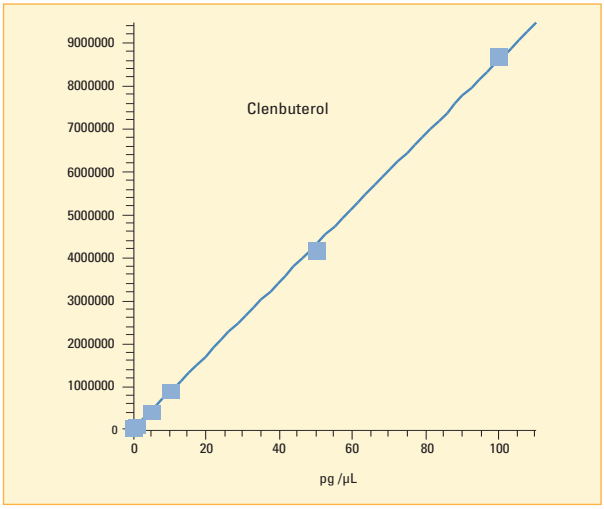

Figure 9: Clenbuterol curve at 0.1 Da FWHM

SAMPLE NAME	AREA	CALC AMT	UNITS	%RSD
Urine blank	0.00	0.00	pg/L	
Urine blank	0.00	0.00	pg/L	
Cal 0.1 pg/L	11245.02	0.10	pg/L	0.2%
Cal 0.1 pg/L	11272.54	0.10	pg/L	0.2%
Cal 0.5 pg/L	41960.02	0.46	pg/L	1.1%
Cal 0.5 pg/L	42592.84	0.46	pg/L	1.1%
Cal 1 pg/L	90353.60	1.02	pg/L	3.4%
Cal 1 pg/L	94633.92	1.07	pg/L	3.4%
Cal 5 pg/L	435920.49	5.04	pg/L	0.4%
Cal 5 pg/L	438538.32	5.07	pg/L	0.4%
Cal 10 pg/L	893656.24	10.36	pg/L	0.9%
Cal 10 pg/L	904758.00	10.49	pg/L	0.9%
Cal 50 pg/L	4120496.02	47.90	pg/L	1.3%
Cal 50 pg/L	4195902.58	48.78	pg/L	.3%
Cal 100 pg/L	8667429.70	100.81	pg/L	0.5%
Cal 100 pg/L	8727427.54	101.50	pg/L	0.5%

Table 2: Calculated standards at 0.1 Da FWHM

The increase in selectivity at a peak width setting of 0.1 Da FWHM is due to the fact that Clenbuterol is a chlorinated compound and thus the negative mass deficiency can be used to eliminate interferents from the urine matrix in SRM mode. This increased selectivity can be achieved without detrimental loss of transmission. Typically only a factor of two to three fold decrease in peak area is observed between analyses performed at 0.7 and 0.1 Da FWHM, however, greater selectivity could then be achieved.

The calibration curves for Clenbuterol concentrations of between 0.1 to 100 pg/µL at resolution settings of 0.1 and 0.7 Da FWHM both demonstrate excellent linearity. The calibration line at 0.7 Da FWHM showed a high intercept due to chemical background in the urine blank. This was significantly reduced by the use of high resolution.

The use of higher resolution to increase selectivity and precision could enable the limit of quantitation of an assay to be lowered and achieves a higher degree of confidence in identification of analytes in biological matrices.

AN62561E 12/07S

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Drugs of Abuse and Pain Management
Drug Analysis for Forensic Toxicology

(Click the Note number to view.)

Poster Note ASMS13_W132: Quantitation of Seven Designer Cathinones in Urine Using Q Exactive Mass Spectrometer

Poster Note ASMS13_W122: Direct Analysis using Paper-Spray Mass Spectrometry: Method Development for the Rapid Screening of Drugs of Abuse for Forensic Toxicology

Poster Note ASMS13_W118: Quantitative Confirmatory Analysis of the NIDA 5 Panel Using Prelude SPLC System and TSQ Quantum Ultra MS

Poster Note ASMS13_M617: High-Resolution, Accurate-Mass Forensic Toxicology Screening in Blood Samples Using a Q Exactive Mass Spectrometer

Poster Note ASMS13_M113: Verification of the Simultaneous Analysis of Heroin Addiction Treatment Compounds Using LC/MS/MS with a New Prelude SPLC™ System

Poster Note ASMS13_M083: Development of a Dilute-and-Shoot LC-MS/MS Method with 21 Opiates in Urine

Poster Note 63786: Quantitative Analysis of THC and Main Metabolites in Whole Blood Using Tandem Mass Spectrometry and Automated Online Sample Preparation

Poster Note 63783: Quantitation of Seven Designer Cathinones in Urine Using Q Exactive Mass Spectrometer

Note 576: Simultaneous Quantitation of 43 Pain Management Drugs in Human Urine with a “Dilute-and-Shoot” LC-MS/MS Method

Note 571: Simultaneous Quantitation of 19 Drugs in Human Plasma and Serum by LC-MS/MS

Note 570: Quantitation of Six Opioids in Urine with Super-Dilution and Microflow LC-MS/MS

Note 561: Quantitation of Amphetamines in Urine for SAMHSA Mandated Workplace Drug Testing Using a Triple Stage Quadrupole LC-MS System

Note 559: Quantitation of Synthetic Cannabinoids in Urine Using a Triple Stage Quadrupole LC-MS System in Forensic Toxicology

Note 556: Antidepressants and Neuroleptics Quantitation Using Tandem Mass Spectrometry and Automated Online Sample Preparation

Note 551: Demonstrating High-Performance Quantitative Analysis of Benzodiazepines using Multiplexed SIM with High-Resolution, Accurate Mass Detection on the Q Exactive LC/MS

Note 548: THC-COOH Quantification in Urine Using Dilute and Shoot LC-MS/MS Method for Forensic Toxicology

Note 547: Quantitation of 14 Benzodiazepines and Benzodiazepine Metabolites in Urine Using a Triple Quadrupole LC-MS System

Note 546: Quantitation of Six Opiates in Urine Using a Triple Quadrupole LC-MS System

Note 545: Quantitation of Six Synthetic Opioids in Urine Using a Triple Quadrupole LC-MS System

Note 541: Software Driven Quantitative LC-MS Analysis of Opioids in Urine for Forensic Laboratories

Note 536: Targeted Screening of Drugs of Abuse and Toxic Compounds with LC-MS/MS Using Triple Stage Quadrupole Technology

Drugs of Abuse and Pain Management
Drug Analysis for Forensic Toxicology (continued)

(Click the Note number to view.)

Note 529: Quantitative LC-MS Analysis of 14 Benzodiazepines in Urine Using TraceFinder 1.1 Software and High Resolution Accurate Mass

Note 527: Screening of 20 Benzodiazepines and Four Metabolites in Whole Blood using UHPLC-MS/MS

Note 524: A Fully Automated LC-MS Screening System using Automated Online Sample Preparation for Forensic Toxicology

Note 517: Screening for Drugs and Toxic Compounds: Comparison between LC-MS/MS, HPLC-DAD, and Immunoassay

Note 512: Screening and Quantification of Multiple Drugs in Urine Using Automated Online Sample Preparation and Tandem Mass Spectrometry

Note 507b: Forensic Toxicology Screening with LC-MS/MS and Automated Online Sample Preparation

Note 499: Quantitative LC-MS Screening for Illicit Drugs Using Ultrahigh Resolution Mass Analysis and Accurate Mass Confirmation

Note 496: Screening in Equine Doping Control Analysis with Ultrahigh Resolution and Accurate Mass

Note 489b: Quantitation of 12 Benzodiazepines and Metabolites in Urine Using Ultrahigh Resolution LC-MS for Forensic Toxicology Use

Note 488b: Quantitation of Urinary Ethyl Glucuronide and Ethyl Sulfate Using Ultrahigh Resolution

Note 486b: Simultaneous Analysis of Opiates and Benzodiazepines in Urine in Under 3 Minutes per Sample Using LC-MS/MS

Note 467: Screening Drugs and Toxic Compounds with LC-MS/MS: An Alternative to LC-UV for Research Toxicology Labs

Note 461b: Forensic Analysis of Opiates in Whole Blood by LC-MS/MS Using Automated, Online Sample Preparation

Note 461: Rapid Analysis of Opiates from Low Volume Whole Blood Samples by LC-MS/MS Utilizing TurboFlow Methods

Note 457: Quantitation of Fentanyl and Norfentanyl from Urine Using On-line High Throughput System

Note 449: A Complete Toxicology Screening Procedure for Drugs and Toxic Compounds in Urine and Plasma Using LC-MS/MS

Note 439: UHPLC/MS: An Efficient Tool for Determination of Illicit Drugs

Note 394: Improved Signal-to-Noise Ratio in the Antidoping Analysis of Clenbuterol in Urine Using LC–FAIMS–H-SRM

Note 390: A Quantitative Test for Multiple Classes of Illicit Drugs and Their Primary Metabolites in Human Biological Fluids by LC-MS/MS for Forensic Use

Note 383: Determination of LSD and Its Metabolites in Human Biological Samples by Liquid Chromatography–Tandem Mass Spectrometry

Note 366: Analysis of Multiple Illicit Drugs, Methadone, and their Metabolites in Oral Fluid Using a Linear Ion Trap Mass Spectrometer

Note 350: Rapid Quantitative and Confirmational Screening for Drugs in Race Horse Urine by ESI-LC-MS/MS and MS3

Note 346: MS/MS as an LC Detector for the Screening of Drugs and Their Metabolites in Race Horse Urine

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Quantitation of Seven Designer Cathinones in Urine Using Q Exactive Mass Spectrometer

Marta Kozak,[1] Kristine Van Natta,[1] and Shijun Sheng[2]
[1]Thermo Fisher Scientific, San Jose, CA; [2]Thermo Fisher Scientific, Franklin, MA

Thermo SCIENTIFIC

Overview

Purpose: To evaluate various scan modes available through high-resolution, accurate-mass analysis to determine suitability for *in vitro* plasma protein binding assay analysis.

Methods: An *in vitro* plasma protein binding assay was analyzed using various scan modes available to a high-resolution, accurate-mass analysis LC-MS system and the results compared to data obtained using a triple quadrupole mass spectrometer.

Results: The lower limit of detection was found to be between 5 nM and 50 nM in full scan mode. The 5 nM was detected for a majority of the samples analyzed using full scan mode. The signal response was determined to be linear across 3 orders of magnitude for most test compound calibration curves. The results for the calculated amount of the free fraction remaining (% Free) for the binding assay demonstrated a good correlation between the results for the high-resolution, accurate-mass analysis and the results collected using LC-MS/MS analysis. Sample analysis performed using SIM mode provided a lower limit of detection of 5 nM for all compounds in the assay calibration curve demonstrating an improvement in sensitivity for several compounds in the more targeted scan mode.

Introduction

High-resolution mass spectrometers are becoming increasingly more powerful and capable of sophisticated scanning experiments that offer new solutions to complex challenges. Additionally, assays that fall into a well defined and routine workspace, such as *in vitro* screening assay in early drug discovery, will also benefit from the ease of use and high performance of high-resolution mass spectrometric analysis but do not require all available scan capabilities needed for more complex applications. In this evaluation several different full scan and SIM analyses were used to analyze a protein plasma binding assay with an Thermo Scientific™ Orbitrap™ mass analyzer and the results compared to previous analysis performed using traditional LC-MS/MS on a triple quadrupole mass spectrometer.

Methods

Sample Preparation

A set of 24 of commercially available drug compounds was selected based on reported binding properties and molecular weight and incubated in an *in vitro* plasma protein binding assay in triplicate at a concentration of 10 µM. Samples were incubated for 6.5 hours in a dialysis block followed by protein precipitation. Protein precipitation was performed by first adding 150 mL of acetonitrile containing internal standard compound (Alprenolol) to a 96-well 340-mL V-bottomed storage plate followed by addition of 50 mL of each of the assay samples. Calibration curves were also generated for each compound. A working stock solution of 50 mM in DMSO was first made for each compound. A five-point standard curve at concentrations of 5, 50, 500, 1000 and 2000 nM was prepared for each compound by serial dilution from the working stock solution into a blank mixed matrix using an eight channel pipette[1].

Liquid Chromatography

Gradient elution was accomplished using water (A) + 0.1% Formic Acid (v/v) and Acetonitrile (B) + 0.1% Formic Acid (v/v). The gradient was held at 98% aqueous for 0.25 minutes, ramped to 98% B over 0.35 minutes, and held at 98% B for 0.2 minutes before returning to the starting conditions at 2% B for a 0.4 minute equilibration time.

Chromatographic separation was performed using a C18, 2.1 x 30 mm, 3µm column with 5uL injections made for each sample. All injections were completed using a Thermo Scientific™ Accela™ Open system with DLW (Dynamic Load and Wash) and with Thermo Scientific™ Accela™ 1250 pumps at a flow rate of 900 µL/min.

Mass Spectrometry

Samples were analyzed using both a Thermo Scientific™ Exactive™ Plus mass spectrometer in Full Scan mode (*m/z* 220 – 900) and a Thermo Scientific™ Q Exactive™ mass spectrometer in both Full Scan (*m/z* 220 – 900) and SIM mode with each using a resolution setting of 35,000 (FWHM) at *m/z 200* and a spectral speed of 7 Hz. Generic ion source conditions were used for all sample collection including vaporizer temperature (350 °C), capillary temperature (300 °C), sheath gas of 45 arbitrary units, and an auxiliary gas of 10 arbitrary units. The instrument was calibrated in positive ion mode before sample acquisition using Thermo Scientific™ Pierce™ LTQ Velos™ ESI Positive Ion Calibration Solution.

Data Analysis

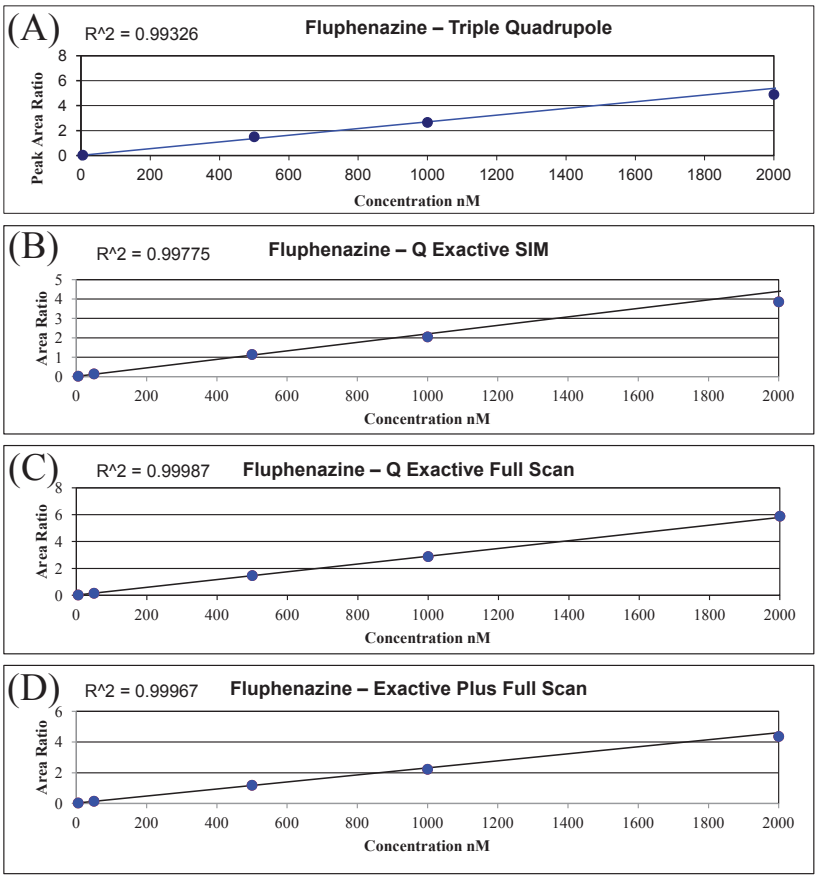
Data was acquired using Thermo Scientific™ Xcalibur™ 2.2 and Exactive Tune 2.1 software. Chromatographic data review and calibration curve generation was performed and reported using Thermo Scientific™ QuickCalc software (powered by Gubbs Inc., GMSU Gubbs™ Mass Spec Utilities, Atlanta, GA). Peak area measurements in the buffer chamber of the dialysis plate were compared to the peak area measurement in the serum chamber of the dialysis plate to calculate the percent of unbound compound (% Free) at assay equilibrium[1]. The average % Free for each compound replicate was reported for each analysis scan type and compared to values obtained using a triple quadrupole mass spectrometer. The coefficient of variation of the % Free values for each scan mode was also calculated for each compound analyzed.

Results

Scan Mode Signal Response

Each compound analyzed in the plasma protein binding (PPB) assay was evaluated in a concentration curve to evaluate overall sensitivity and linear dynamic range. All compounds were serially diluted using PPB matrix blank solution with concentrations ranging from 5 nM to 2000 nM concentration and analyzed using full scan and SIM analysis. The calibration curves for all compounds were generated using a linear regression and $1/x^2$ weighting. Individual calibration points exceeding a % difference of more than 20% of the regression line fit were excluded from the calibration curve. The majority of the compounds analyzed in full scan and SIM mode analysis exhibit the required sensitivity and linear dynamic range across the full range of the serial dilution and correlate well to the results collected using MS/MS analysis with a triple quadrupole mass spectrometer. Example calibration curves for each evaluated scan mode is displayed below for Fluphenazine (Figure 1).

FIGURE 1. Calibration curve of Fluphenazine in each scan mode. (A) MS/MS analysis, (B) Q Exactive SIM analysis, (C) Q Exactive Full Scan Analysis, (D) Exactive Plus Full Scan Analysis



The calibration curves for twenty-three of the twenty-four compounds analyzed using MS/MS analysis were linear across the full range of the calibration curve. One compound calibration curve in the MS/MS analysis required the exclusion of the 2000 nM calibration point due to signal saturation. Six of the twenty-four compounds analyzed using full scan and SIM mode analysis required the exclusion of the 2000 nM calibration point due to signal saturation (Figure 2). High-resolution analysis using an Orbitrap mass analyzer enables a user-definable parameter for the amount of target ions collected for each scan during analysis. An increase in the amount of ions collected during each scan should limit the effects of signal saturation for future analysis. Due to sample volume limitations, optimization of the ion collection target could not be performed for this experiment. Full scan analysis of the compound calibration curves demonstrated adequate sensitivity for the analysis of the calibration curves for twenty of twenty-four compounds or 83%. One compound demonstrated improved sensitivity in full scan mode using the Q Exactive Orbitrap MS, while all other calibration curve signal responses were consistent for full scan analysis across both high-resolution platforms.

FIGURE 2. Heat map display of compound calibration curve points included and excluded for each scan mode used for analysis. Calibration points with a % Difference greater than 20% were excluded from the linear regression. Excluded calibration points common to 3 scan modes are labeled in yellow. Excluded calibration points in 2 or fewer scan modes are labeled in red.

	Exactive Plus Full					Q Exactive Full				
Compound	5 nM	50 nM	500 nM	1000 nM	2000 nM	5 nM	50 nM	500 nM	1000 nM	2000 nM
Propranolol										
Diltiazem										
Imipramine										
Halperidol										
Carbamazpine										
Chlorpheniramine										
Phentolamine										
Buspirone										
Verapamil										
Desipramine										
Clozapine										
Acebutolol										
Retonavir										
Thioridazine										
Nefazadone										
Timolol										
Minaprine										
Fluphenazine										
Metoprolol										
Ticlopidine										
Compound A										
Erythromycin										
Clomipramin										
Bendamustine										

	Q Exactive SIM					Triple Quadrupole				
Compound	5 nM	50 nM	500 nM	1000 nM	2000 nM	5 nM	50 nM	500 nM	1000 nM	2000 nM
Propranolol										
Diltiazem										
Imipramine										
Halperidol										
Carbamazpine										
Chlorpheniramine										
Phentolamine										
Buspirone										
Verapamil										
Desipramine										
Clozapine										
Acebutolol										
Retonavir										
Thioridazine										
Nefazadone										
Timolol										
Minaprine										
Fluphenazine										
Metoprolol										
Ticlopidine										
Compound A										
Erythromycin										
Clomipramin										
Bendamustine										

	Incuded in Curve		Excluded from curve %Diff > 20%
	Excluded from curve %Diff > 20% and observed in 2 or fewer of the scan modes		

Analysis in SIM mode using the Q Exactive MS provided adequate sensitivity for all compounds analyzed and provided a sensitivity improvement for some compounds over full scan analysis (Figure 2).

PPB % Free Calculation

Percent free or unbound amount of compound in the protein binding assay was calculated for each scan mode used for analysis[1]. The coefficient of variation of the % Free across each scan mode was calculated for each compound and the results were listed in a table and sorted from lowest to highest by %CV (Table 1).

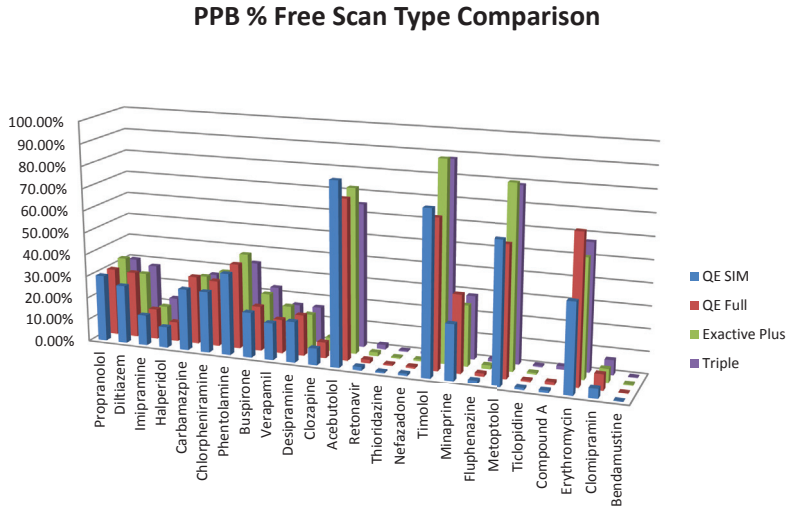
Cells highlighted in red in Table 1 denote a scan mode that did not provide sufficient signal for a specific compound to generate a % Free value and were excluded from the %CV calculation for the respective compound.

Table 1. % Free for analyzed compounds in each scan mode and %CV across scan modes. Cells highlighted in red denote scan modes with no results due to lack of analyte signal.

Q Exactive SIM	QE SIM	QE Full	E Plus Full	Triple			
Compound	% Free	% Free	% Free	% Free	Avg(%)	StdDev(%)	% CV
Propranolol	30.03	30.45	33.01	30.00	30.87	1.44	**4.66**
Diltiazem	26.36	29.80	26.72	27.70	27.64	1.54	**5.58**
Imipramine	13.73	13.67	12.11	13.10	13.15	0.75	**5.69**
Halperidol	9.38	8.73	10.04	9.50	9.97	1.56	**5.70**
Carbamazpine	27.75	30.74	28.28	26.40	28.29	1.81	**6.40**
Chlorpheniramine	27.60	29.79	31.40	27.00	25.06	6.26	**7.00**
Phentolamine	36.84	38.43	40.39	33.80	37.36	2.79	**7.46**
Buspirone	20.33	20.22	23.04	23.30	20.12	2.73	**7.71**
Verapamil	16.66	15.29	18.43	16.20	16.64	1.32	**7.92**
Desipramine	18.37	18.32	15.76	16.10	17.14	1.40	**8.18**
Clozapine	7.45	7.10	6.10	6.70	6.88	0.58	**8.42**
Acebutolol	82.00	72.00	74.52	65.10	73.41	6.98	**9.51**
Retonavir	1.61	1.59	1.70	2.00	1.58	0.36	**11.01**
Thioridazine	0.60	0.69	0.58	0.50	0.64	0.13	**12.93**
Nefazadone	1.00	0.80	0.73	0.90	0.86	0.12	**13.73**
Timolol	73.40	67.20	90.13	88.10	79.71	11.19	**14.03**
Minaprine	25.05	34.90	27.30	28.60	21.97	7.32	**14.57**
Fluphenazine	1.53	1.38	1.70	1.20	1.51	0.31	**14.64**
Metoptolol	62.92	58.52	82.10	79.00	70.64	11.66	**16.51**
Ticlopidine	0.91	0.75	0.73	0.60	0.75	0.13	**16.74**
Compound A	1.00	1.20		1.50	1.24	0.25	**20.22**
Erythromycin	40.00	66.49	53.13	57.10	54.18	10.99	**20.28**
Clomipramin	4.31	7.10	6.08	6.70	5.66	1.99	**20.38**
Bendamustine	0.18	0.19		0.20	0.26	0.13	**5.41**

The calculated % Free values for each compound were plotted in a bar chart to illustrate differences in the % Free values across each scan mode for the PPB analysis.(Figure 3).

Figure 3. % Free for individual compounds across each scan mode used for assay analysis. Twenty-two of twenty-four compounds analyzed demonstrate a %CV of less than 25% across the various scan modes while providing adequate sensitivity for assay analysis across all scan modes.



Twenty-two of the twenty-four compounds analyzed in the protein binding assay provide a %CV of less than 25% across the various scan modes while providing adequate sensitivity for analyte analysis in the binding assay. Although four compounds did not provide enough signal in the calibration curve analysis only two did not provide enough signal for % Free calculation in the PPB assay itself. 92% of the compounds analyzed provided sufficient signal in both full scan and SIM mode with a %CV of less than 25%. The two compounds that did not provide enough sensitivity to generate a % Free value in the binding assay were challenging in full scan on the Exactive Plus only and not on the Q Exactive. One explanation for this observation maybe due to the generic mass spec and chromatographic conditions used for data analysis. Although both instruments collected data in full scan mode, the Q Exactive filters all ions outside of the specified full scan mass range. While the Exactive Plus does filter some ions at the s-lens, additional ions outside the specified mass range are also collected and injected into the Orbitrap Mass Analyzer. Further optimization of the ion target amount collected per scan in the mass spec method along with optimized chromatographic clean up of the assay samples in the generic method may improve signal response in full scan mode in the absence of true ion filtering with a quadrupole and will be evaluated in future work.

Conclusion

- 83% of compounds analyzed met the assay calibration curve LOQ of 5nM.
- 92% of the compounds provided sufficient signal in the assay for calculation of the % Free in all scan modes evaluated.
- Full scan analysis using high resolution accurate mass provided adequate signal response and linear dynamic range to accurately measure 92% compounds analyzed in the PPB assay.
- Additional sensitivity and linear dynamic range may be achieved through further method optimization.

References

1. Zhang J, Shou WZ, Vath M, Kieltyka K, Maloney J, Elvebak L, Stewart J, Herbst J, Weller HN., Rapid Commun Mass Spectrom. 2010 Dec 30;24(24):3593

Acknowledgements

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Direct Analysis using Paper-Spray Mass Spectrometry: Method Development for the Rapid Screening of Drugs of Abuse for Forensic Toxicology

Maria C. Prieto Conaway,[1] Nicholas E. Manicke,[2] and Marta Kozak[1]
[1]Thermo Fisher Scientific, San Jose CA; [2]Department of Chemistry, Purdue University, West Lafayette, IN

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Overview

Purpose: Method development for the rapid screening of drugs of abuse in forensic toxicology using paper spray mass spectrometry.

Methods: Bovine blood spiked with common drugs of abuse and analyzed as dried blood spots by paper spray ionization/Orbitrap mass spectrometry. Accurate mass full MS and All Ion Fragmentation experiments for the identification and confirmation of drugs from dried blood spot samples. Thermo Scientific™ TraceFinder™ 3.0 software for data analysis.

Results: Able to identify six drugs of abuse tested down to 100 ng/mL from dried blood spots. Limit of detection on single drug analysis to 10 ng/mL from dried blood spots. Paper spray is easy to use, requires no sample preparation and no prior chromatography, making for a quick technique with the potential to identify compounds in seconds. The Thermo Scientific™ Exactive Plus system is ideally suited for coupling to paper spray ionization.

Introduction

Paper spray is a direct ionization technique that simplifies the mass spectrometric analysis of dried blood spots (DBS). Paper-spray technology is therefore attractive for forensic toxicology screening for drugs of abuse. The sample collection and storage of DBS in a simple paper cassette make shipment of samples to the forensic toxicology lab safe and convenient. Both qualitative and quantitative analysis of small molecules from complex matrices such as blood or other biological fluids is possible without time consuming sample preparation and chromatography.

Single-component quantitation of DBS samples with paper-spray MS is fairly well established. While previous work used a Thermo Scientific triple quadrupole mass spectrometer and monitored specific MS/MS transitions, full-MS instruments with Thermo Scientific™ Orbitrap™ analyzers are ideally suited as rapid screening tools. Orbitrap analyzers provide high resolution, accurate mass (HR/AM) full MS spectra for high confidence identification, allow for unlimited number of analytes in the method and retrospective data analysis.

In this work, the ability of paper spray coupled to a very sensitive and fast Orbitrap analyzer is explored for its potential as a forensic toxicology screening tool.

Methods

Sample Preparation

- Mixtures of drugs (Cerilliant, TX) were spiked in blood (bovine blood, Lampire Biologicals, New Jersey) stabilized with K2-EDTA.
- Blood sample integrity maintained by not exceeding 5% of solvent in blood (v/v).
- Single drug quantitation used a deuterated analog (500 ng/mL) as internal standard.
- Twelve microliters of spiked blood sample were loaded to paper cartridges, allowed to dry for two hours at room temperature and loaded into stackers that hold up to 40 cassettes.
- Solvent is automatically dispensed to the DBS before analysis and an applied high voltage (3-5 kV) induces electrospray from the sharp tip of the paper (Figure 1).
- The extraction solvent used in this work is 95/5 (v/v) methanol/water at pH 4.5.

Mass Spectrometry

- The paper-spray source was coupled to a Thermo Scientific Exactive Plus benchtop Orbitrap mass spectrometer.
- The Exactive Plus™ instrument was operated at various resolving power settings, from 17,500 to 140,000 (FWHM at m/z 200) and in positive ionization MS mode.
- An automated experiment for drug screening consisted of 30 sec data collection, switching back between full scan and All Ion Fragmentation (AIF) experiments.
- For screening, the MS Full scan data was acquired at 70,000 resolving power and the AIF at 17,500 resolving power with a collision energy of 43 eV.
- All data acquisition used Xcalibur™ sequences and contact closure trigger from the paper spray.

Data Analysis

- QualBrowser and QuanBrowser software from the Xcalibur platform were used for viewing and single compound quantitative analysis, respectively. TraceFinder™ 3.0 software was used for the automated identification and confirmation in the targeted screening of drugs.
- Potential mass fragments were generated using Thermo Scientific™ MassFrontier™ 7.0 software.

FIGURE 1. Prototype paper spray ion source (QuantIon Technologies, Inc., IN) showing, clockwise from top left: paper spray ion source, mechanism for dispensing solvent to the sample, paper cassette indicating sample deposition and DBS-spotted paper cassette electrospraying into mass spectrometer inlet.

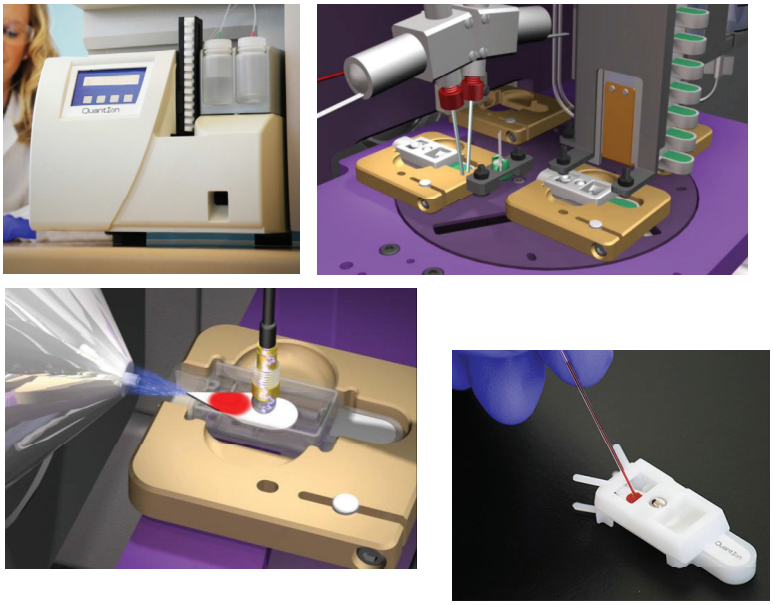


FIGURE 2a. MS spectra for the $[M+H]^+$ ion of amitriptyline at various concentrations from DBS samples. Acquired at 70,000 resolving power.

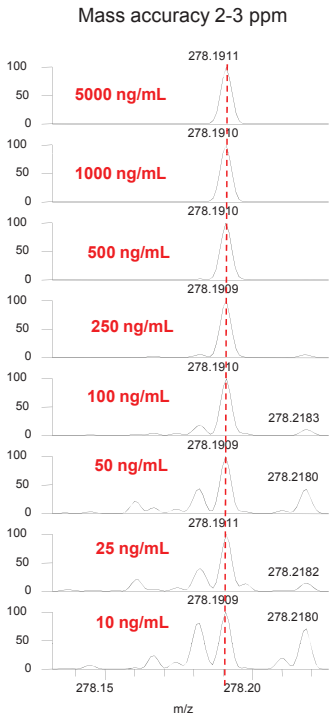
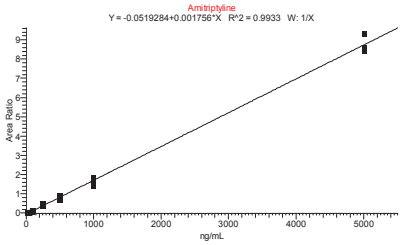


FIGURE 2b. Quantitative results for amitriptyline normalized by internal standard from DBS samples. Calibration curve and %RSD variability (n=3) shown.



Level (ng/mL)	% RSD
5000	5.4
1000	11.9
500	15.6
250	9.7
100	*
50	13.2
25	0.3

* No replicates available

Results

Quantitative - Single drug

- Amitriptyline spiked in bovine blood and evaluated at various concentrations using amitriptyline-d_3 as the internal standard.
- Amitriptyline-spiked in MeOH/water (data not shown) and blood (10–5,000 ng/mL) yielded limits of quantitation (LOQ) of 10 ng/mL for drug in solvent and LOQ of 25 ng/mL for samples in blood.
- Variability in terms of %RSD (Std Dev/Mean*100) is between <1 to 13% for drug out of solvent. Figure 2 displays amitriptyline data for dried blood spots.

Screening for drugs of abuse: resolving power, accurate mass for compound identification

- Figure 3 shows that high and ultrahigh resolving powers (70,000 and 140,000 FWHM at *m/z* 200) are required when evaluating samples from complex matrices with no sample preparation and no prior chromatographic separation.
- Results from TraceFinder, which can be used for targeted or unknown screening analysis, are neatly summarized in Figure 4. All six drugs are positively identified from a dry blood spot sample.

'All Ion Fragmentation' and isotopic pattern matching for compound confirmation

- Accurate mass m/z values were used for identification of screened drugs. Isotopic pattern matching and two fragments from the AIF experiment were used for drug confirmation (TraceFinder table Fig. 4).
- Figure 5 shows accurate mass fragmentation spectra by AIF for a DBS sample containing a mixture of 6 drugs.
- Fragments unique for a particular drug can be identified, examples shown for codeine and cocaine.
- Other fragments are shared by a few of the drugs present, for example amphetamine and methamphetamine, whose structures differ only by a methyl group.
- Mass Frontier™ 7.0 software was used to generate potential fragments for each drug using the "Generate Fragments and Mechanisms" tool which were then compared to the MS spectra.

Screening for drugs of abuse at various concentrations

- A drug mixture of six compounds was analyzed at 100, 500, 1000 and 2500 ng/mL for forensic toxicology screening. Amphetamine, methamphetamine, cocaine, cocaethylene, codeine and PCP are shown in this work.
- This group of samples were detected by full MS down to 100 ng/mL levels (Figure 6).

FIGURE 3. The use of higher resolving power, *e.g.*, 70,000 and 140,000 (FWHM at *m/z* 200), is required for the identification of drugs from DBS due to matrix interference in MS experiment. Bovine blood spiked with six drugs, four drugs shown below. Resolving power from 17,500, 35,000, 70,000 and 140,000 top to bottom. The $[M+H]^+$ ion is highlighted by red line.

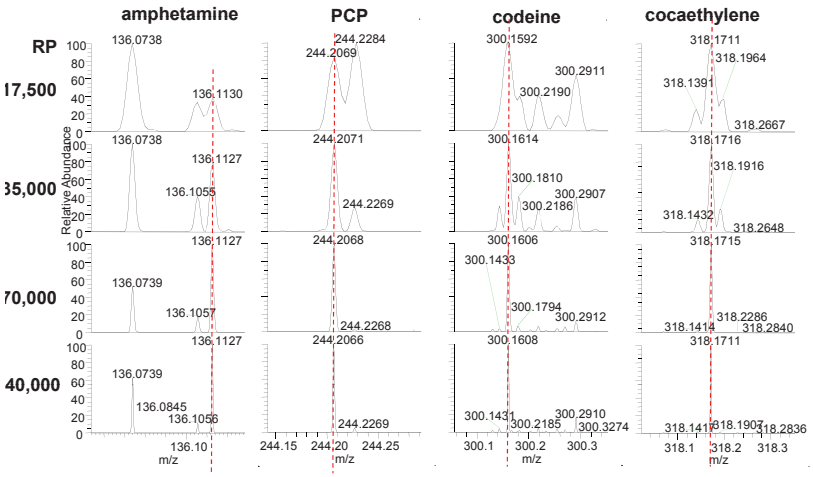


FIGURE 4. TraceFinder 3.0 software results shown below. Data processed in targeted screening analysis mode. All analytes in the mix are positively identified by exact m/z values and confirmed by isotopic pattern and the presence of two fragments from the AIF experiment. Each analyte appears twice in the table below because acquired paper spray peak width is twice wider than maximum peak width supported by data processing software.

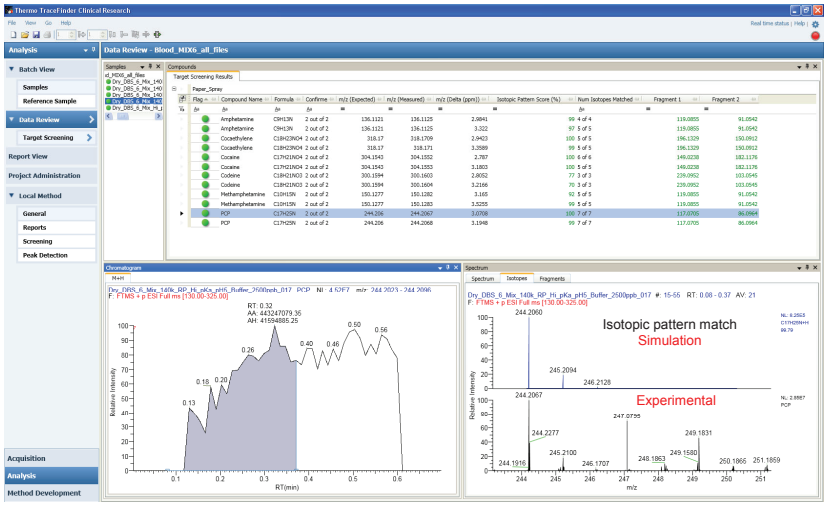


FIGURE 5. Accurate mass fragments acquired through an 'All Ion Fragmentation' experiment provide compound confirmation in the screening of drugs. Sample is a mixture of six drugs analyzed from DBS. Examples of two unique fragments and two that are common to more than one drug are shown.

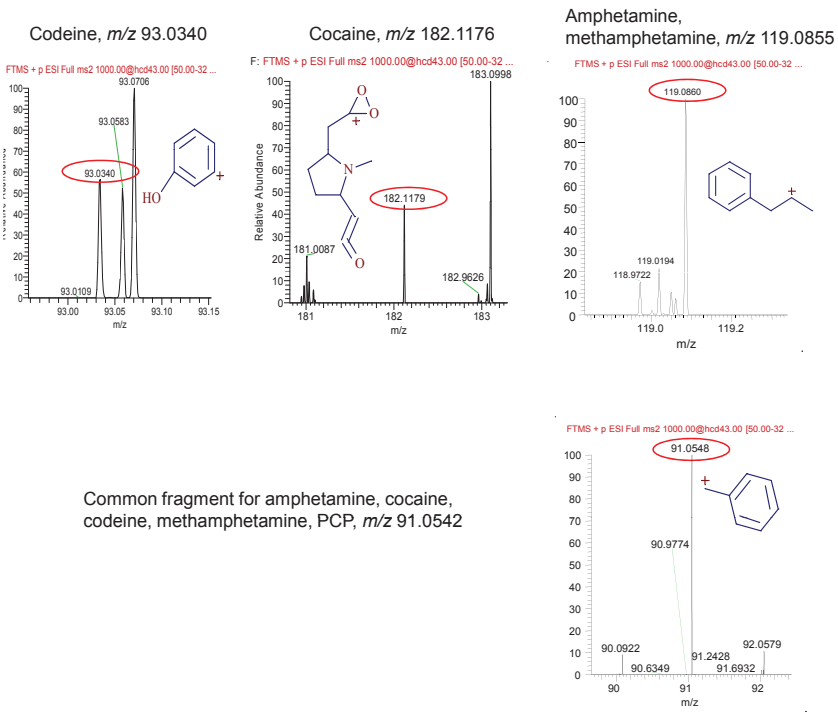
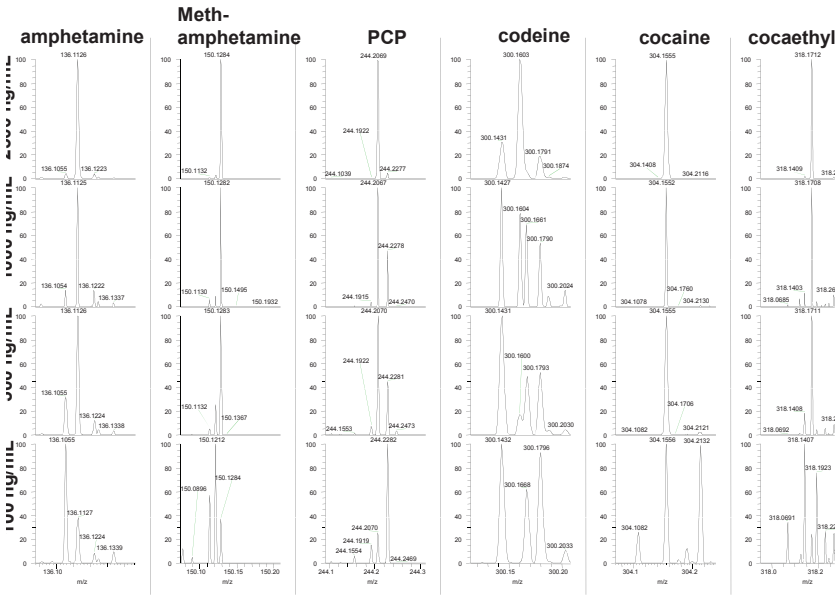


FIGURE 6. Accurate mass (3-4 ppm) MS spectra at 70,000 resolving power showin drugs detected down to 100 ng/mL. Sample contained six drugs analyzed from DB



Conclusion

- We have shown an easy to use technique (no sample preparation, no chromatography) that shows extraordinary potential for screening drugs of abuse in forensic toxicology.
- The paper sample cassette allows for direct sample deposition, safe sample handling and storage after drying. Once dry, the sample is stable for convenient shipping.
- Quantitation of drug out of dry blood spot sample is demonstrated with full MS experiment in the Exactive Plus mass spectrometer. Analyte normalized by labeled internal standard.
- High resolution and accurate mass are crucial techniques for analyzing complex samples by MS and nicely complement the paper spray technique in the screening of drugs from dried blood spots.

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Quantitative Confirmatory Analysis of the NIDA 5 Panel Using Prelude SPLC System and TSQ Quantum Ultra MS

Haiqiang (Bill) Yu, Kristine Van Natta, Marta Kozak
Thermo Fisher Scientific, San Jose, CA, USA

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Overview

Purpose: Develop and validate a simple and efficient quantitative LC-MS/MS method for SAMHSA-compliant confirmatory analysis of 5 panel drug using novel HPLC system.

Methods: Human urine containing the drugs were spiked with internal standards, enzymatically hydrolyzed, and diluted.

Results: The LC-MS/MS method was developed and validated to comply with SAMHSA guidelines.

Introduction

Effective on October 2011, the new SAMHSA/NIDA guidelines allow implementation of LC-MS technique to perform NIDA-5 panel, urine quantitative confirmatory analysis. LC-MS/MS methods are often less complicated than the previously implemented GC-MS/MS methods because they do not require derivatization. The NIDA-5 panel requires 6 separate quantitative methods for analysis of THCA, opiates, amphetamines, cocaine, phencyclidine and 6-MAM to confirm immunomethod positive samples. Here we developed 6 methods using a single sample preparation procedure, analytical column, mobile phase and instrument configuration. The methods are implemented on new Thermo Scientific™ dual channel Prelude ™ SPLC online sample preparation-liquid chromatography system, which allows method execution in parallel with a different method on each channel or the same method on both channels multiplexed to a single mass spectrometer.

Serial MS detection of multiplexed methods improves mass spectrometer utilization time, increases laboratory throughput and reduces analysis cost. The syringe pumps and high-pressure, low-volume gradient mixing used in the Prelude SPLC system provide enhanced LC performance including improved peak shape and resolution, stable retention times and reduced solvent consumption.

Methods

Sample Preparation

The sample prep procedure includes glucuronide hydrolysis followed by dilution. For each sample a 200-µL aliquot of urine was spiked with 10 µL of internal standard solution and 100 µL of β-glucuronidase enzyme in ammonium acetate buffer, pH=5.0. The samples were incubated at 60 °C for 2 hours. A 200-µL aliquot of methanol was added to each sample to stop enzymatic reaction. Samples were cooled down, centrifuged and diluted 20-fold with water, except for THCA, which was diluted 2-fold with water. Then 20 µL of sample was injected onto the LC-MS/MS system.

Liquid Chromatography

Chromatographic separations were performed with the Prelude SPLC system by direct injection onto a Thermo Scientific™ Accucore™ PFP 50x2.1mm, 2.6 µm analytical column. The column was maintained at room temperature. Mobile phases A and B consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol, respectively. Separate methods were set up to analyze 6-MAM, BE, PCP, and THCA. One method was set up for the combination of amphetamine, methamphetamine, MDA, MDEA and MDMA. A final method was used for the opiates morphine and codeine along with hydromorphone, hydrocodone, oxymorphone and oxycodone. Figure 1 shows the LC method for analyzing the opiates.

Mass Spectrometry

MS/MS analysis was carried out on a Thermo Scientific™ Quantum Ultra™ triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. MRM transitions for each compound are listed in Table 1.

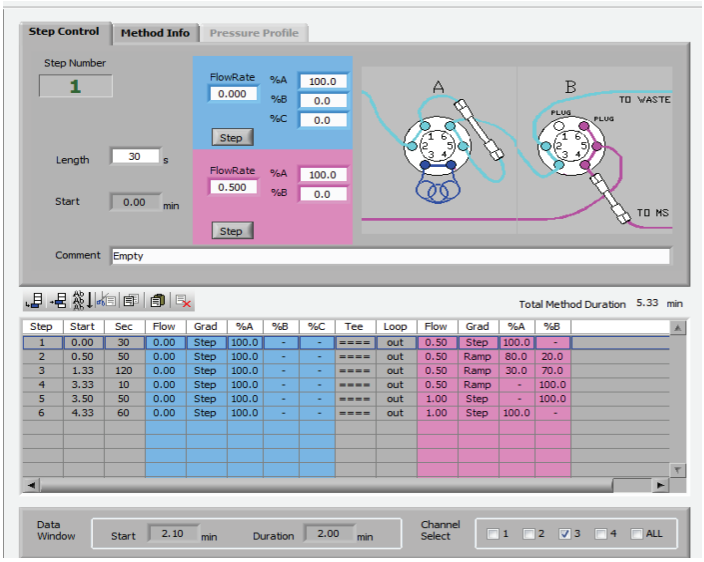
Validation

The calibration standards and quality control (QC) samples were prepared by spiking compounds into blank urine. Samples were processed as described in the Sample Preparation section. Methods were validated in multiplexed mode. Intra- and inter- method precision and accuracy were determined by analyzing a calibration curve along with replicate QCs on three different days. Matrix effects were determined by comparing peak area of samples processed in multiple lots of urine to that of one process in water. Additionally for the opiates, we were able to correlate results obtained with this method to those from a toxicology laboratory validated method.

Data Analysis

Thermo Scientific™ TraceFinder™ software was used for data acquisition and processing. Data were processed with ion ratio confirmation.

FIGURE 1. LC method for separating morphine and codeine.



Results

For each method, performance was within SAMHSA/NIDA guidelines. The quantitation limits (LOQ) for some compounds were lower than required to demonstrate method capability. The linear ranges were 2.5–2000 ng/mL for PCP and THCA; 5–2000 ng/mL for methamphetamine, BE and 6-MAM; 10–2000 ng/mL for morphine, codeine, amphetamine, MDA, and MDMA (Figure 2). The intra-method precision was <13.5%, <3.5%, <14.1%, <6.9%, < 9.6%, <15.9% for PCP, BE, 6-MAM, THCA, opiates and amphetamines respectively. The inter-method precision was <8.9%, <3.6%, <10.9%, <8.8%, <7.0%, <15.3% for PCP, BE, 6-MAM, THCA, opiates and amphetamines respectively. These results are summarized in Table 2. Limited matrix effects were seen and those were largely mediated by deuterated internal standards. The percent recovery for 8 spiked urine donor samples was in range of 80–120% (Table 3). Data collected for opiates with developed methods correlated well with toxicology laboratory data with coefficient of correlation >0.99 (Figure 4). Implementation of the dual channel Prelude SPLC system with syringe pumps improved retention time precision, chromatographic peaks shape and resolution, thus allowing for short, small solvent consumption LC methods while still keeping good data quality.

TABLE 1. List of NIDA 5 compounds MRM transitions, cutoff requirements, LOQ and Linear range

Drug	MRM (Q: Quantifier)	Cutoff (ng/mL)	LOQ (ng/mL)	Linear Range
Amphetamine	136.1–91.3 (Q), 136.2–119.3	250	10	10–5000
Methamphetamine	150.2–91.2 (Q), 150.2–119.2	250	5	5–5000
MDA	180.2–135.2 (Q), 180.2–163.2	250	10	10–5000
MDMA	194.1–163.1 (Q), 194.1–135.1	250	10	10–5000
MDEA	208.1–163.1 (Q), 208.1–135.2	250	10	10–5000
Benzoylecgonine	290.1–168.1 (Q), 290.1–105.1	100	5	5–2000
THCA	354.3–336.3 (Q), 354.3–308.3	15	2.5	2.5–2000
Phencyclidine	244.2 –159.1 (Q), 290.1–105.1	25	2.5	2.5–2000
Morphine	286.11–152.1 (Q), 286.11–165.1	2000	10	10–6000
Codeine	300.2–152.1 (Q), 300.2–165.1	2000	10	10–6000
6-Acetylmorphine	328.1–165.1 (Q), 328.1–211.1	10	5	5–2000

FIGURE 2. Representative calibration curves for BE, THCA, 6-MAM and PCP.

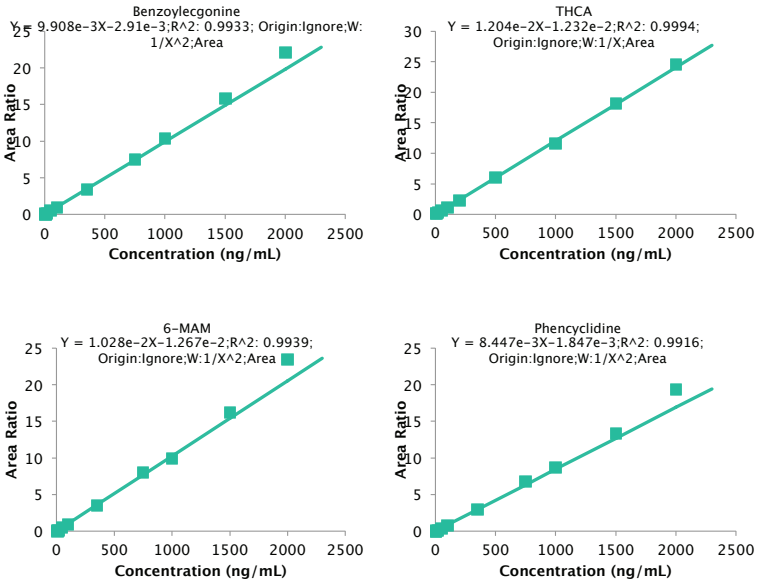


FIGURE 3. Example chromatograms for each method at respective LOQs.

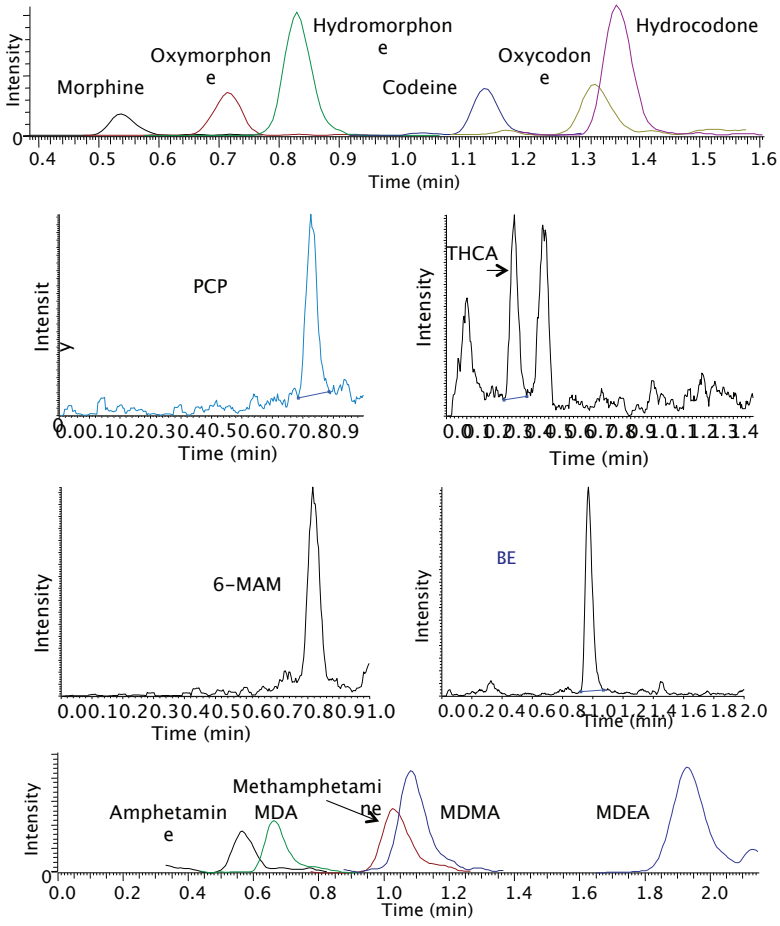


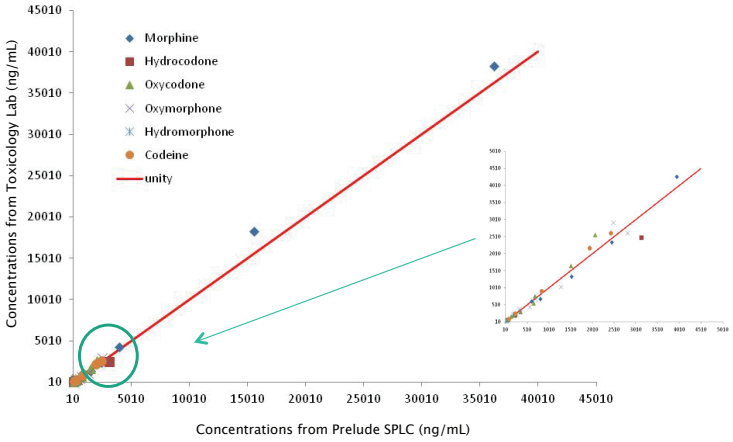
TABLE 2. Intra-method and Inter-method Precision.

Compound	Precision (RSD%)					
	Intra-method			Inter-method		
	LQC	MQC	HQC	LQC	MQC	HQC
Amphetamine	<15.9	<3.68	<2.86	15.33	3.23	2.32
MDEA	<5.33	<3.46	<5.24	3.65	2.88	3.62
MDA	<6.66	<4.15	<11.89	5.84	2.83	2.52
MDMA	<5.52	<4.34	<3.26	4.68	3.31	3.46
Methamphetamine	<5.47	<4.52	<16.63	6.2	4.33	3.79
Benzoylecgonine	<2.21	<2.35	<2.53	1.84	1.8	2.2
Phencyclidine	<6.88	<3.56	<4.33	8.8	3.57	3.63
6-Acetylmorphine	<5.87	<3.39	<4.11	4.69	3.51	3.67
THCA	<7	<2.8	<2.3	8.3	2.5	3.3
Morphine	<8.2	<10.8	<2.2	8.2	4.8	3
Codeine	<7.35	<5.20	<3.68	5.8	3.99	3.77

TABLE 3. Recovery of 11 drugs in 6 different urine lots.

Urine Lot	1	2	3	4	5	6
Amphetamine	100	103	98.3	95.8	101	103
MDEA	94.8	99.9	101	98.3	98.5	94.9
MDA	99.6	107	101	100	102	98.7
MDMA	101	100	97.9	99.3	103	102
Methamphetamine	99.8	101	102	96.1	105	98.5
Benzoylecgonine	106	111	97.6	107	109	106
Phencyclidine	88	84.2	81	83.5	85.6	85.9
6-Acetylmorphine	117	109	104	108	104	105
THCA	95.8	90.2	91.2	93.7	97.8	106
Morphine	96.1	99.8	91	90.7	93.9	92.3
Codeine	102	100	102	103	99.7	104

FIGURE 4. Correlation of data acquired with Prelude-Ultra method compared with data from a toxicology research laboratory validated method.



Conclusion

- An LC-MS/MS method for confirmatory analysis of the 11 drugs in the NIDA 5 panel using the Prelude SPLC and TSQ Quantum Ultra MSwas developed and validated.
- The method has LOQs that satisfy the SAMSHA cutoff requirements for these 11 drugs.
- No matrix interference were observed.
- The method is simple and fast.
- Two-channel multiplexing on Prelude SPLC would allow two different methods multiplexing in two channels and 3 minutes for a sample.

Acknowledgements

We would like to thank Kent Johnson from Pacific Hospital of Long Beach for supplying the comparison samples.

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High-Resolution, Accurate-Mass Forensic Toxicology Screening in Blood Samples Using a Q Exactive Mass Spectrometer

Isabelle Morel,[1] Sylvie Lepage,[1] Benedicte Duretz[2]
[1]Forensic Toxicology Laboratory, Rennes Hospital, Rennes, France;
[2]Thermo Fisher Scientific , Les Ulis, France

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Overview

Purpose: To evaluate the Thermo Scientific™ Q Exactive™ High-Resolution Mass Spectrometer in Forensic Toxicology Screening for whole blood analysis and make a comparison with Targeted Screening on a Triple Quadrupole MS using the SRM (Selected Reaction Monitoring) mode and also UPLC/Diode Array Detection (DAD).

Methods: Blood samples were spiked with internal standards and extracted with TOXI-TUBES™ A (Agilent Technologies, Santa Clara, CA). LC separation was performed with a 30 minute gradient. Mass spectrometry data were acquired in Full Scan and MS^2 mode using the Q Exactive MS.

Results: Data collected show benefits of high-resolution screening over both the triple quadrupole approach and DAD detection.

Introduction

Forensic scientists and forensic toxicologists need to identify an unlimited number of compounds in complex matrixes with the capability of retrospective data analysis for quick and confident analysis. The major challenge is to separate the analytes of interest from the matrix and accurately identify them. Here we evaluated the Q Exactive MS, a bench-top quadrupole-Orbitrap™ ultra-high resolution mass spectrometer routinely capable of better than 5 ppm mass accuracy and 140,000 FWHM resolution, with Thermo Scientific™ ExactFinder™ data processing software, for forensic toxicology screening in blood samples. We will also compare the results with those obtained by forensic targeted screening using an SRM approach and DAD detection.

Methods

Sample Preparation

500 µl of each blood sample was spiked with 20 µl of an internal standard solution (Flurazepam at1 mg/L) and extracted with TOXI-TUBES A™ (Agilent Technologies). The organic layers were transferred, evaporated to dryness, reconstituted in 2.5 ml of a mixture containing 70% of mobile phase A and 30% of mobile phase B, and injected onto the Q Exactive MS. For triple quadrupole analysis and DAD detection, the sample was reconstituted in 500 µl and 100 µl, respectively, of the mixture described above.

Liquid Chromatography

The U-HPLC comprises Thermo Scientific™ Accela™ 1250 pumps with an Accela Autosampler. Mobile phases are 10 mM Ammonium formate and 0.1% Formic acid in water (A) and 0.1% Formic acid in Acetonitrile (B). The LC separation was performed on a Thermo Scientific™ Hypersil™ GOLD PFP column 150 x 2.1 mm 3µm.

FIGURE 1. HPLC Gradient Method

Start (min)	Flow (mL/min)	%A	%B
0.00	0.2	95	5
5	0.2	55	45
18	0.2	30	70
20	0.2	5	95
27	0.2	5	95
27.1	0.2	95	5
32	0.2	95	5

Mass Spectrometry

Compounds are detected on a Q Exactive mass spectrometer equipped with an Orbitrap mass analyzer. A schematic diagram of the Q Exactive MS is illustrated in Figure 2. A Heated Electrospray Source Ionization (HESI) probe was used as an ion source. The instrument was operating in alternating positive and negative full scan mode. Each Full Scan was followed by 8 high-resolution MS^2 scans in positive mode and 3 high-resolution MS^2 scans in negative mode. Precursor selection was done in the data-dependent operation mode where the most intense ion of the previous scan was selected for fragmentation. Resolution was set to 70,000 FWHM for each full scan mode and 17,500 FWHM for MS^2 scan acquisition.

MS^2 spectra were acquired with a Normalized Collision Energy (NCE) of 70. Relevant scan and source parameters are shown in Figures 3 and 4.

DAD Detection

Data have been acquired on a UPLC-Acquity™ (Waters Corporation, Milford, MA) equipped with a DAD detector. The library contains 612 molecules. Acquisition is performed using a 15 minute LC gradient.

Triple Quadrupole Detection

Six different targeted LC/MSMS methods have been used to acquire data in SRM (Selected Reaction Monitoring) mode. This method includes 97 molecules.

FIGURE 2. Schematic diagram of the Q Exactive High-Resolution, Accurate-Mass Instrument.

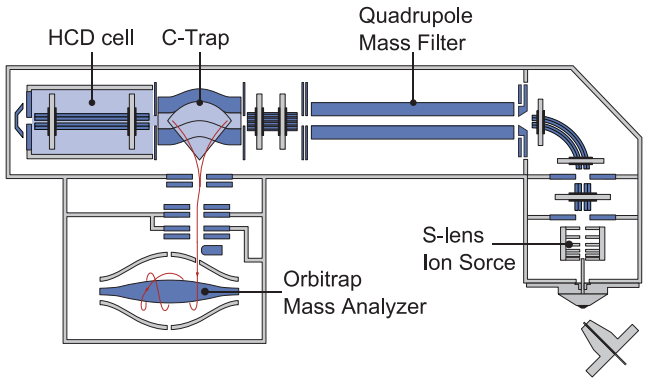


FIGURE 3. Scan Parameters for Q Exactive Mass Spectrometer

Parameter	Value
Full MS	
Microscans	1
Resolution (FWHM)	70,000
AGC Target	1e6
Maximum IT	250 msec
Scan Range	150-800 m/z
MS^2 Experiments	
Microscans	1
Resolution	17,500
AGC Target	1e5
Maximum IT	250 msec
NCE	70.0

FIGURE 4. Source Parameters for HESI Probe.

Parameter	Value
Sheath Gas	30
Aux gas	15
Spray voltage (V)	3500
Capillary temp (°C)	320
Vaporizer Temp (°C)	350

* Parameters are the same for positive and negative modes

Data Analysis

All MS data have been processed using ExactFinder 2.0 software. Identification of the analytes is performed using the exact mass of the precursor, the retention time, the isotopic distribution and the fragment exact masses.

Results

Data Processing

Chromatograms were reconstructed with a 5 ppm mass accuracy. The method was set to identify compounds based on the exact mass of the parent and the retention time. Confirmation was performed using the isotopic pattern and up to 5 fragment ions obtained from each precursor. A database containing up to 650 analytes was selected for processing. Figure 5 shows an example of the results page showing the XIC (extracted ion chromatogram) for Nordiazepam reconstructed with 5 ppm mass accuracy (a), isotopic pattern (b) and fragment ion confirmation (c).

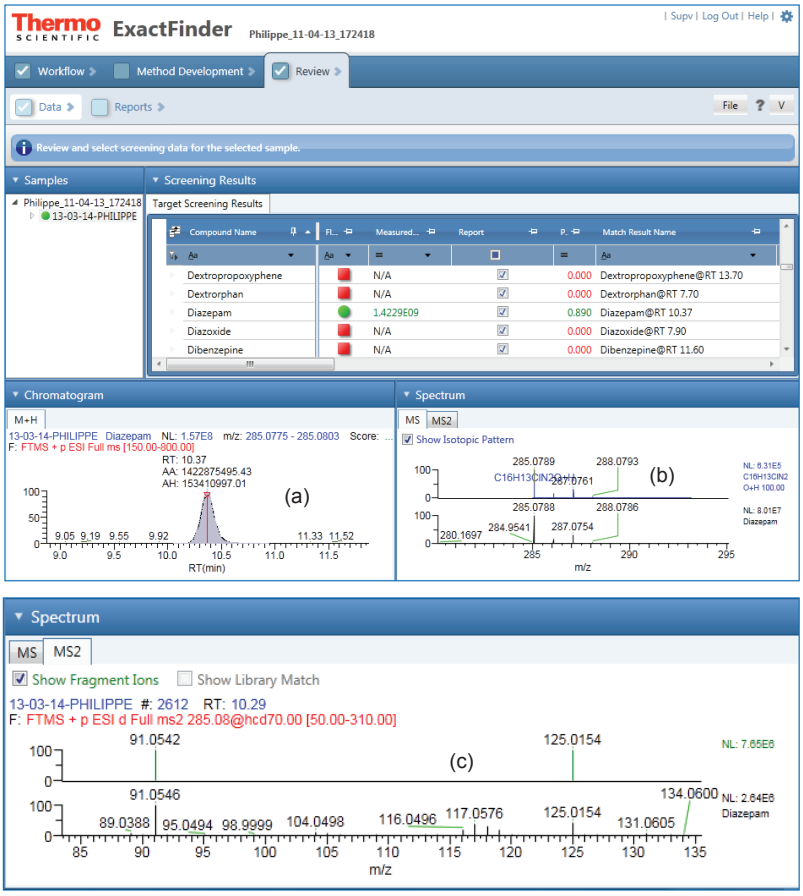
Results are reported using flags of different colors :

(green circle): When the sample/compound/peak combination is identified and fully confirmed.

(yellow triangle): When the sample/compound/peak combination is identified but not fully confirmed.

(red square): When the sample/compound/peak combination is not identified.

FIGURE 5. ExactFinder results page showing XIC chromatogram for Diazepam reconstructed with 5 ppm mass window (a), isotopic pattern (b) and fragment ion confirmation (c).



Metabolite Identification

In addition to compound identification, it is possible to confirm the results by identifying potential metabolites present in the sample. The approach is simple. As the acquisition is performed in Full Scan mode, identification of metabolites can be realized with the same HR-MS analysis by only extracting theroretical *m/z* values for predicted biotransformations. Figure 6 shows an example of metabolites identified from a single sample. The main compound identified is methadone and we have also been able to identify two major metabolites: EDDP and EMDP.

FIGURE 6. ExactFinder results page showing XIC chromatogram for EDDP (a), EMDP (b) and Methadone (c) reconstructed with 5 ppm mass window.

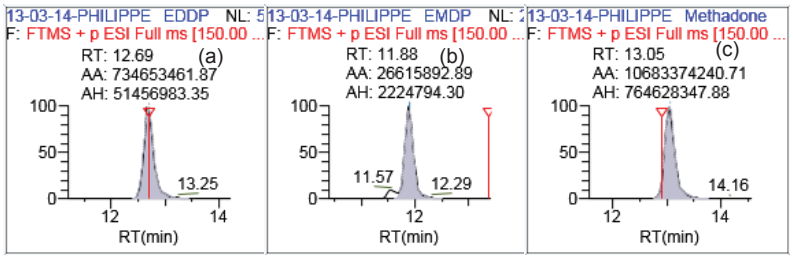
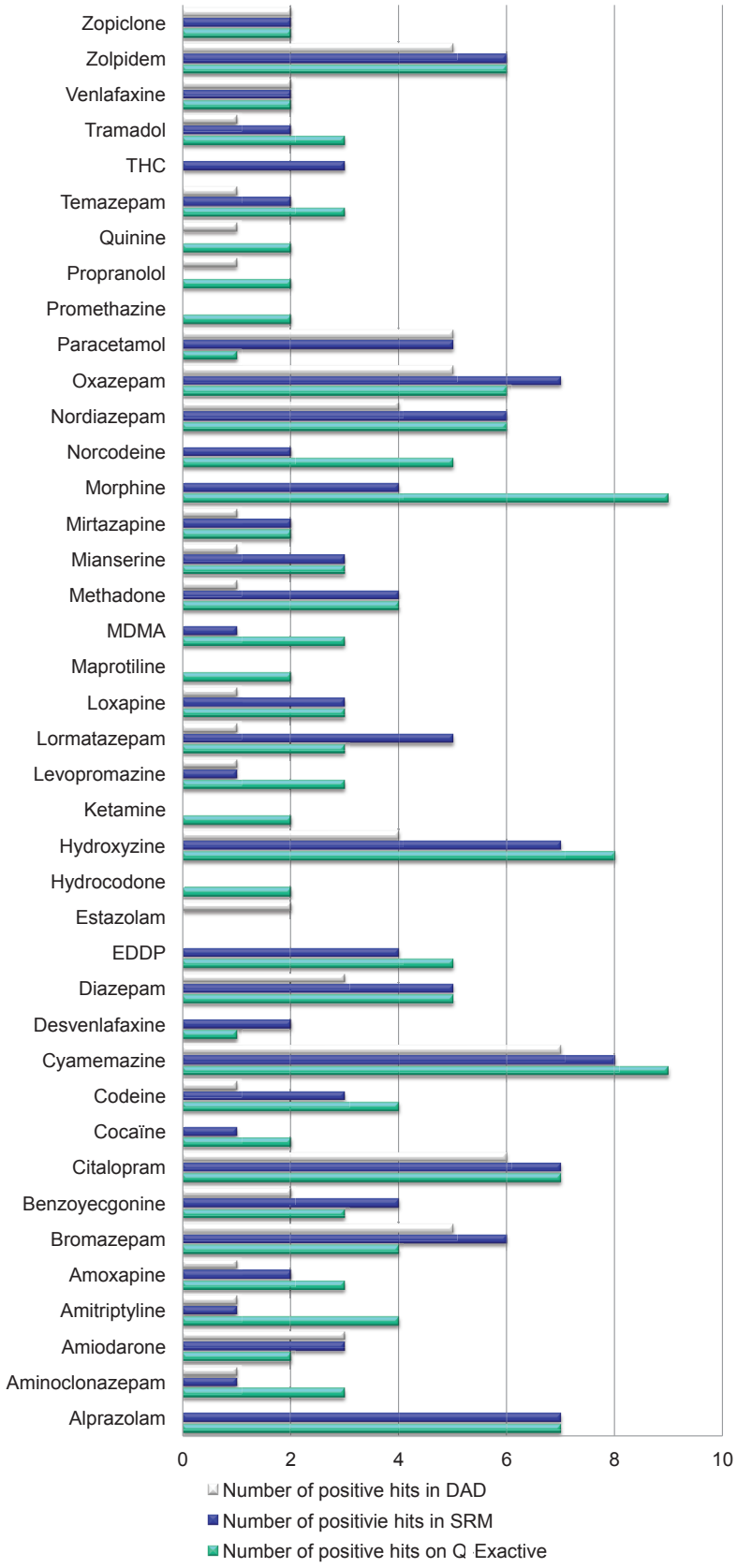


FIGURE 7. List of analytes that have been identified among 39 samples and confirmed using the 3 approaches: targeted screening in SRM, DAD and Q Exactive screening.



Comparison between the different approaches: DAD detection, targeted screening using a triple quadrupole, HRAM screening using the Orbitrap technology

We've analyzed and compared 39 samples using the 3 different technologies. Overall, the HRAM approach allowed identification of a higher number of analytes than the other approaches. We have been able to identify 143 compounds with the HRAM approach, 121 with the six targeted forensic screening methods performed on the triple quadrupole MS and 69 compounds using the DAD. Some of the results are reported in Figure 7 where we compare for 40 analytes (among the 77 identified) the number of positive hits obtained for each approach.

DAD Approach

Fewer analytes have been identified using this approach despite the size of the library (612 analytes). Sensitivity is certainly the main concern with this technique. Moreover, DAD may provide in some cases some false positive results. For example estazolam has been identified in DAD but not confirmed using the MS technologies. This approach is well known for its poor sensitivity in benzodiazepines analysis. As reported in Figure 7, alprazolam is not detected with DAD but is confirmed using the other two approaches.

Triple Quadrupole Approach Using the Six Targeted SRM Methods.

This approach gives good results in terms of positive hits identified. THC was identified using this approach as the sample preparation was done in acidic conditions unlike the other approaches where basic conditions were used. There are still some limitations. The identification is confirmed using six different SRM methods which means that we may have to inject the same sample several times. Moreover these six methods contain only 97 analytes. The run is performed in SRM mode and for this reason there is no capability for retrospective analysis and potential metabolite identification.

HRAM Approach Using the Q Exactive MS

This approach is able to identify the largest number of analytes with the 650 analytes library. But there are still some limitations to overcome. Precursor selection was done in the data-dependent operation mode where the most intense ion of the previous scan was selected for fragmentation. So we may, in some cases, have to add the compounds in the inclusion list in order to not miss the MS^2 acquisition. Some of the analytes listed are isomers (eg: maprotiline, paroxetine and EDDP). As they have exactly the same exact mass, we have to make sure they present different fragment ions in MS^2 or elute at different retention times. All data have been processed though ExactFinder 2.0 software with a 5 ppm mass accuracy. In this version of the software, the mass accuracy is set and can't be adjusted. For this reason, low mass fragments like the one we have with paracetamol at *m/z* 110.0595 are in some cases not properly identified with an accuracy of 5 ppm. This limitation is nevertheless going to be overcome with the launch of Thermo Scientific™ TraceFinder™ 3.0 where the mass accuracy is set by the user and can be expressed in ppm or milli-amu.

Conclusion

- The Q Exactive MS provides high confidence with high-resolution capabilities (up to 140,000 FWHM) for forensic screening.
- Data processing is performed using ExactFinder 2.0 software. Compounds are identified and confirmed using the exact mass of the precursor, the isotopic distribution, the retention time and the exact mass of up to 5 fragment ions.
- HRAM LC/MSMS method identified more compounds for forensic toxicology than Diode Array Detection and Triple Quadrupole Targeted SRMs methods.
- Additional information such as metabolites identification can be easily obtained by extracting the theoretical *m/z* values for predicted biotransformations
- This HRAM method also allows for retrospective data analysis.
- A new HRAM database (https://www.mzcloud.org/) will soon be available to perform targeted and also unknown identification.

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Verification of the Simultaneous Analysis of Heroin Addiction Treatment Compounds Using LC/MS/MS with a New Prelude SPLC™ System

Sarah Fair, Joseph Herman, and Kerry Hassell
Thermofisher Scientific, Franklin, MA

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Overview

Purpose: There are several compounds used for the treatment of heroin addiction. These compounds include methadone, buprenorphine, norbuprenorphine, naloxone, naltrexone , and their metabolites. The metabolites of interest are 2-ethylidine-1,5 dimethyl-3,3-diphenylpyrrolidine (methadone metabolite, aka EDDP), buprenorphine glucuronide (buprenorphine metabolite), and norbuprenorphine glucuronide (norbuprenorphine metabolite). All total, the analysis of these compounds for research includes 8 analytes with 4 internal standards, that are commonly used in the treatment of heroin addiction.

Methods: Samples for this analysis were prepared in human urine. After the addition of internal standard, they were injected for analysis using the Thermo Scientific™ Prelude™ SPLC sample preparation-liquid chromatography system. This system was fitted with a Thermo Scientific™ Accucore™ 100x3.0, 2.6 µm particle size column for separation. Additionally, a Thermo Scientific™ TSQ Vantage™ mass spectrometer in positive ion mode was used for analyte detection.

Results: All 8 compounds were simultaneously verified using the Prelude SPLC system and the TSQ Vantage MS. The resulting chromatography, correlation coefficients, standard curve linearity, quality control data, and analyte transitions are explained in the following sections to illustrate the success of this analysis.

Introduction

Several different compounds are currently used in the treatment of heroin addiction. These compounds and their metabolites were analyzed for research using the new Prelude SPLC system and a TSQ Vantage MS. This workflow takes advantage of a low system volume to decrease solvent consumption and successfully quantify methadone, 2-ethylidine-1,5 dimethyl-3,3-diphenylpyrrolidine (EDDP), buprenorphine, buprenorphine glucuronide, norbuprenorphine, norbuprenorphine glucuronide, naloxone, and naltrexone. This work verifies a heroin treatment panel method performed on the Prelude SPLC system. In order for this system to be evaluated, it must fall within certain acceptance criteria. These set parameters are designed to determine the success or failure of a particular LC-MS/MS workflow. These parameters include, but are not limited to:

1) The lower limit of quantitation (LLOQ) and low quality control need to be ±20% of the expected concentration.
2) All of the remaining calibrators and controls need to be ±15% in order for the instrument to be successfully validated.
3) All of these requirements must be met for three consecutive days so that interday and intraday accuracy and precision can be determined.
4) The signal in the blank following the highest standard may not exceed 20% of the LLOQ signal. This factor is often called carryover.

Methods

Sample Preparation

Human urine was spiked with all 8 analytes and then serial diluted into a calibration curve. Buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide had an analytical measurement range of 1.0 ng/mL to 100 ng/mL. Methadone, EDDP, naloxone, and naltrexone had an analytical measurement range of 5.0 ng/mL to 500 ng/mL. Quality controls were also prepared in human urine at three different levels. The urine aliquots were diluted with a combination of water and methanol that contained internal standards. These samples are then injected onto the system for analysis.

Liquid Chromatography

Chromatographic separations of all compounds were performed using Prelude SPLC system, seen in Figure 1, equipped with an Accucore 100x3.0mm C18 analytical column with 2.6 µm particle size. The system mobile phases consisted of 10mM ammonium formate, 0.05% formic acid in water and 10mM ammonium formate, 0.05% formic acid in methanol. The system needle washes were 60% water, 40% methanol, and 0.5% formic (aqueous) and 45% isopropanol, 45% acetonitrile, and 10% acetone (organic).

Mass Spectrometry and Data Analysis

The detector was a TSQ Vantage triple-stage quadrupole mass spectrometer with HESI-II ionization probe in positive ion mode. Quantitation of results was performed using Thermo Scientific™ LCQUAN™ software.

FIGURE 1. Prelude SPLC system

Results

Analyte result summary

Buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide were all prepared at a range of 1.0 ng/mL to 100 ng/mL with quality control concentrations at 3.0, 40.0, and 80.0 ng/mL. Methadone, EDDP, naloxone, and naltrexone were prepared at a range of 5.0 ng/mL to 500 ng/mL with quality control concentrations of 15.0, 200, and 500 ng/mL. Deuterated internal standards were used for each analyte. Methadone-d9 was used for the quantitation of methadone and EDDP. Naloxone-d5 was used for the quantitation of naloxone and naltrexone. Buprenorphine-d4 was used for buprenorphine and buprenorphine glucuronide, and norbuprenorphine-d3 for norbuprenorphine and norbuprenorphine glucuronide. The transitions used for the analytes and internal standards can be seen in Table 1.

Table 1. Analyte Transitions

Compound	Transition
methadone	310→265
EDDP	278→219
naloxone	328→212
naltrexone	342→270
buprenorphine	468→396
norbuprenorphine	414→187
buprenorphine glucuronide	644→468
norbuprenorphine glucuronide	590→414
methadone-d9	319→268
naloxone-d5	333→212
buprenorphine-d4	472→400
norbuprenorphine-d3	417→187

All analytes had linear calibration curves which are illustrated in Figure 2. The x-axis of each block is the area ratio of the analyte to the internal standard. The y-axis is the concentration in ng/mL. Additionally, near the top of each block the correlation coefficient values are posted. These values are also summarized in Table 2 for easier viewing. These r^2 values range from 0.9924 to 0.9995 for all compounds.

Figure 3 show the lower limit of quantitation (LLOQ) chromatograms for each of the 8 analytes. Buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide all have an LLOQ of 1.0 ng/mL while methadone, EDDP, naloxone, and naltrexone have an LLOQ of 5.0 ng/mL.

Figure 2. Calibration curve linearity for all analytes.

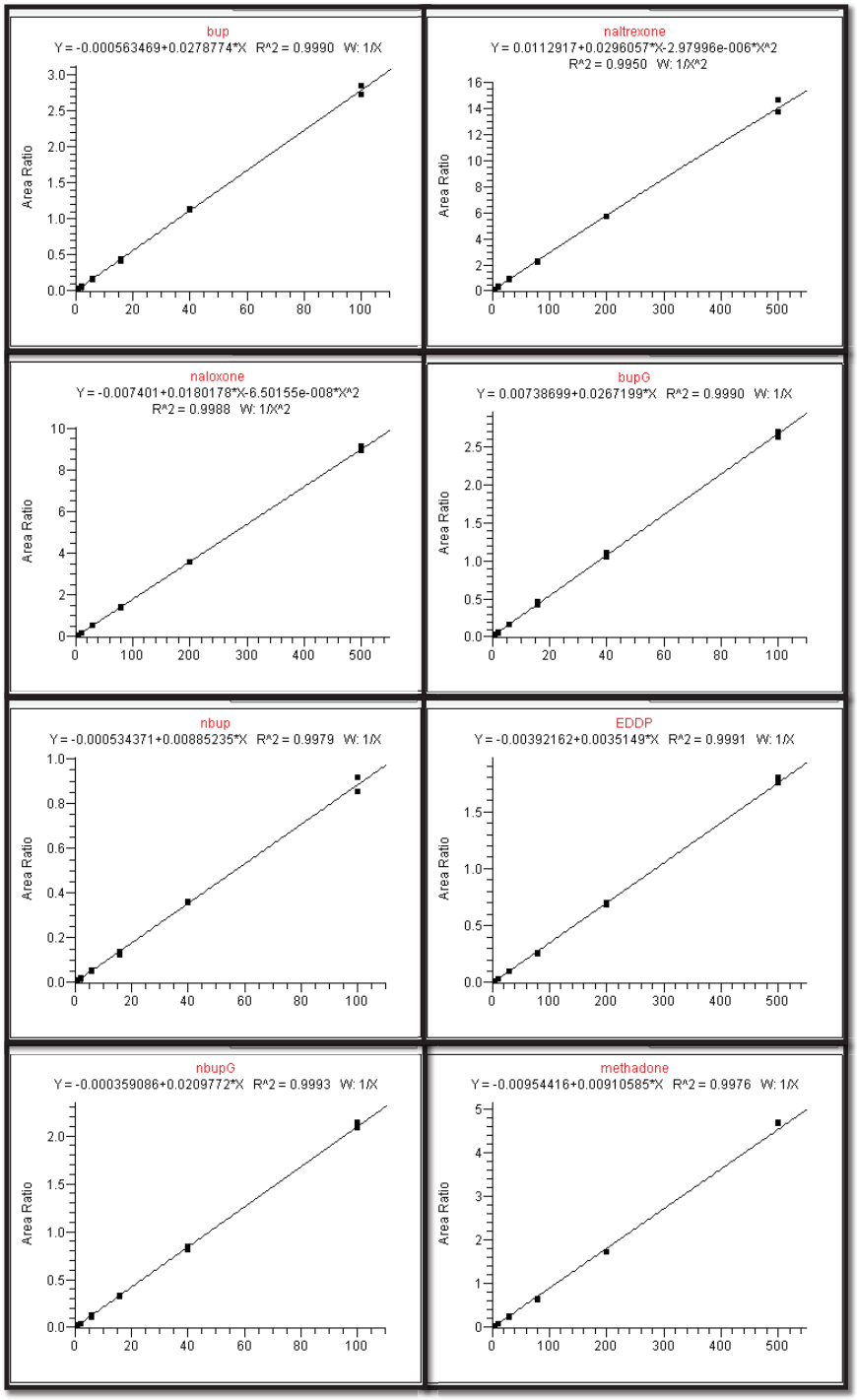

Table 2. Correlation coefficient values for all analytes

Analyte	r^2 day 1	r^2 day 2	r^2 day 3
buprenorphine	0.9949	0.9976	0.9983
norbuprenorphine	0.9985	0.9969	0.9979
buprenorphine glucuronide	0.9974	0.9982	0.9990
norbuprenorphine glucuronide	0.9993	0.9993	0.9993
methadone	0.9974	0.9976	0.9994
EDDP	0.9995	0.9991	0.9986
naloxone	0.9985	0.9942	0.9988
naltrexone	0.9951	0.9924	0.9950

Figure 3. Lower limit of quanititation (LLOQ) for all analytes.

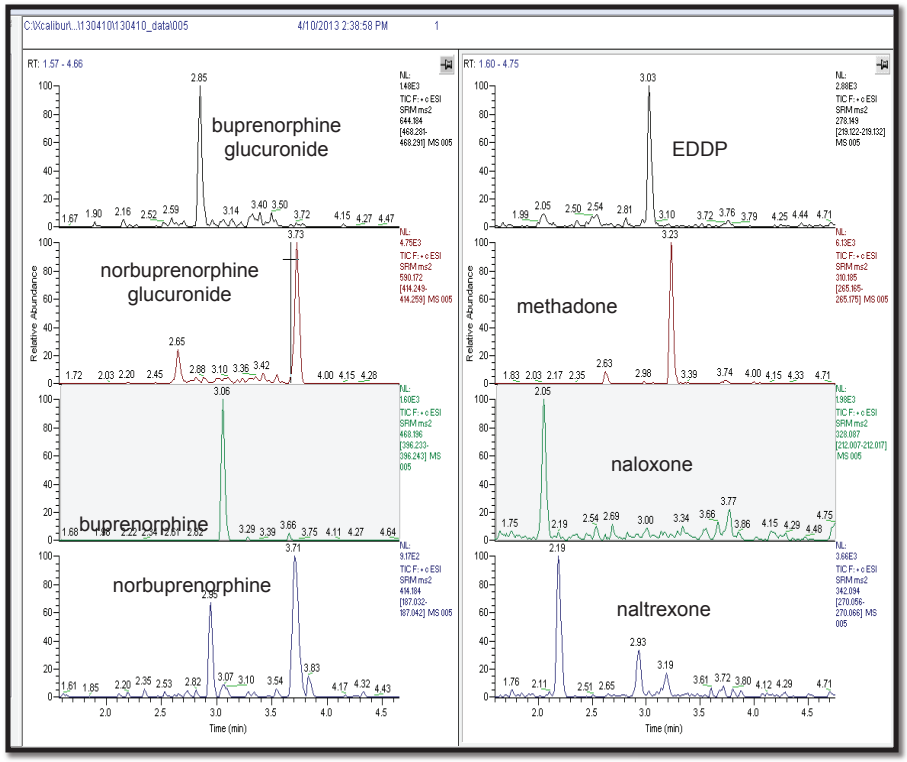

Figure 4 shows the matrix blank that is injected after the highest standard in the calibration curve often referred to as the upper limit of quantitation (ULOQ). This matrix blank (n=2) is used to assess the level of carryover for each analyte. The signal in the matrix blank cannot be greater than 20% of the LLOQ signal. All analytes have zero carryover at the retention time of interest with one exception: methadone has an average carryover of about 4.7%, but this is still well within the allowance of 20% of the LLOQ.

Figure 4. Carryover as shown in the matrix blanks injected after the ULOQ.

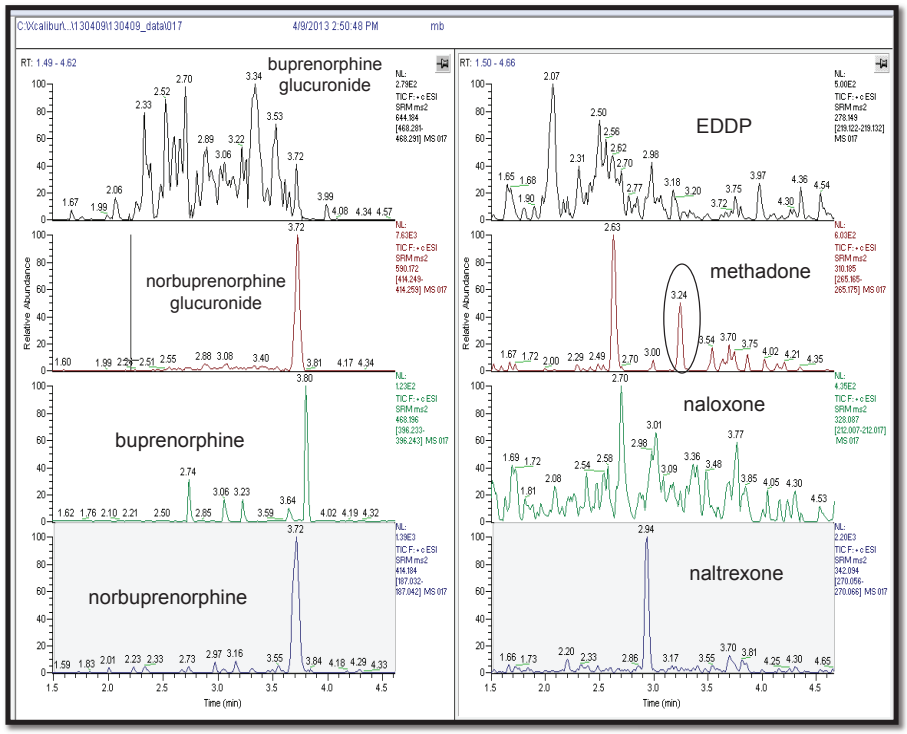

Table 3 shows the resulting quality control data from the interday and intraday accuracy and precision. Three consecutive days of runs were summarized to show the ending RSD percentages. All compounds had RSD values of ≤10% of the expected concentrations showing excellent accuracy and precision. The third column in the table shows the expected QC value with column 4, 5, and 6, showing the QC averages (run in replicates of 5) for each day. Then, in column 7, the overall average is calculated along with the standard deviation (SD) in column 8. Lastly, the %RSD can be seen in column 9.

Table 3. Quality control data summary.

Analyte	(ng/mL)	Expected	Day 1	Day 2	Day 3	Average	SD	%RSD
buprenorphine	Low QC	3.00	3.05	2.81	2.79	3.0	0.1	3.3
	Mid QC	40.0	36.4	38.7	40.0	38.0	1.8	4.7
	High QC	80.0	71.0	79.3	79.8	77.0	4.9	6.4
norbuprenorphine	Low QC	3.00	2.89	3.18	3.27	3.0	0.2	6.7
	Mid QC	40.0	37.4	39.1	37.8	38.0	0.9	2.4
	High QC	80.0	76.5	76.6	77.6	77.0	0.6	0.8
buprenorphine	Low QC	3.00	3.31	2.88	2.86	3.0	0.3	10.0
glucuronide	Mid QC	40.0	36.3	39.4	39.5	38.0	1.8	4.7
	High QC	80.0	74.3	79.8	80.9	78.0	3.5	4.5
norbuprenorphine	Low QC	3.00	2.74	3.18	3.01	3.0	0.2	6.7
glucuronide	Mid QC	40.0	37.8	38.8	38.7	38.0	0.5	1.3
	High QC	80.0	77.1	76.8	79.2	78.0	1.3	1.7
methadone	Low QC	15.0	14.8	15.6	15.1	15.0	0.4	2.7
	Mid QC	200	197	202	191	197	5.6	2.8
	High QC	400	418	412	409	413	4.9	1.2
EDDP	Low QC	15.0	14.8	14.5	14.6	15.0	0.2	1.3
	Mid QC	200	192	200	190	194	5.3	2.7
	High QC	400	398	411	399	403	7.1	1.8
naloxone	Low QC	15.0	14.7	15.5	16.5	16.0	0.9	5.6
	Mid QC	200	207	196	198	200	6.1	3.1
	High QC	400	396	415	387	399	14.6	3.7
naltrexone	Low QC	15.0	14.7	15.4	15.2	15.0	0.4	2.7
	Mid QC	200	201	192	194	195	4.8	2.5
	High QC	400	383	405	382	390	12.9	3.3

Conclusion

- All 8 compounds show excellent verification results using the Prelude SPLC system in combination with the TSQ Vantage MS. With quality control RSD percentages less than 10% and correlation coefficient values of 0.9924 to 0.9995, these verification analyses are proven to be very successful.
- Due to the low volume and low solvent consumption capabilities of the Prelude SPLC system, these compounds were analyzed for research in less time, using less solvent, and with reduced cost to a standard HPLC system
- The design of the Prelude SPLC system allows for efficient online sample clean-up that demonstrates reproducible, reliable data for all analytes, with a total injection time that less than 6 minutes.

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Development of a Dilute-and-Shoot LC-MS/MS Method with 21 Opiates in Urine

Simon Sheng,[1] Kate Evans,[2] Francois Espourteille[3]
[1]Thermo Fisher Scientific, San Jose, CA; [2]Thermo Fisher Scientific, San Diego, CA;
[3]Thermo Fisher Scientific, Franklin, MA, USA

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Introduction

Opiates are primarily central nervous system depressants and narcotic analgesics, which can be abused and often lead to addiction. Pain management physicians need to verify that patients are taking their prescribed medications as directed to monitor patients for compliance. The number of new pain management forensic laboratories in operation are increasing worldwide to keep up with sample demand.

Traditionally, urine opiates forensic testing has been by competitive immunoassays. While this method is fast and relatively inexpensive, it often lacks specificity, since the antibodies are directed against drug groups or classes, not at specific drug compounds. Other more specific methods, such as GC/MS, were used to identify or quantitate the concentration of a specific opiate. But GC/MS requires extensive sample preparation (derivatization) and suffers from long analytical run times.

LC-MS/MS is emerging as a new "gold standard" for opiates testing in current pain management forensic laboratories. A typical workflow includes acid or enzyme hydrolysis of opiates glucuronides into free opiates prior to LC-MS/MS analysis. This strategy might simplify the numbers of drugs to be analyzed, but also suffers from disadvantages such as long incubation times (2 hours+) and poor analyte recovery. Detection of opiate metabolites can also provide important evidence that the parent drug was possibly being abused. This work demonstrates a simple dilute-and-shoot quantitative method of 21 opiates, metabolites and common glucuronides by liquid chromatography tandem mass spectrometry (LC-MS/MS), without the limitation of time-consuming hydrolysis. This method provides broad drug coverage, fast turn-around time and compares favorably to hydrolysis analysis.

Methods

Urine specimens were diluted 4-fold with high purity water and 25 µl injected directly into The LC-MS/MS system (with addition of Internal Standard [IS}). A Thermo Scientific™ Transcend™ TLX-2 HPLC coupled with Thermo Scientific™ TSQ Quantum Ultra™ mass spectrometer was used for LC-MS/MS analysis in Selected Reaction Monitoring (SRM) mode. Two Thermo Scientific™ Syncronis™ C18, 3x100 mm 5 µm columns were used to achieve multiplexing capability. The LC run time is 8 minutes with MS data collection window of 3.5 minutes for all 21 Opiates, metabolites and select glucuronides.

All calibration standards, synthetic urine and deuterated internal standards used for quantitation were purchased from Cerilliant (Roundrock, TX). Surine was used as urine matrix to make calibration standards. Pilot study urine samples were obtained from commercial reference lab.

HPLC Method Parameters
LC: TLX-2 HPLC
Analytical Column: Syncronis C18, 3x100 mm, 5 µm column
Solvent A 0.1% formic acid in water
Solvent B 0.1% formic acid in methanol

Mass Spectrometer Parameters
MS: Thermo Scientific™ TSQ Quantum Ultra™ triple stage quadrupole
Polarity: Positive mode
MS Ionization Source: Heated electrospray (HESI)
Spray Voltage: 4000 V
Sheath Gas Pressure (N2): 60 arbitrary units
Auxiliary Gas Pressure (N2): 15 arbitrary units
Vaporizer Temperature: 600 C
Capillary Temperature: 350 C
Collision Gas Pressure: 1.5 mTorr
Q1 Peak Width: 0.7 Da
Q3 Peak Width: 0.7 Da

Data Acquisition and Processing
Thermo Scientific™ Xcalibur™ and Thermo Scientific LCQuan™ software

Quality Control
Each analyte and internal standard were identified with ion ratio. All compounds have to pass pre-set target ratios (qualifier ion/quantitation ion) at all calibration levels. The ion ratios criteria is as follows:

Target ratio(%)	Window(+/-%)
>50%	+/-20%
20-50%	+/-25%
10-20%	+/-30%
<10%	+/-50%

Results and Discussions

A multiplex quantitative method suitable for use in the forensic environment has been established. The 21 drugs included: fentanyl, norfentanyl, cis-tramadol, meperidine, normeperidine, O-desmethyltramadol, methadone, EDDP, codeine, hydrocodone , oxycodone, oxymorphone, morphine, hydromorphone , noroxycodone, norhydrocodone, Morphine-3-beta-D-glucuronide, morphine-6-beta-D-glucuronide, codeine-6-beta-D-glucuronide, oxymorphone-3beta-D-glucuronide, and hydromorphone-3-beta-D-glucuronide. Some of the drugs are isomers with the same parent mass and fragmentation ions. The accurate quantitation of these drugs can only be achieved by complete chromatographic separation.

We have optimized the LC separation by using several different column chemistries, mobile phases and pH conditions. Figures 1 and 2 show the chromatogram layout of 16 opiates and 5 glucuronides by using the Thermo Scientific™ Syncronis™ C18, 3x100 mm, 5 μm columns.

FIGURE 1. Chromatogram layout of 16 opiates.

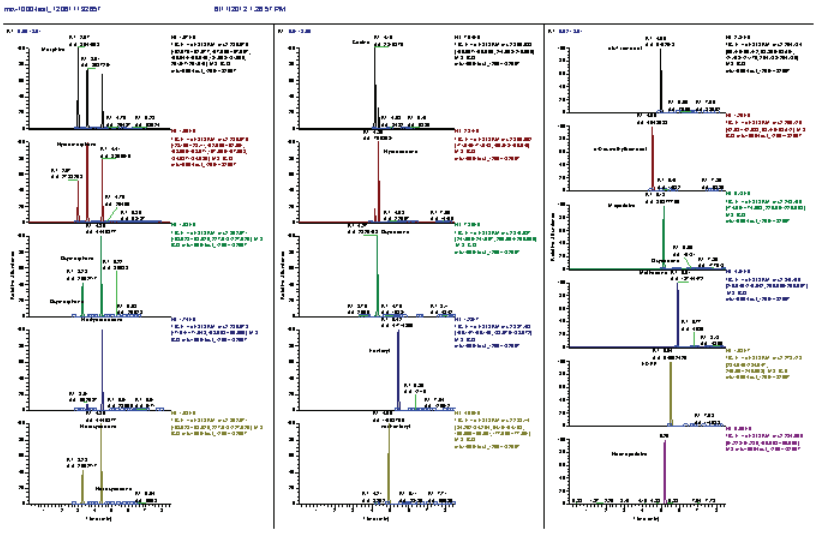
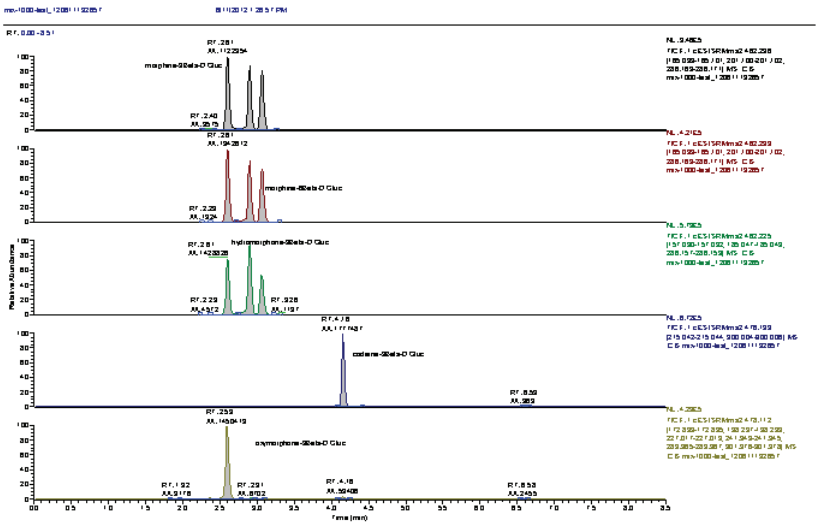
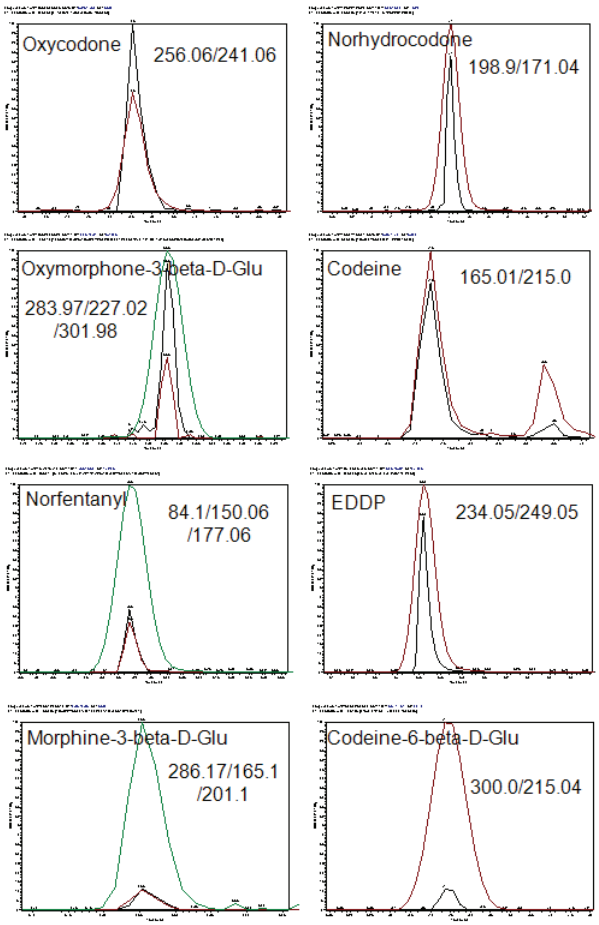


FIGURE 2. Chromatogram layout of 5 glucuronides



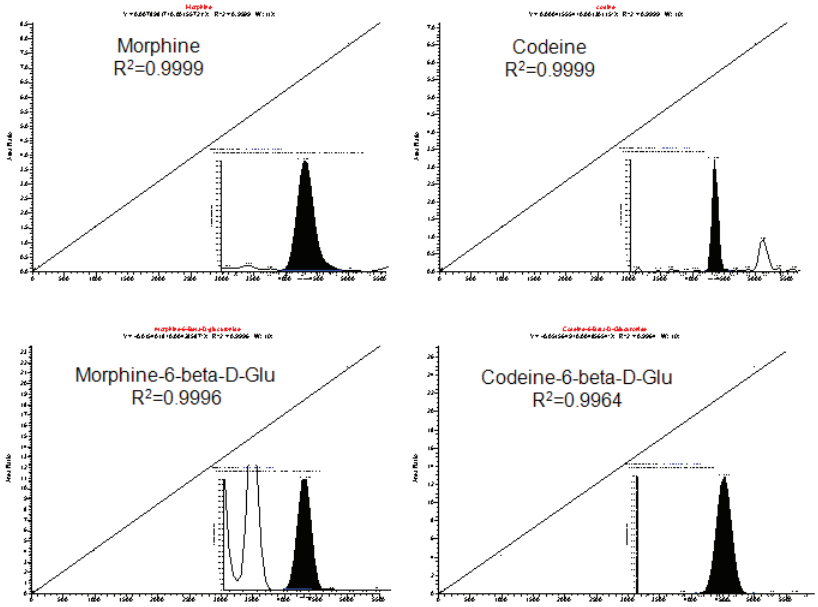
Stringent ion ratio criteria were used to ensure the specificity of MRM measurements in urine matrix. All compounds need to pass pre-set target ion ratios at all levels (Figure 3).

FIGURE 3. Example of chromatogram overlay of quantitative ion/qualifier ion at 25 ng/ml level



Prior to determining the calibration curve and range, the matrix blank was screened to ensure there was no detectable opiates. Surine was used as urine matrix to make calibration standards, which are made from serial dilution of Cerilliant stock standards. The deuterated drug compounds were spiked into specimens as internal standards. Calibration standards are in the range of 25-5000 ng/ml, with linear response of three orders of magnitude (R2 > 0.99) for all analyzed drugs. Figure 4 shows the representative calibration curves of selected analytes.

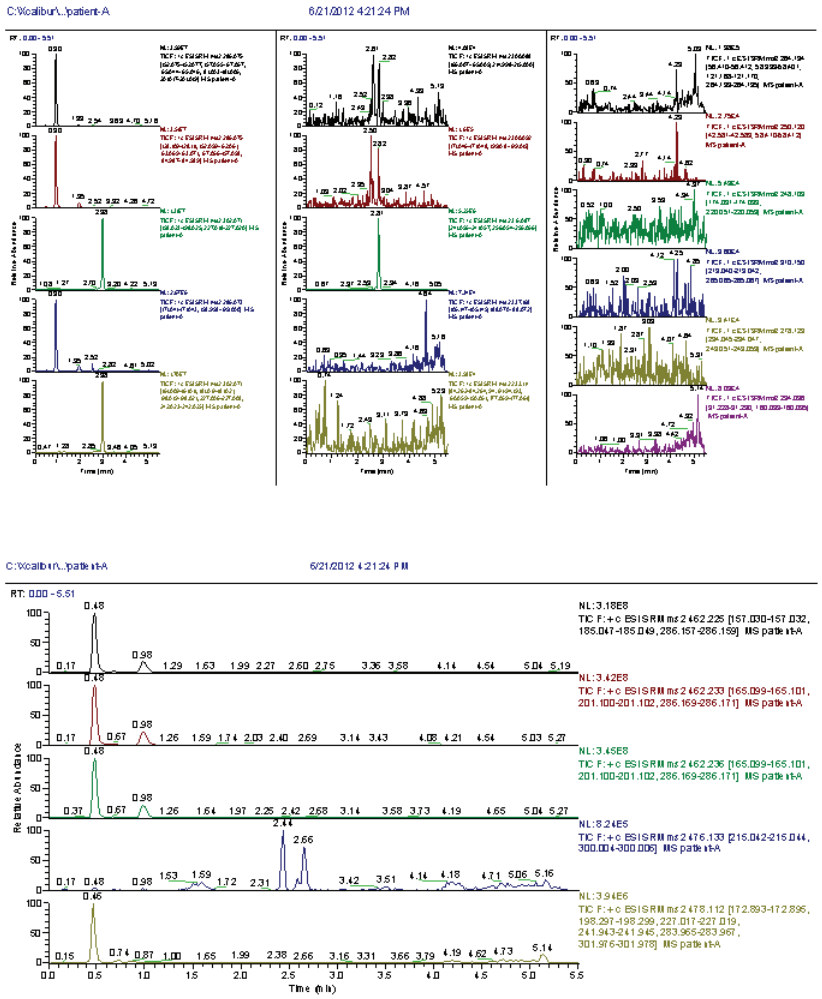
FIGURE 4. Calibration curves of Morphine, Codeine, morphine-6-beta-D-glucuronide and codeine-6-beta-D-glucuronide. The lower left insert of the chromatogram display shows the lowest calibrator 25 ng/ml.



The limits of quantitation (LOQ) were determined at 25 ng/ml for all drugs. All coefficients of variation (CV) (n=20) were less than 20% for the LLOQ and less than 10% for all other points of the curve. The CV% of retention times across two channels is less than 1%.

Ten pilot study urine samples that tested positive for opiates (from immunoassay screening) were used to test the correlation of the dilute-and-shoot method with an acid hydrolysis method provided by a reference lab. Variation of all positive opiate hits are within 20%, except for Morphine, which was <30%. Further investigation of morphine discrepancy was undertaken. One possible explanation is that some of the prescription morphine drug is actually made from morphine sulphate, which is not included in the original analyte list. Figure 5 shows the opiates chromatogram layout of one pilot study sample.

FIGURE 5. Opiates chromatogram layout of pilot study urine sample A



Conclusion

A quick, automated multiplex LC-MS/MS method has been developed for monitoring opiates in pain management forensic laboratories. This work demonstrates a technically simple but robust quantitative method to simultaneously measure 21 opiates, metabolites and select glucuronides in human urine. This dilute-and-shoot SRM LC-MS/MS methodology is suitable for use in high-throughput forensic laboratories where sample volume is high with minimized amount of sample preparation.

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Quantitative Analysis of THC and Main Metabolites in Whole Blood Using Tandem Mass Spectrometry and Automated Online Sample Preparation

Valérie Thibert, Bénédicte Duretz Thermo Fisher Scientific, Courtaboeuf, France
Christophe Petit, Martine Lachambre Analysis Expertise, Epinal, France

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Overview

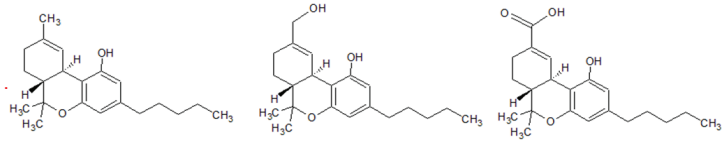
Purpose: Sensitive quantification of THC, 11-OH-THC and THC-COOH from whole blood with Thermo Scientific TurboFlow technology. For confirmation purposes, expected limit of quantification must be close to 0.5 ng/mL.
Methods: Blood samples were treated by protein precipitation followed by an online extraction and analysis by Reverse Phase Liquid Chromatography (RP-LC) coupled to mass spectrometry.

Results: This method was linear from 0.5-100 ng/mL for THC and its metabolites with good repeatability and sensitivity.

Introduction

Cannabis is the most highly used illicit substance around the world, and due to its psychoactive effects, it is of great importance to have analytical procedures for the assessment of the extent of its abuse. The major psychoactive constituent product of cannabis is Δ^9-tetrahydrocannabinol (THC) that is rapidly metabolized mainly in11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and then in 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), chemical structures are presented on figure 1.

FIGURE 1. Molecular structures of Δ^9-tetrahydrocannabinol (THC) and main metabolites,11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH).



To have a better understanding of the effects of cannabis abuse, blood analysis is recommended. Nevertheless, THC and 11-OH-THC have short windows of detection in this matrix, and therefore limits of detection for their analysis are often settled to concentrations as low as 0.5 ng/mL.

In recent years, LC-MS has gained ground to GC-MS as a reference method for the analysis and confirmation of drugs of abuse in biological matrices in clinical and forensic toxicology. In the case of cannabinoids, it is particularly interesting to attain high sensitivities without a need for derivatization, but one of the key parameters to achieve sensitivity requirements is the choice of an appropriate sample treatment prior to the LC-MS method.

Thermo Scientific TurboFlow technology is an automated online sample preparation technique that has been coupled to LC-MS/MS for the quantitative analysis of biological samples. Our goal is to develop a method to measure THC and its metabolites by reducing method time while attaining good analytical performances.

Methods

Sample Preparation

A 0.2-mL sample (whole blood) was spiked with internal standards (IS) and then mixed with 0.4 mL of 0.1% formic acid in acetonitrile (v/v) . The mixture was vortexed and stored at 0 °C for 10 min. After a 2 minutes sonication, the mixture was centrifuged at 10,000 rpm for 10 min, and 90 µL of supernatant was injected for LC-MS/MS analysis.

TurboFlow and LC method

The TurboFlow™ method was performed in Focus mode (figure 2) with a Thermo Scientific TurboFlow Cyclone-P column. Analytical separation was carried out on a Thermo Scientific Accucore C18 column (50×2.1 mm, 2.6-µm particle size) . The mobile phases were as follows: loading A : 0.1% formic acid in water; loading C: 0.1% formic acid in acetonitrile; loading D : mixture of isopropanol, acetonitrile, and acetone (40/40/20 v/v/v) ; elutingC : 10mM ammonium formate + 0.1% formic acid in water; elutingD : 0.1% formic acid in methanol. The total LC runtime was 10.4 min (Figure 3).

FIGURE 2. . "Focus Mode Technical" diagram of TurboFlow Technology.

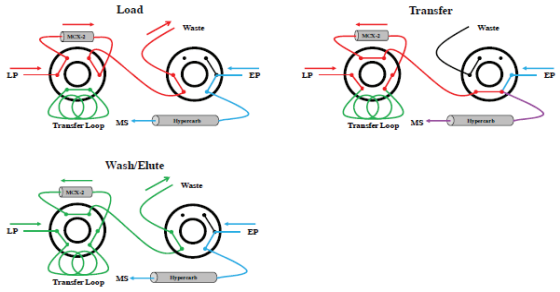


FIGURE 3. TurboFlow and LC method conditions.

Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
0.00	45	1.50	Step	80.0	-	20.0	-	====	out	0.40	Step	-	-	80.0	20.0
0.75	75	0.10	Step	90.0	-	10.0	-	T	in	0.30	Step	-	-	80.0	20.0
2.00	119	1.50	Step	-	-	100.0	-	====	out	0.40	Ramp	-	-	2.0	98.0
3.98	100	1.00	Step	-	-	-	100.0	====	out	0.40	Step	-	-	2.0	98.0
5.65	15	0.50	Step	-	-	-	100.0	T	out	0.01	Step	-	-	2.0	98.0
5.90	30	1.50	Step	-	-	100.0	-	====	out	0.40	Step	-	-	2.0	98.0
6.40	90	1.50	Step	20.0	-	80.0	-	====	in	0.40	Step	-	-	80.0	20.0
7.90	150	1.00	Step	80.0	-	20.0	-	====	out	0.40	Step	-	-	80.0	20.0

TurboFlow method conditions (Loading Pump)

LC gradient conditions (Eluting Pump)

Mass Spectrometry

A Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode for THC and 11-OH-THC and in negative ionization mode for THC-COOH. Data were acquired in the selected reaction monitoring (SRM) mode (Figure 4).

FIGURE 4. MS source parameters and SRM transitions.

Ionization	HESI-II
Spray voltage (V)	3500 (+) 2700 (-)
Vaporizer Temp (°C)	330
Capillary Temp (°C)	270
Sheath gas (AU)	35
Auxilliary gas (AU)	25
Ion sweep gas (AU)	5
Collision gas pressure (mTorr)	25
Q1 (FWMH)	0.4
Q3 (FWMH)	0.7

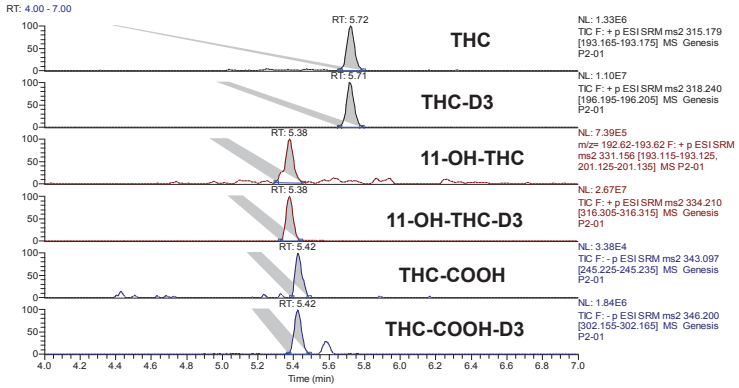
Compound	Parent Ion (m/z)	Product Ion (m/z)	S-Lens	CE	Polarity
THC	315.2	193.2	89	20	+
THC-D3	318.2	196.2	89	23	+
11-OH-THC	331.1	193.1	83	24	+
11-OH-THC-D3	334.2	316.3	83	15	+
THC-COOH	343.1	245.2	118	28	-
THC-COOH-D3	346.2	302.2	119	21	-

Results

Method Development

Different TurboFlow columns (Cyclone, Cyclone P, Fluoro, Phenyl-Hexyl) were evaluated with different loading conditions. Also different separation columns were evaluated (Accucore C18, Hypersil Gold C18, Accucore PFP and Accucore aQ) with different gradients. And finally, transfer optimization was also studied. The final chromatogram is shown in Figure 5.

FIGURE 5. SRM chromatograms of THC, 11-OH-THC and THC-COOH as well as deuterated standards (D3) from a blood sample spiked at 0.5 ng/mL.



Recovery and matrix effects

Precipitation Recovery was obtained by comparing an injection of whole blood spiked with the analytes and then crashed, against whole blood crashed first and then spiked.

On-line extraction Recovery was evaluated by comparing a direct injection of a standard solution to the analytical column against an injection to the TurboFlow column.

Matrix Effects were evaluated by comparing an injection of standard solution to the TurboFlow column against an injection of blood spiked at the same concentration.

Overall recovery was obtained considering both recovery and matrix effects. Results are presented on figure 6.

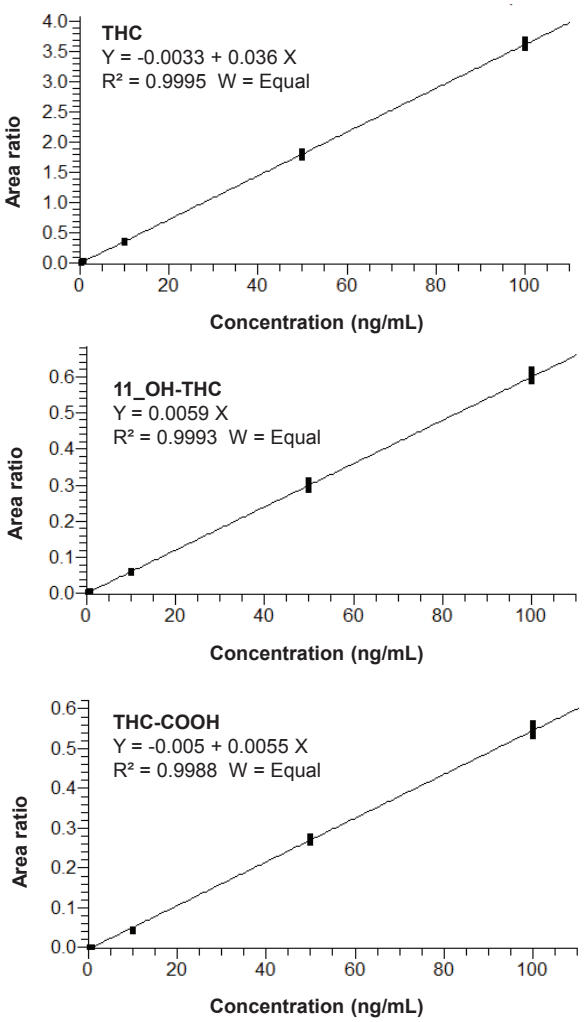
FIGURE 6. Method recovery and matrix effects.

The concentration was 7.5 ng/mL in standard , crashed whole blood and whole blood samples. Injection volume was set to 20µL in all cases and 5 injections were performed in each condition.

Compound	Precipitation recovery	On-line extraction Recovery	Matrix effects	Overall recovery
THC	97%	61%	+ 50%	92%
11-OH-THC	94%	82%	+ 36%	112%
THC-COOH	88%	76%	- 5%	73%

Calibration curves were generated with LCQuan 2.7 SP1 software by injecting whole blood samples spiked with THC, 11-OH-THC and THC-COOH. And crashed before injection Their deuterated (D3) compounds were used as internal standards. With a concentration of 17ng/mL The calibration model was linear with an equal weighting. In these conditions, curves were linear through the calibration range, from 0.5ng/mL to 100ng/mL. The calibration curves are presented in figure 7.

FIGURE 7. Calibration curves for THC, 11-OH-THC and THC-COOH from spiked and crashed whole blood. Calibration ranges goes from 0.5ng/mL to 100ng/mL.



Each calibration point was injected 10 times. The mean calculated concentration, the accuracy (%Diff) and the repeatability (%RSD) for each calibration point are presented in figure 8.

FIGURE 8. Accuracy (%Diff) and repeatability (%RSD) obtained for each calibrator (n=10)

Conc (ng/mL)		0.5	1	10	50	100
THC	Mean	0.49	1.02	10.03	49.5	94.8
	%Diff	-2	+2	+0.3	-1	-5.2
	%RSD	5	3	2	2	2
11-OH-THC	Mean	0.50	1.00	9.97	49.7	94.6
	%Diff	0	0	-0.3	-0.6	-5.4
	%RSD	5	4	3	3	2
THC-COOH	Mean	0.50	1.00	9.95	50.4	94.2
	%Diff	0	0	-0.5	+0.8	-5.8
	%RSD	9	7	2	2	2

Limits of quantification were determined as the lowest concentration for which a 20% RSD is obtained as well as a bias inferior to 20%. The results are presented on figure9.

FIGURE 9. Limits of quantification for THC, OH-THC and THC-COOH in spiked and crashed whole blood samples.

Compound	Concentration (ng/mL)	% RSD (n=10)	Bias (Mean +/- RSD)
THC	0.5	5	0.49 +/- 0.02
11-OH-THC	0.5	5	0.50 +/- 0.03
THC-COOH	0.5	9	0.50 +/- 0.04

The limits of quantification satisfy the requirements for cannabis analysis in whole blood, considering that the limits of detection are expected to be close to 0.5 ng/mL.

Conclusion

- A fast, automated, and analytically sensitive LC-MS/MS method was developed to quantify THC and its metabolites in crashed whole blood.
- The total online extraction and analytical LC runtime was 10.4 minutes. This throughput could be increased by multiplexing this method on a Thermo Scientific Transcend TLX system.
- This method was linear from 0.5 to 100 ng/mL.
- The lower limit of quantitation was at least of 0.5 ng/mL for THC and its metabolites. Good repeatability was obtained for the different calibration levels with %RSD inferior to 10%.
- Correlation between GC-MS and this analytical method is being performed by Analysis – Expertise laboratory.

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3. Peters F., Drummer O., Musshoff F. – Forensic Science International 165 (2007) 216-224

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Quantitation of Seven Designer Cathinones in Urine Using Q Exactive Mass Spectrometer

Marta Kozak,[1] Kristine Van Natta,[1] Shijun Sheng[2]
[1]Thermo Fisher Scientific, San Jose, CA, USA
[2]Thermo Fisher Scientific, Franklin, MA, USA

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Overview

Purpose: To develop an HPLC-MS method for the forensic toxicological analysis of the three Schedule I cathinones: MDPV, methylone and mephedrone, as well as other substituted cathinones: methedrone, ethylone, butylone and naphyrone in urine with limits of quantitation (LOQs) of 0.5 ng/mL.

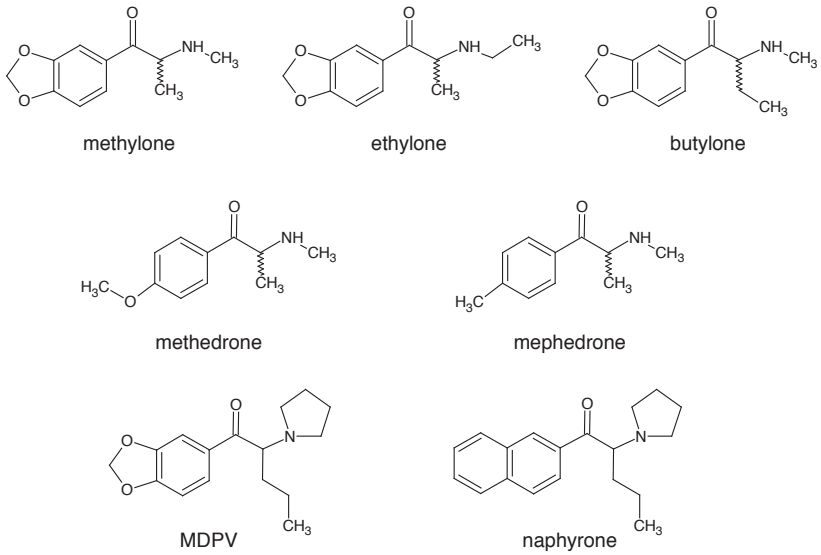
Methods: Liquid/liquid extraction followed by HPLC/MS/MS analysis on a Thermo Scientific™ Q Exactive™ benchtop Orbitrap mass spectrometer.

Results: We achieved LOQs of 0.5 ng/mL with good reproducibility and accuracy for MDPV, mephedrone, methylone, methylone, ethylone and butylone. Naphyrone showed more variability and is considered qualitative using this method.

Introduction

Substituted cathinones, or "Bath Salts," have become the latest abused designer drugs. Based on cathinone, a substance found in the African *Catha edulis* (khat) plant, substituted cathinones are stimulants with amphetamine- and cocaine-like effects. As with many designer drugs classes, variations on base structure abound (Figure 1). On October 21, 2011 the United States Drug Enforcement Agency (US DEA) listed three of the most common chemicals – methylenedioxy pyrovalerone (MDPV), methylone and mephedrone – as Schedule I drugs, thereby making them illegal. As these drugs are not detected by current ELISA drug screening tests, new methods are needed to detect and quantitate these compounds.

FIGURE 1. Structures of designer cathinones

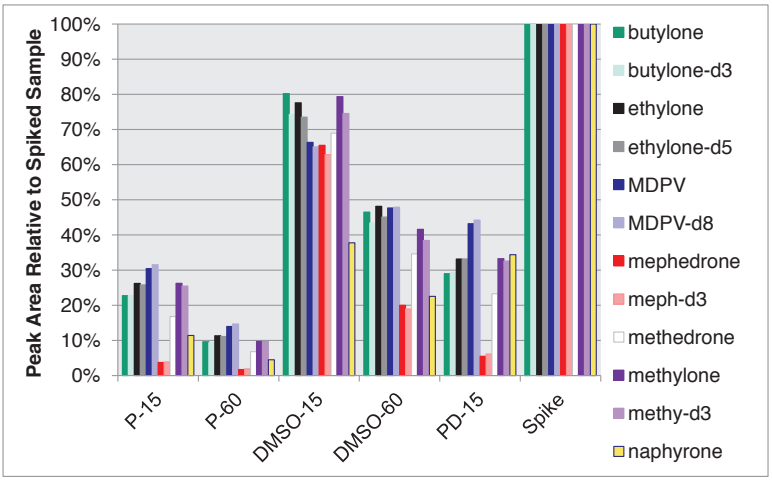


Methods

Method Development

This assay was originally developed as a urine dilution method. When a lower limit of quantitation (LOQ) was desired, liquid/liquid extraction was developed to concentrate the samples. Initial experiments showed good linearity and detection limits, but also low recovery and highly variable internal standard responses. To investigate and mediate the possible loss of analytes during the evaporation step, the following experiments were performed: 1 & 2) Evaporate samples for either 15 minutes or 60 minutes; 3 & 4) Add 20 µL of DMSO to the tubes before evaporation to prevent samples from evaporating to dryness, again for 15 or 60 minutes; 5) Add 20 µL DMSO to tubes after evaporation to determine if solubility is an issue; and 6) Spike a blank processed sample with analytes after evaporation as 100% recovery. Results shown in Figure 2 indicate that evaporation time is critical, especially for mephedrone, the smallest molecule tested, and solubility might be an issue for naphyrone.

FIGURE 2. Results for method development experiments to determine effects of evaporation step in sample processing. P = plain tubes after -15 and -60 min evaporation; DMSO = tubes with DMSO added prior to -15 and -60 min evaporation; PD = evaporated without DMSO, add DMSO after 15 min evaporation; Spike = compounds spiked after 15 min evaporation



Sample Preparation

Deuterated internal standards were available for all compounds except methedrone and naphyrone. Butylone-d3 was used as internal standard for methedrone and MDPV-d8 was used for naphyrone. Samples preparation is a liquid-liquid extraction (LLE). 200 µL of urine and 10 µL of internal standard mix solution (2 µg/mL of each deuterated IS) were basified with 100 µL of 1 N NaOH. Extraction was performed by adding 1 mL of ethylacetate:hexane (1:1), mixing and centrifuging. 800 µL of the resulting supernatant was transferred to a clean test tube containing 20 µL of DMSO to prevent complete evaporation of solvent. Analytes are small and slightly volatile, and will evaporate if left too long in the evaporator. Solvent was evaporated at 37 °C under nitrogen for 15 minutes. 200 µL of 5% methanol was added, mixed and transferred to an HPLC vial with limited-volume insert. 20 µL was then injected onto HPLC-MS.

Liquid Chromatography

Chromatographic analysis was performed using the Thermo Scientific™ Accela™ 600 HPLC pump and a Thermo Scientific™ Hypersil™ GOLD C18 column (50 x 2.1 mm, 3 µm particle size) under gradient conditions (Figure 3). Mobile phases A and B consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol, respectively. Mobile phase C was acetonitrile:1-propanol:acetone (45:45:10). The total run time was 5 minutes.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific Q Exactive bench-top Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe (Figure 5). The Q Exactive was operated in t-MS^2 mode at a resolution of 17,500 (@*m/z*=200). Exact masses, collision energies and fragment ions are listed in Figure 6.

FIGURE 3. HPLC gradient for cathinone analysis.

Time (min)	%A	%B	%C	Flow (µL/min)
0	90	10	0	500
0.15	90	10	0	500
2.15	5	95	0	500
2.45	5	95	0	500
2.46	0	0	100	500
3.30	0	0	100	500
3.31	90	10	0	500
5.00	90	10	0	500

FIGURE 4. Mass spectrometer source conditions.

Parameter	Value
Sheath Gas	35
Aux gas	15
Sweep gas	1
Discharge current	4
Capillary temp	320
S-Lens RF Level	60
Vaporizer Temp	350

Data Analysis

Data acquisition and processing were performed using Thermo Scientific™ TraceFinder™ software.

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at low (LQC), middle (MQC) and high (HQC) concentrations. Intra-run variability and robustness were determined by processing six replicates of each QC level along with a calibration curve as outlined in the Sample Preparation section on three different days. Matrix effects were investigated by comparing peak areas of analyte at 10 ng/mL, and internal standard prepared in 12 different lots of urine to those of a sample prepared in water.

FIGURE 5. Diagram of Q Exactive Mass Spectrometer

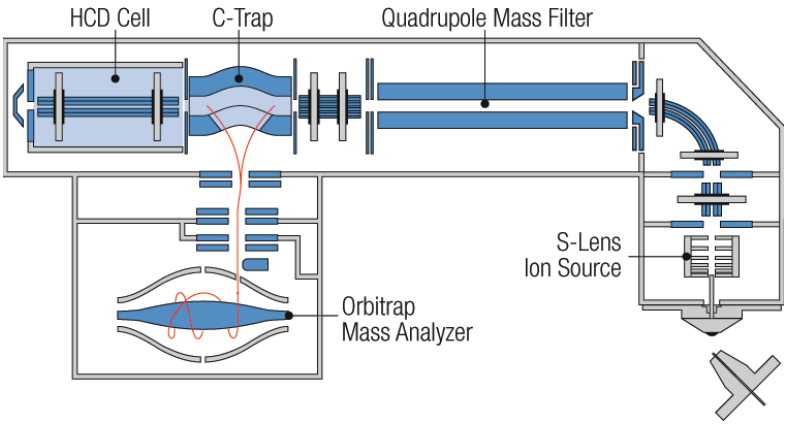


FIGURE 6. Exact masses and normalized collision energies (NCE) for cathinones

Analyte	m/z	NCE	Quantifier m/z	Qualifier m/z
Mephedrone	178.1226	35%	160.1119	145.0885
Mephedrone-d3	181.1415	35%	163.1306	148.1072
Methylone	208.0968	35%	160.0756	190.0861
Methylone-d3	211.1156	35%	163.0943	193.1049
MDPV	276.1594	60%	126.1278	135.0440
MDPV-d8	284.2096	60%	134.1779	135.0440
Naphyrone	282.1852	45%	141.0697	211.1115
Ethylone	222.1125	45%	174.0911	204.1016
Ethylone-d5	227.1438	45%	179.1224	209.1329
Butylone	222.1125	45%	174.0912	204.1017
Butylone-d3	225.1313	45%	177.1099	207.1205
Methedrone	194.1176	35%	176.1069	161.0834

Results

MDPV, methylone, mephedrone, methedrone, ethylone and butylone were all linear from 0.5 to 1000 ng/mL. Figure 7 shows representative calibration curves for all compounds. Figure 8 shows representative chromatogram at 0.5 ng/mL for all compounds tested. Inter-assay quality control statistics shown in Figure 9 demonstrate the method to be reproducible across the calibration range for the above compounds. Limited matrix effects were seen for the above compounds, and those were largely mediated by deuterated internal standards. The absolute recoveries of all cathinones tested in various lots of urine compared to a sample prepared in water ranged from 89% to 163%. Relative recoveries ranged from 105% to 136%. Precision across all lots also improved when deuterated internal standards were used.

Although naphyrone was detected at 0.5 ng/mL, it showed more variability than the other compounds and a greater matrix effect from lot to lot. Absolute recoveries for naphyrone ranged from 146% to 754% while relative recoveries using MDPV-d8 as internal standard ranged from 150% to 596%. All available internal standards were tried, and MDPV-d8 showed the best results. A lack of a deuterated analog for naphyrone does not allow for matrix effect corrections and negatively effects method precision. In this assay, naphyrone should be considered qualitative.

FIGURE 7. Representative calibration curves of cathinones in urine.

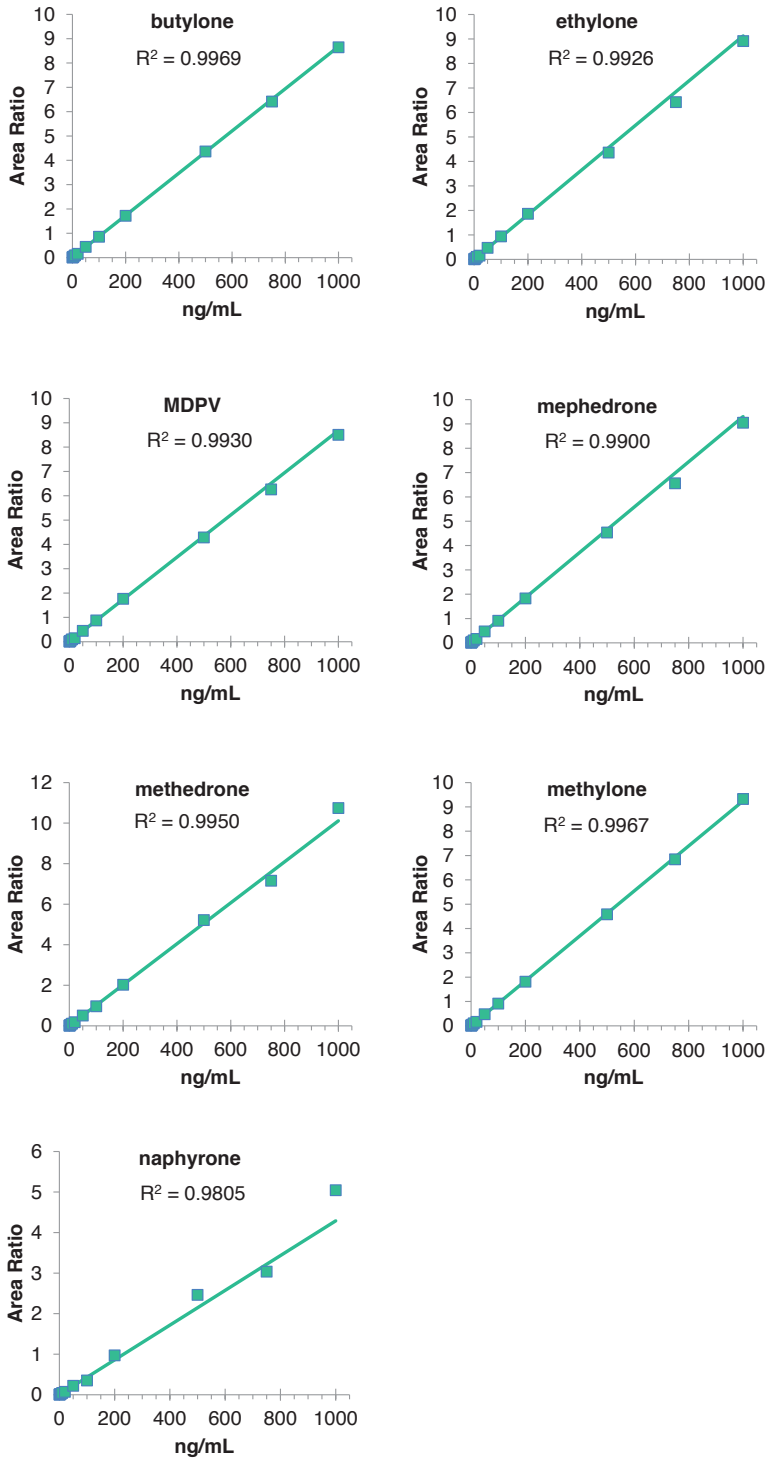


FIGURE 8. Representative chromatogram of cathinones at 0.5 ng/mL in urine reconstructed at 5 ppm mass accuracy.

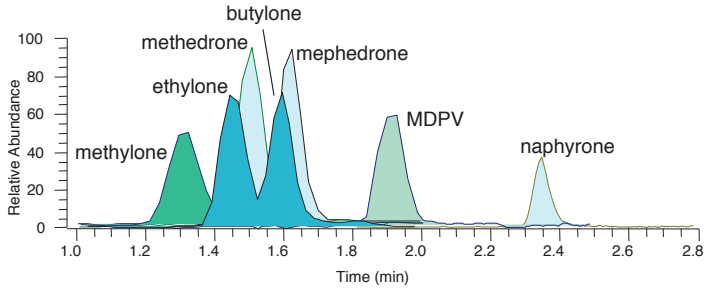


FIGURE 9. Inter-assay QC results

	butylone			ethylone		
	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL
Mean	2.28	26.0	99.0	2.31	25.9	99.6
%Bias	-8.7	4.1	-1.0	-7.4	3.4	-0.4
%CV	3.7	3.8	4.4	3.4	3.0	1.7

	MDPV			mephedrone		
	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL
Mean	2.30	25.7	98.4	2.38	26.7	97.8
%Bias	-8.1	2.9	-1.5	-4.7	6.8	-2.2
%CV	5.8	6.3	5.1	7.1	1.9	2.7

	methedrone			methylone		
	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL
Mean	2.49	28.1	105	2.34	26.1	99.6
%Bias	-0.3	12.5	5.0	-6.5	4.6	-0.4
%CV	4.4	6.4	3.1	5.1	1.8	3.1

	naphyrone		
	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL
Mean	2.55	22.9	103
%Bias	2.1	-8.3	3.0
%CV	22	8.5	5.2

Conclusion

We achieved our goal of a 0.5-ng/mL LOQ for the three newly-regulated cathinones, MDPV, mephedrone and methylone, as well as methylone, ethylone and butylone in urine. Naphyrone, which shows greater variability, can be detected down to 0.5 ng/mL in a qualitative manner. Deuterated internal standards are essential for rigorous quantitation of these compounds.

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Simultaneous Quantitation of 43 Drugs in Human Urine with a "Dilute-and-Shoot" LC-MS/MS Method

Xiang He and Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Application Note 576

Key Words

TSQ Quantum Access MAX, forensic toxicology, drugs of abuse, pain management drugs, urine, quantitation

Goal

The goal of this work was to develop a simple "dilute-and-shoot" liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantitation of 43 drugs of abuse, including pain management drugs, in human urine for forensic toxicology purposes. The drugs to be analyzed included opioids, amphetamines, benzodiazepines, cocaine, buprenorphine, methadone, and some of their metabolites. An additional objective was to use ultra-high-pressure liquid chromatography (UHPLC) to improve throughput and sensitivity of the method.

Introduction

LC-MS/MS has become more accepted as the tool for quantitative analysis of drugs in forensic toxicology laboratories. This technique enables simultaneous detection of multiple analytes of interests and is compatible with a simple "dilute-and-shoot" sample preparation method for urine samples.

Methods

Sample Preparation

Nine individual human urine and pure water samples were spiked with 20 and 200 ng/mL of the 43 drugs of abuse, pain management drugs, and with internal standards (IS). The samples were then mixed with β-glucuronidase and incubated at 60 °C for hydrolysis. Methanol was added to the mixture and the supernatant was diluted with water. The final dilution factor was 20. The mixture was centrifuged at 17,000 *g* for 5 minutes. Fifty microliter injections of the supernatant were analyzed by LC-MS/MS.

Blank human urine was used as the matrix for calibration samples. The concentrations of the calibrators were 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL. Concentration of the internal standards in all samples was 250 ng/mL.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific™ Accela™ 1250 pump and Accela Open autosampler coupled to a Thermo Scientific TSQ Quantum Access MAX™ triple stage quadrupole mass spectrometer. The analytical column was a Thermo Scientific Accucore™ PFP column (50 × 2.1 mm, 2.6 µm particle size) maintained at room temperature. Details of the LC gradient and mobile phases (MP) are as follows:

Time (min)	Flow rate (mL/min)	Gradient	MPA (%)	MPB (%)	MPC (%)
0.00	0.75	Step	95	5	0
0.50	0.75	Ramp	60	40	0
2.60	0.75	Ramp	5	95	0
4.50	1.00	Step	0	100	0
5.50	1.00	Step	0	0	100
5.75	1.00	Step	95	5	0

MPA: 10 mM NH_4Ac and 0.1% formic acid in water
MPB: 10 mM NH_4Ac and 0.1% formic acid in methanol
MPC: acetonitrile/isopropanol/acetone 9:9:2 (v/v/v)

The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. The MS conditions were as follows:

Spray voltage (V)	4000
Vaporizer temperature (°C)	300
Sheath gas pressure (arbitrary units)	50
Auxiliary gas pressure (arbitrary units)	15
Capillary temperature (°C)	300

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Data were acquired in selected-reaction monitoring (SRM) mode. SRM transitions for the 43 drugs and their internal standards are shown in Table 1. For each analyte and internal standard, two SRM transitions were monitored. One of transition was used as the quantifier and the other as the qualifier. The signal ratio between the qualifier and the quantifier was used to evaluate the validity of the results.

Validation

The validation procedure included tests for the following: 1) matrix effects; 2) lower limit of quantitation (LLOQ), linear range, accuracy, and precision; and 3) carryover.

Table 1. Drug analytes, their corresponding internal standards, and the SRM transitions for both analytes and internal standards

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)	Ion Ratio (%)	Corresponding Internal Standard	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)
6-MAM	328.10	165.10	211.00	86.0	6-MAM-d3	331.20	165.10	211.10
7-Amino-clonazepam	286.00	222.10	250.10	95.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
7-Amino-flunitrazepam	284.10	135.10	226.10	52.0	7-Amino-flunitrazepam-d7	291.11	138.20	230.10
7-Aminonitrazepam	252.10	121.10	224.10	16.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
α-Hydroxy-alprazolam	325.10	216.10	297.10	52.0	α-Hydroxy-alprazolam-d5	330.10	221.10	302.10
Alprazolam	309.40	205.00	281.00	76.0	Temazepam-d5	306.10	260.10	288.10
Amphetamine	136.10	65.30	91.20	10.0	Amphetamine-d5	141.10	92.20	93.20
Benzoylecgonine	290.10	105.10	168.10	30.0	Benzoylecgonine-d3	293.10	105.10	171.10
Benzylpiperazine	177.10	65.30	91.20	16.0	Benzylpiperazine-d7	184.10	70.20	98.20
Buprenorphine	468.30	396.30	414.30	120.0	Diazepam-d5	290.12	198.10	227.10
Carisprodol	261.20	62.10	97.10	58.5	α-Hydroxy-alprazolam-d5	330.10	221.10	302.10
Clonazepam	315.90	214.00	270.00	26.6	Temazepam-d5	306.10	260.10	288.10
Cocaine	304.10	82.20	182.10	17.0	Amphetamine-d5	141.10	92.20	93.20
Codeine	300.10	165.00	215.00	91.0	Codeine-d3	303.20	165.10	215.10
Diazepam	285.10	193.10	222.10	72.0	Diazepam-d5	290.12	198.10	227.10
EDDP	279.20	235.20	250.20	54.5	Temazepam-d5	306.10	260.10	288.10
Fentanyl	337.20	105.20	188.20	67.0	Temazepam-d5	306.10	260.10	288.10
Flunitrazepam	314.40	239.10	268.10	32.5	Temazepam-d5	306.10	260.10	288.10
Flurazepam	388.10	288.10	315.10	11.5	Temazepam-d5	306.10	260.10	288.10
Hydrocodone	300.20	171.00	199.00	34.5	MDA-d5	185.10	110.20	137.10
Hydromorphone	286.11	157.10	185.10	64.0	Benzylpiperazine-d7	184.10	70.20	98.20
Lorazepam	321.00	275.00	303.00	64.0	α-Hydroxy-alprazolam-d5	330.10	221.10	302.10
MDA	180.10	105.20	135.10	79.0	MDA-d5	185.10	110.20	137.10
MDEA	208.10	135.10	163.00	24.0	Nordiazepam-d5	276.10	165.00	213.10
MDMA	194.10	135.10	163.10	40.0	MDMA-d5	199.10	135.10	165.10
Meperidine	248.20	174.20	220.10	28.0	Diazepam-d5	290.12	198.10	227.10
Methadone	310.20	105.10	265.10	29.0	Diazepam-d5	290.12	198.10	227.10
Methamphetamine	150.10	65.30	91.20	9.5	Methamphetamine-d5	155.10	91.20	92.20
Midazolam	326.10	249.20	291.20	28.0	Diazepam-d5	290.12	198.10	227.10
Morphine	286.10	152.10	165.00	78.0	Morphine-d3	289.10	152.10	165.10
Naloxone	328.21	212.00	310.10	23.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
Naltrexone	342.20	270.10	324.20	16.0	MDA-d5	185.10	110.20	137.10
Norbuprenorphine	414.30	187.10	340.30	99.0	Temazepam-d5	306.10	260.10	288.10
Nordiazepam	271.00	140.10	208.10	100.5	Nordiazepam-d5	276.10	165.00	213.10
Norfentanyl	233.20	55.30	84.30	16.0	MDMA-d5	199.10	135.10	165.10
Normeperidine	234.20	111.10	160.10	0.3	Temazepam-d5	306.10	260.10	288.10
Oxazepam	287.00	241.00	269.00	82.0	Oxazepam-d5	292.10	246.10	274.10
Oxycodone	316.20	241.20	298.20	22.5	Benzoylecgonine-d3	293.10	105.10	171.10
Oxymorphone	302.10	227.10	284.20	35.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
PCP	244.20	86.20	159.10	84.5	Diazepam-d5	290.12	198.10	227.10
Propoxyphene	340.20	58.20	91.10	15.0	Diazepam-d5	290.12	198.10	227.10
Temazepam	301.00	255.00	283.00	36.0	Temazepam-d5	306.10	260.10	288.10
Tramadol	264.20	58.30	246.10	3.0	Temazepam-d5	306.10	260.10	288.10

Results and Discussion

Matrix Effects

Matrix effects were assessed with the nine individual human urine samples. Absolute recovery was determined by comparing the signals of unlabeled drugs in urine and water samples. Relative recovery was determined by comparing the analyte/IS ratio in urine and water samples. The recovery/matrix effects results are summarized in Table 2. All 43 drugs had almost full absolute recovery (between 80% and 120%), except morphine for which the matrix effect was compensated by the use of its internal standard, morphine-d3. The observed precision from the nine individual human urine samples was below 15% for most of the 43 drugs.

Table 2. Summary of matrix effects

Drug	Average Absolute Recovery (%, n=9)		CV (%, n=9)		Average Relative Recovery (%, n=9)		CV (%, n=9)	
	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL
6-MAM	86.7	92.3	16.2	12.2	95.1	100.2	5.6	5.7
7-Amino-clonazepam	96.4	108.4	11.0	11.6	90.3	103.7	6.1	5.8
7-Amino-flunitrazepam	86.8	90.5	11.4	8.4	97.1	102.1	6.3	5.1
7-Aminonitrazepam	86.0	85.5	12.3	9.6	80.6	81.9	9.9	8.2
α-Hydroxy-alprazolam	87.4	87.4	12.9	6.7	99.4	96.2	10.4	4.1
Alprazolam	94.0	89.1	26.0	16.6	95.1	84.8	22.9	13.9
Amphetamine	109.5	112.3	11.8	8.0	112.4	110.8	16.7	3.8
Benzoylecgonine	82.7	85.7	13.0	12.7	98.7	100.9	4.6	3.6
Benzylpiperazine	87.3	85.4	10.0	7.2	100.4	100.6	8.7	7.5
Buprenorphine	108.4	96.9	15.0	6.2	118.1	97.2	14.6	5.4
Carisprodol	88.0	96.3	13.1	11.0	100.5	105.8	13.6	8.4
Clonazepam	100.7	98.4	9.5	6.9	103.4	94.4	13.6	9.5
Cocaine	93.6	93.5	7.4	8.2	95.5	92.2	5.3	5.0
Codeine	93.9	98.9	8.6	8.2	99.3	98.0	3.3	7.2
Diazepam	98.0	96.6	14.0	9.1	106.5	96.7	11.7	6.5
EDDP	103.8	99.2	6.8	2.9	106.8	95.0	13.7	6.2
Fentanyl	98.6	100.9	4.1	2.8	101.4	96.7	10.7	5.8
Flunitrazepam	85.7	86.9	18.8	14.7	87.1	82.9	14.6	12.1
Flurazepam	97.5	103.1	4.2	3.9	100.2	98.8	11.7	5.8
Hydrocodone	91.5	96.4	15.1	13.5	95.2	97.9	7.8	9.6
Hydromorphone	91.2	94.5	11.0	10.4	104.6	110.8	7.2	5.4
Lorazepam	105.7	90.5	16.5	6.0	120.7	99.7	17.2	5.7
MDA	96.6	105.8	16.1	9.6	100.6	107.9	8.7	6.4
MDEA	95.6	94.0	11.8	10.1	99.0	82.8	9.7	10.6
MDMA	92.3	94.3	9.3	7.8	106.1	102.4	2.4	4.1
Meperidine	88.4	88.4	9.8	9.9	96.2	88.5	7.4	7.8
Methadone	101.6	103.2	3.2	3.4	111.1	103.6	8.9	5.4
Methamphetamine	94.6	86.2	12.3	11.1	105.5	94.1	8.4	5.6
Midazolam	98.4	97.4	9.5	5.5	107.1	97.6	6.0	3.5
Morphine	48.1	53.8	6.0	8.2	90.5	98.4	6.9	5.4
Naloxone	124.2	129.4	17.9	16.1	116.1	123.5	9.9	7.7
Naltrexone	96.1	100.2	12.6	10.9	100.3	101.9	5.2	6.1
Norbuprenorphine	76.9	104.6	19.4	14.2	78.9	99.9	20.4	11.6
Nordiazepam	102.8	107.1	21.3	7.3	106.3	94.2	19.8	7.5
Norfentanyl	89.5	92.2	11.4	8.2	103.2	100.1	12.4	4.5
Normeperidine	81.7	92.0	11.9	11.6	83.1	87.7	7.8	8.4
Oxazepam	93.8	91.3	10.8	5.1	113.4	102.1	6.8	4.7
Oxycodone	80.4	84.7	8.8	10.9	97.0	100.1	9.7	6.1
Oxymorphone	107.0	101.4	15.2	12.9	100.1	97.0	8.2	9.0
PCP	100.8	100.5	4.2	4.3	110.3	100.9	8.8	5.1
Propoxyphene	101.3	103.8	6.8	5.8	111.2	104.1	15.3	4.4
Temazepam	95.3	102.2	14.2	7.1	97.3	97.7	12.3	4.6
Tramadol	77.8	84.8	14.1	12.9	78.9	80.8	10.1	10.4

Lower Limit of Quantitation, Linear Range, Accuracy, and Precision

The LLOQ of these 43 drugs and other aspects of analytical performances of this method are summarized in Table 3. Linear fit with 1/X weighting was used for calibration curves of all the 43 drugs. The LLOQ for these 43 drugs was determined to be between 2 and 20 ng/mL except for tramadol, which was 50 ng/mL. At the LLOQ, the accuracy ranged between 89.9% and 118.4%, and precision ranged between 3.6% and 19.5%. The method was linear to 1000 ng/mL for all the drugs. Figure 1 shows the calibration curves of six typical pain management drugs in human urine.

Table 3. Lower limit of quantitation, linear range, accuracy, and precision

Drug	Retention Time (min)	LLOQ (ng/mL)	Accuracy at LLOQ (%, n=4)	CV at LLOQ (%, n=4)	Linear Range (ng/mL)	R^2	Precision 20 ng/mL (%, n=6)	Precision 200 ng/mL (%, n=6)
6-MAM	2.97	2	95.0	14.6	2–1000	0.9955	5.8	2.8
7-Amino-clonazepam	2.76	5	95.1	10.1	5–1000	0.9988	3.4	4.0
7-Amino-flunitrazepam	3.31	5	101.0	13.7	5–1000	0.9980	5.3	4.0
7-Aminonitrazepam	2.51	2	102.0	9.6	2–1000	0.9972	3.7	3.2
α-Hydroxy-alprazolam	3.87	20	94.0	10.0	20–1000	0.9972	6.9	5.9
Alprazolam	4.11	5	94.1	13.1	5–1000	0.9950	2.7	1.2
Amphetamine	2.97	20	94.9	7.7	20–1000	0.9944	5.4	5.5
Benzoylecgonine	2.99	5	92.3	3.6	5–1000	0.9990	2.7	2.0
Benzylpiperazine	2.70	10	96.0	10.4	10–1000	0.9979	9.5	5.0
Buprenorphine	4.50	20	94.7	17.3	20–1000	0.9976	6.0	6.1
Carisprodol	3.80	10	104.5	11.3	10–1000	0.9903	9.5	6.3
Clonazepam	4.00	20	92.7	7.7	20–1000	0.9954	9.1	6.1
Cocaine	4.23	5	101.2	7.4	5–1000	0.9969	4.0	3.7
Codeine	2.82	10	110.4	18.3	10–1000	0.9978	6.6	3.8
Diazepam	4.24	5	93.0	11.9	5–1000	0.9979	7.0	3.4
EDDP	4.90	10	106.5	3.9	10–1000	0.9944	4.5	2.2
Fentanyl	4.62	2	108.9	3.7	2–1000	0.9975	4.8	1.8
Flunitrazepam	4.12	20	93.7	17.2	20–1000	0.9904	6.4	4.4
Flurazepam	4.57	2	118.4	3.6	2–1000	0.9961	4.9	2.4
Hydrocodone	3.16	2	106.6	9.6	2–1000	0.9988	7.8	2.8
Hydromorphone	2.25	2	89.9	13.2	2–1000	0.9979	8.2	3.1
Lorazepam	3.86	20	92.5	17.3	20–1000	0.9943	2.2	9.6
MDA	3.16	10	93.1	6.8	10–1000	0.9974	1.1	3.1
MDEA	3.97	2	104.3	4.5	2–1000	0.9937	7.5	4.4
MDMA	3.61	5	97.3	4.3	5–1000	0.9975	7.6	2.2
Meperidine	4.20	5	101.2	9.6	5–1000	0.9986	5.5	4.6
Methadone	4.95	5	100.3	3.8	5–1000	0.9982	4.2	3.0
Methamphetamine	3.51	5	106.0	5.1	5–1000	0.9979	5.0	4.0
Midazolam	4.48	2	117.1	12.7	2–1000	0.9983	7.0	4.3
Morphine	1.71	5	93.0	13.6	5–1000	0.9990	5.0	3.3
Naloxone	2.86	10	102.3	10.9	10–1000	0.9944	3.3	2.9
Naltrexone	3.11	5	101.9	7.0	5–1000	0.9985	5.1	1.6
Norbuprenorphine	4.13	20	101.4	14.4	20–1000	0.9955	3.9	8.4
Nordiazepam	4.06	10	97.1	19.5	10–1000	0.9948	8.4	3.8
Norfentanyl	3.68	10	102.5	6.3	10–1000	0.9985	7.1	2.3
Normeperidine	4.00	2	116.2	11.2	2–1000	0.9982	7.3	4.2
Oxazepam	3.88	20	108.0	15.0	20–1000	0.9970	10.9	6.4
Oxycodone	3.03	5	91.9	11.7	5–1000	0.9982	2.6	2.3
Oxymorphone	2.01	2	93.3	9.5	2–1000	0.9946	10.0	2.6
PCP	4.83	2	100.9	4.0	2–1000	0.9981	7.8	3.0
Propoxyphene	4.70	10	113.6	4.1	10–1000	0.9978	7.3	5.2
Temazepam	4.05	5	104.6	16.9	5–1000	0.9981	5.6	2.2
Tramadol	4.04	50	98.8	2.5	50–1000	0.9970	NA	2.5

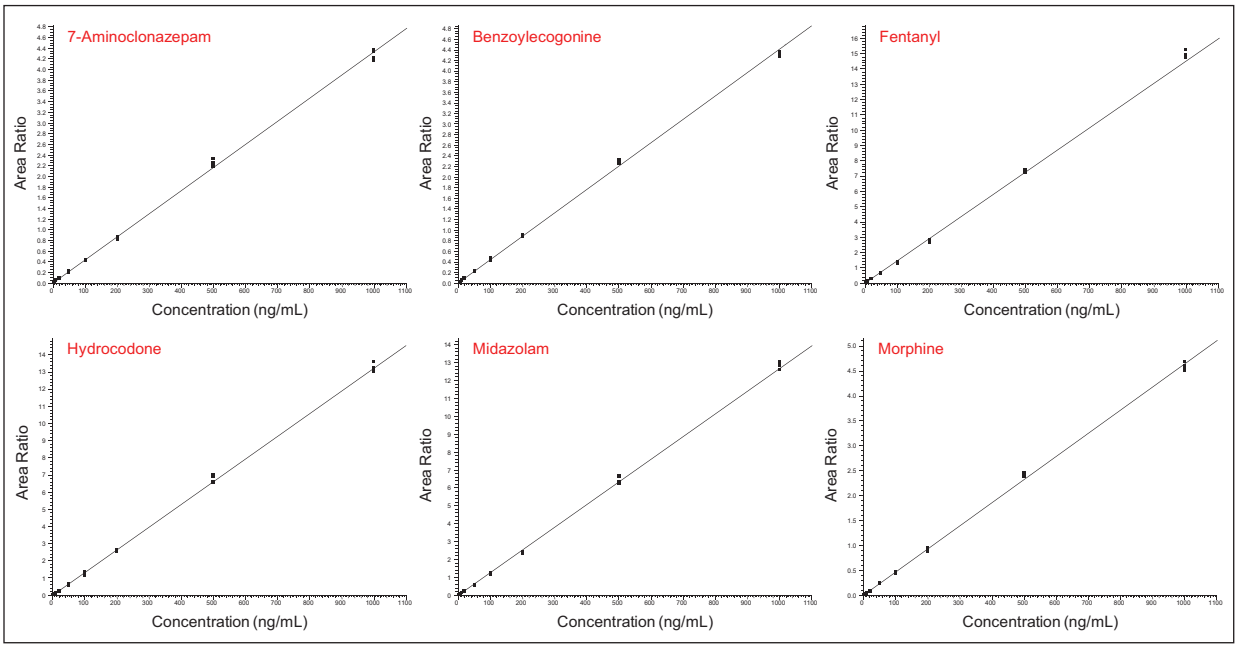

Figure 1. Calibration curves of six selected drugs in spiked human urine

Method precision was also assessed with spiked human urine samples at low and high quality control (QC) concentrations of 20 and 200 ng/mL, respectively (Table 2). Precision values at low (20 ng/mL) and high (200 ng/mL) quality control concentrations ranged between 1.1% and 10.9% (Table 2). Figure 2 shows both the quantifier and qualifier SRM chromatograms of 20 selected pain management drugs spiked at 20 ng/mL in human urine.

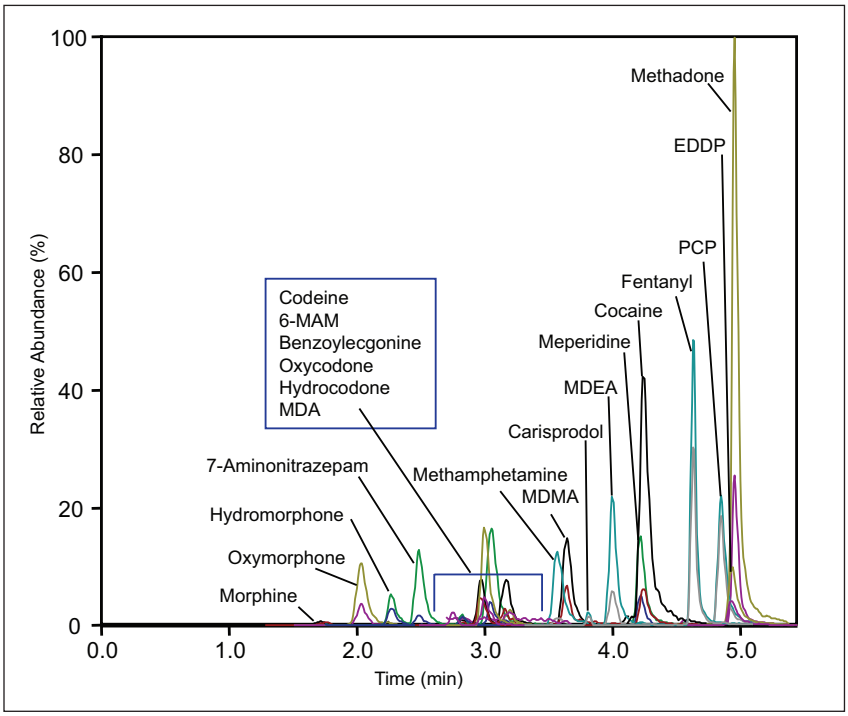

Figure 2. SRM chromatograms of 20 selected drugs at 20 ng/mL in spiked human urine

Carryover

The lowest calibrator was analyzed after the highest calibrator. No carryover causing elevated measurements of the drugs in the lowest calibrator was observed.

Conclusion

The developed method provides a simple, fast, and sensitive way for forensic toxicology labs to simultaneously quantify 43 drugs of abuse, including pain management drugs, in human urine by LC-MS/MS. The method provided LLOQ values of 2–20 ng/mL for 42 of the 43 drugs, and was linear to 1000 ng/mL. Minimal ion suppression and no carryover were observed in matrix samples. At the LLOQ, the accuracy ranged between 89.9% and 118.4%. Method precision ranged between 1.1% and 10.9% at low and high QC samples.

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Simultaneous Quantitation of 19 Drugs in Human Plasma and Serum by LC-MS/MS

Xiang He and Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Application Note 571

Key Words

TSQ Vantage, drug monitoring research, clinical research, CSS, plasma, serum

Goal

To develop a simple, fast, and sensitive LC-MS/MS method for the simultaneous quantitation of 19 drugs in human plasma and serum.

Introduction

Liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) has become an accepted tool for quantitative analysis of drugs in clinical research laboratories. LC-MS/MS enables simultaneous, sensitive detection and quantitation of multiple analytes of interest. In this study, 19 drugs of various types, including antipsychotics, antiepileptics/anticonvulsants, antianginals, and antidepressants, were monitored and simultaneously quantitated using LC-MS/MS.

Experimental

Sample Preparation

Nineteen drugs (Table 1) and 15 isotopically labeled internal standards of the drugs were used in this research.

Table 1. Drug analytes

Analytes		
Amitriptyline	Dothiepin	Nortriptyline
Bromazepam	Doxepin	Oxazepam
Clobazam	Flunitrazepam	Perhexilline
Clomipramine	Imipramine	Temazepam
Clonazepam	Lamotrigine	Trimipramine
Clozapine	Levetiracetam	
Diazepam	Nitrazepam	

To assess signal recovery and determine the best dilution factor, 9 randomly chosen individual human-donor plasma samples were spiked with the 19 drugs at 40 ng/mL and 15 isotopically labeled internal standards at 100 ng/mL. These samples were mixed (1:3, v/v) with a 1:1 methanol/acetonitrile mixture. The samples were vigorously vortexed and stored at -30 °C for 30 min. The samples were then centrifuged at 17,000 *g* for 5 min. Supernatant (20 µL) was drawn off and diluted 10-fold, 20-fold, and 50-fold with 10% methanol in water to final dilution factors of 40x, 80x, and 200x.

Calibration and linearity standards were prepared by spiking a matrix of charcoal-stripped human serum (CSS) with the 15 internal standards at 100 ng/mL and the 19 drug analytes at 4, 10, 20, 40, 100, 200, and 400 ng/mL. The samples were processed as above and diluted to a final dilution factor of 200x.

For accuracy and precision testing, CSS samples were spiked with the 15 isotopically labeled internal standards at 100 ng/mL and the 19 drugs at both 40 ng/mL and 200 ng/mL. The samples were processed as above and diluted to a final dilution factor of 200x.

Also for accuracy and precision testing, 9 individual human-donor plasma samples were spiked with the 15 isotopically labeled internal standards at 100 ng/mL and the 19 drugs at 40 ng/mL. The samples were processed as above and diluted to a final dilution factor of 200x.

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Liquid Chromatography

Chromatographic separations were performed with a Thermo Scientific Accela 1250 pump and Accela Open autosampler. The analytical column was a Thermo Scientific Accucore PFP column (50 × 2.1 mm, 2.6 µm particle size). The column was maintained at room temperature. Details of the LC gradient and information on the mobile phases (MP) are shown in Table 2. The injection volume was 40 µL.

Table 2. LC gradient

Time (min)	Flow rate (mL/min)	Gradient	MPA (%)	MPB (%)	MPC (%)
0.00	0.4	Step	95	5	0
0.50	0.4	Step	90	10	0
1.50	0.4	Ramp	50	50	0
2.00	0.4	Ramp	5	95	0
6.50	0.4	Step	0	100	0
7.75	0.6	Step	0	0	100
8.00	0.6	Step	95	5	0

MPA: 10 mM ammonium acetate and 0.1% formic acid in water
MPB: 10 mM ammonium acetate and 0.1% formic acid in methanol
MPC: acetonitrile:isopropanol:acetone 9:9:2 (v/v/v)

Mass Spectrometry

MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer. The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. The MS conditions were as follows:

Spray voltage (V):	4000
Vaporizer temperature (°C):	300
Sheath gas pressure (arbitrary units)	50
Auxiliary gas pressure (arbitrary units)	15
Capillary temperature (°C)	300

Data were acquired in selected-reaction monitoring (SRM) mode. Detailed SRM settings for the 19 drugs and their internal standards are shown in Table 3. For each analyte and internal standard, two SRM transitions were monitored. One was used as the quantifier and the other as the qualifier. The signal ratio between the qualifier and the quantifier was used to evaluate the validity of the results. Results that varied by more than 20% of the nominal ratio were considered invalid data points.

The validation procedure included tests for: 1) signal recovery, 2) lower limit of quantitation (LLOQ) and linear range, 3) accuracy and precision, and 4) carryover.

Table 3. SRM settings for the analytes and internal standards

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Collision Energy (V)	Qualifier Ion (*m/z*)	Collision Energy (V)	S-Lens (V)
Amitriptyline	278.10	202.10	56	233.10	16	74
Bromazepam	316.11	182.10	31	209.10	26	95
Clobazam	301.10	259.10	20	224.10	32	90
Clomipramine	315.10	86.00	17	58.00	35	74
Clonazepam	316.00	270.10	25	214.00	37	101
Clozapine	327.10	270.10	23	192.00	42	94
Diazepam	285.10	193.10	32	154.00	27	88
Dothiepin	296.10	202.10	53	221.10	45	71
Doxepin	280.10	165.10	51	107.00	23	80
Flunitrazepam	314.10	268.10	26	239.10	34	92
Imipramine	281.20	86.00	16	58.00	35	69
Lamotrigine	256.00	211.00	26	109.00	49	89
Levetiracetam	171.10	126.10	14	69.00	28	36
Nitrazepam	282.10	236.10	24	207.10	34	97
Nortriptyline	264.20	233.20	13	91.10	32	66
Oxazepam	287.10	269.10	14	104.10	33	81
Perhexilline	278.20	95.10	28	67.00	34	87
Temazepam	301.11	255.10	22	283.10	13	72
Trimipramine	295.20	100.10	16	58.10	35	71
Internal Standards						
Amitriptyline-D3	281.21	91.10	32	233.20	16	85
Clomipramine-D3	318.20	89.10	18	61.10	36	75
Clonazepam-D4	320.10	274.10	26	218.10	35	102
Clozapine-D4	331.20	272.20	25	192.10	45	102
Diazepam-D5	290.10	198.10	31	154.00	26	89
Doxepin-D3	283.20	107.00	23	77.00	46	78
Flunitrazepam-D7	321.10	275.20	26	246.20	35	96
Imipramine-D3	284.20	89.10	16	61.10	35	69
Lamotrigine-13C, 15N4	261.00	214.00	26	109.10	50	104
Levetiracetam-D6	177.10	132.20	14	69.10	30	38
Nitrazepam-D5	287.11	185.10	37	212.10	34	100
Nortriptyline-D3	267.20	91.00	33	233.20	14	66
Oxazepam-D5	292.10	246.10	22	274.10	15	84
Temazepam-D5	306.10	260.10	23	288.10	13	83
Trimipramine-D3	298.20	103.10	16	61.10	35	72

Results and Discussion

Signal Recovery

Plasma and serum are complex matrices. The matrix content in them can significantly affect the detection of drugs by ESI MS. Therefore, three different dilution factors after protein precipitation (40-fold, 80-fold, and 200-fold) were compared. The LC-MS/MS signals of the analytes in the plasma samples were compared to LC-MS/MS signals from solvent blanks with the same spikes. The 200-fold sample dilution produced the best signal recovery and minimum ion suppression (Table 4 and Figures 1 and 2). For all of the subsequent analyses, all samples were prepared with a 200-fold final dilution factor.

Table 4. Absolute mean signal recovery of 19 drugs at 40 ng/mL in 9 human plasma samples diluted 40-fold, 80-fold, and 200-fold, as compared to a similarly spiked solvent blank

Analyte (40 ng/mL)	Absolute mean signal recovery (%)		
	n=9 200x dilution	n=9 80x dilution	n=9 40x dilution
Amitriptyline	107.9	53.9	79.4
Bromazepam	125.7	49.7	56.6
Clobazam	78.6	43.4	54.4
Clomipramine	103.6	57.5	84.1
Clonazepam	65.9	36.0	32.3
Clozapine	81.5	60.4	56.7
Diazepam	78.4	45.6	57.9
Dothiepin	124.6	53.4	83.9
Doxepin	110.8	57.4	84.0
Flunitrazepam	77.8	44.1	51.9
Imipramine	107.2	50.6	82.8
Lamotrigine	71.5	45.1	52.8
Levetiracetam	86.7	48.2	58.3
Nitrazepam	77.8	38.4	41.7
Nortriptyline	83.7	44.5	62.2
Oxazepam	74.5	41.9	52.7
Perhexilline	94.9	152.8	190.0
Temazepam	74.7	44.6	55.1
Trimipramine	98.4	49.1	76.4

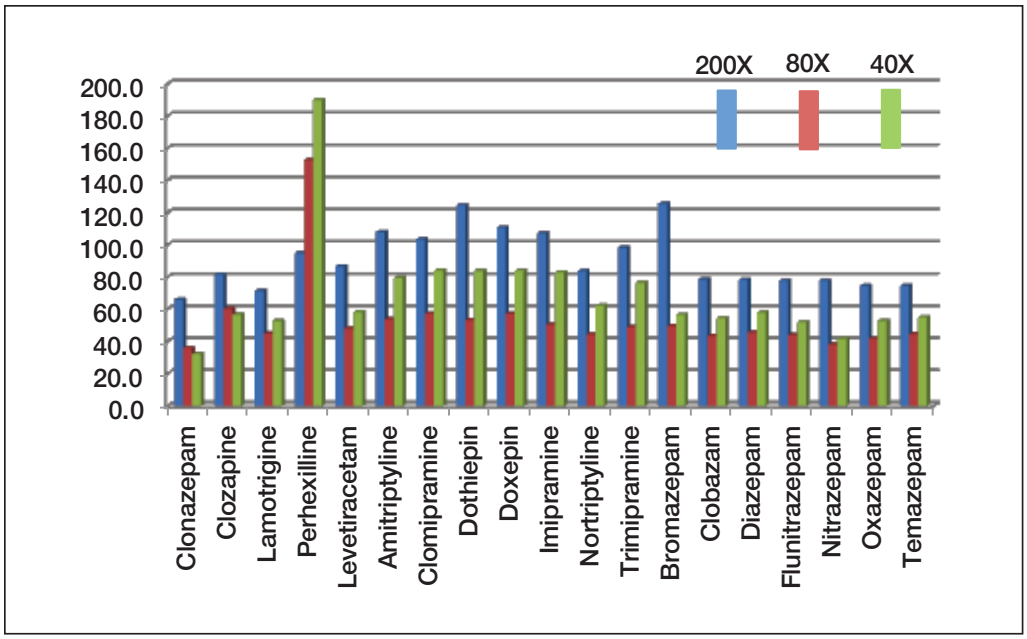

Figure 1. Mean signal recovery of 19 drugs at 40 ng/mL in 9 human plasma samples diluted 40-fold, 80-fold, and 200-fold, as compared to a similarly spiked solvent blank

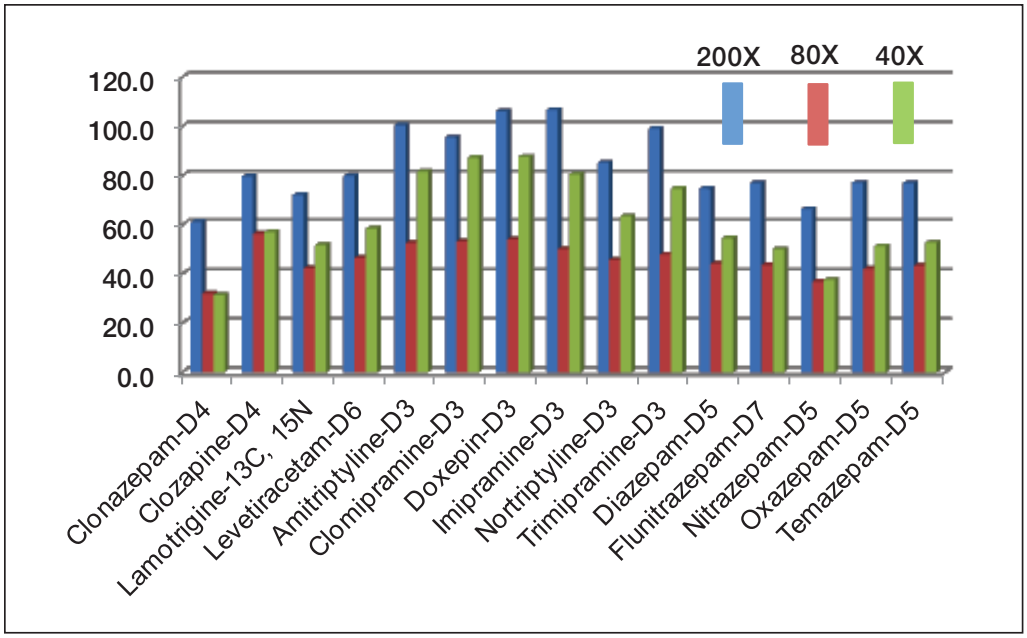

Figure 2. Mean signal recovery of 15 internal standards at 100 ng/mL in 9 human plasma samples diluted 40-fold, 80-fold, and 200-fold, as compared to a similarly spiked solvent blank

Lower Limit of Quantitation and Linear Range

The lower limit of quantitation (LLOQ), linearity, and ion ratio test parameters for the 19 drugs are summarized in Table 5. For calibration curves, a linear fit with 1/X weighting was used. The LLOQ for these 19 drugs were determined to be between 4 and 20 ng/mL. The method was linear to 400 ng/mL for all the drugs. Figure 3 shows the calibration curve of clozapine in CSS. Figure 4 shows the overlaid SRM chromatograms (quantifier and qualifier) of all the 19 drugs at 20 ng/mL in CSS.

Table 5. LLOQ and linearity summary for 19 drugs

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)	Ion Ratio (%)	Ion Ration Window (±%)	LLOQ (ng/mL)	Linear Range (ng/mL)	R^2
Amitriptyline	278.10	202.10	233.10	105	21	4	4–400	0.9941
Bromazepam	316.11	182.10	209.10	90	18	10	10–400	0.9955
Clobazam	301.10	259.10	224.10	37	7	10	10–400	0.9967
Clomipramine	315.10	86.00	58.00	35	7	4	4–400	0.9933
Clonazepam	316.00	270.10	214.00	35	7	10	10–400	0.9960
Clozapine	327.10	270.10	192.00	70	14	4	10–400	0.9974
Diazepam	285.10	193.10	154.00	67	13	4	4–400	0.9951
Dothiepin	296.10	202.10	221.10	84	17	10	10–400	0.9937
Doxepin	280.10	165.10	107.00	180	36	4	4–400	0.9955
Flunitrazepam	314.10	268.10	239.10	39	8	4	4–400	0.9973
Imipramine	281.20	86.00	58.00	35	7	4	4–400	0.9972
Lamotrigine	256.00	211.00	109.00	50	10	10	10–400	0.9881
Levetiracetam	171.10	126.10	98.10	4.6	2	10	10–400	0.9945
Nitrazepam	282.10	236.10	207.10	35	7	4	4–400	0.9980
Nortriptyline	264.20	233.20	91.10	73	15	4	4–400	0.9948
Oxazepam	287.10	269.10	104.10	13	4	10	10–400	0.9943
Perhexilline	278.20	95.10	67.00	66	13	20	20–400	0.9755
Temazepam	301.11	255.10	283.10	25	5	4	4–400	0.9948
Trimipramine	295.20	100.10	58.10	44	9	4	4–400	0.9968

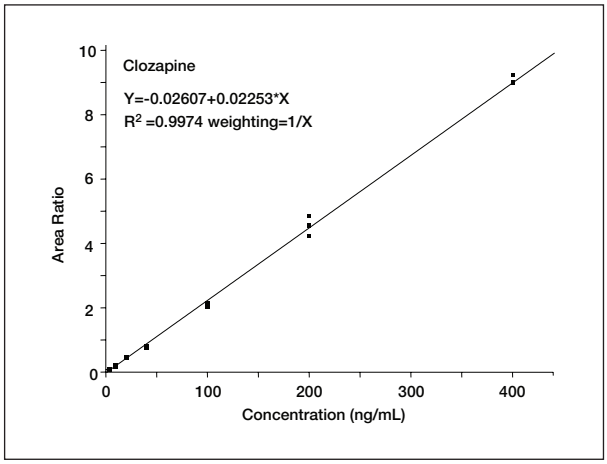

Figure 3. Calibration curve of clozapine in CSS

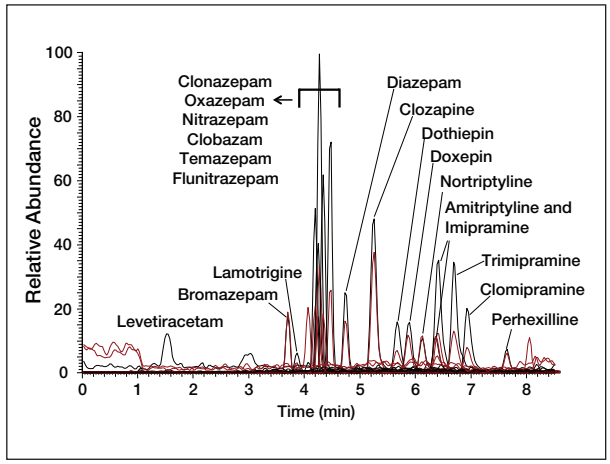

Figure 4. SRM chromatograms of all 19 drugs at 20 ng/mL in CSS after 200-fold dilution

Accuracy and Precision

Accuracy and precision were first assessed with CSS spiked at concentrations of 40 and 200 ng/mL (Table 6). Overall accuracy ranged between 82.4% and 111.3%. Inter- and intra-batch precision (coefficient of variation) values at low (40 ng/mL) and high (200 ng/mL) concentrations varied between 1.4% and 13.5%. Accuracy and intra-batch precision were also assessed in the 9 individual human-donor plasma samples spiked with 40 ng/mL drugs. The results were satisfactory (Table 7).

Table 6. Accuracy and precision summary for analysis of 19 drugs in CSS

Analyte	40 ng/mL					200 ng/mL				
	Precision				Accuracy	Precision				Accuracy
	Intra1 (%) n=5	Intra2 (%) n=5	Intra3 (%) n=5	Inter (%) n=15	Inter (%) n=15	Intra1 (%) n=5	Intra2 (%) n=5	Intra3 (%) n=5	Inter (%) n=15	Inter (%) n=15
Amitriptyline	8.5	10.4	11.5	9.7	87.8	4.6	3.8	9.7	6.3	100.7
Bromazepam	10.1	2.9	3.8	6.9	89.9	3.1	4.0	2.1	3.3	104.2
Clobazam	2.5	3.4	8.6	5.1	90.6	5.5	4.0	4.3	4.6	101.7
Clomipramine	8.3	8.1	6.4	8.0	106.3	3.2	3.1	3.1	4.8	109.4
Clonazepam	3.6	6.4	6.1	5.2	101.4	5.6	2.1	3.3	4.4	107.4
Clozapine	5.7	3.4	5.1	5.4	96.5	4.4	4.3	2.4	3.6	111.3
Diazepam	4.9	6.9	5.9	5.9	88.8	2.7	4.7	3.6	3.6	101.7
Dothiepin	3.7	8.9	5.4	6.1	99.5	4.2	2.5	4.0	4.9	108.2
Doxepin	5.8	10.8	11.9	10.0	96.4	4.5	4.5	2.9	4.5	108.8
Flunitrazepam	1.4	7.0	4.2	5.1	82.4	4.7	4.2	4.3	4.5	100.8
Imipramine	3.1	2.9	2.0	2.8	87.0	1.6	3.1	3.2	2.9	102.2
Lamotrigine	7.0	5.2	8.9	7.2	96.9	3.9	2.5	3.4	3.8	105.8
Levetiracetam	10.9	3.9	9.5	8.3	99.1	5.4	3.0	8.9	5.9	107.8
Nitrazepam	3.8	4.1	6.0	5.2	85.1	5.7	3.8	5.4	4.7	97.3
Nortriptyline	6.9	4.9	4.6	5.2	97.7	2.3	3.9	4.3	3.9	110.5
Oxazepam	8.3	5.5	9.2	7.6	96.5	5.0	7.1	1.7	5.2	106.3
Perhexilline	8.0	12.7	12.7	13.5	86.5	2.2	1.9	6.9	4.4	107.7
Temazepam	7.7	5.5	3.7	6.1	95.3	2.7	2.5	4.8	3.4	104.7
Trimipramine	3.6	3.0	6.1	4.1	89.0	2.9	3.7	3.9	3.7	103.4

Table 7. Accuracy and precision summary for analysis of 19 drugs in 9 individual human-donor plasma samples

Analyte (40 ng/mL)	Mean Measured (ng/mL), n=9	Accuracy (%) n=9	Precision (%) n=9
Amitriptyline	47.2	118.1	7.2
Bromazepam	33.7	84.3	18.0
Clobazam	42.8	107.0	15.0
Clomipramine	41.7	104.2	12.9
Clonazepam	41.4	103.4	13.4
Clozapine	38.6	96.4	9.0
Diazepam	37.2	93.1	8.8
Dothiepin	38.3	95.8	8.1
Doxepin	41.2	102.9	18.5
Flunitrazepam	34.8	87.0	7.7
Imipramine	37.4	93.4	6.5
Levetiracetam	40.0	100.1	7.7
Lamotrigine	37.2	93.0	18.2
Nitrazepam	38.9	97.3	7.0
Nortriptyline	36.2	90.5	6.2
Oxazepam	35.3	88.2	7.2
Perhexilline	42.8	106.9	9.6
Temazepam	36.1	90.3	9.1
Trimipramine	35.9	89.8	7.9

Carryover

The lowest calibrator was analyzed after the highest calibrator, and we did not observe any carryover causing elevated measurements of the drugs in the lowest calibrator.

Conclusion

We have developed a simple, fast, and sensitive LC-MS/MS clinical research method for simultaneously quantitation of 19 drugs in human plasma. The method had LLOQ values of 4–20 ng/mL for all 19 drugs and was linear to 400 ng/mL. Ion suppression was not observed in matrix samples. Accuracy and precision of the method were successfully accessed in both CSS and human plasma samples.

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Quantitation of Six Opioids in Urine with Super-Dilution and Microflow LC-MS/MS

Xiang He and Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Application Note 570

Key Words

TSQ Vantage, Microflow, LC-MS/MS, Forensic Toxicology

Goal

To quantitate six opioids in urine with 500-fold urine dilution and microflow LC-MS/MS for forensic toxicology use, using the Thermo Scientific Dionex UltiMate 3000 RSLCnano LC system and the Thermo Scientific TSQ Vantage mass spectrometer.

Introduction

Morphine, codeine, hydromorphone, hydrocodone, oxymorphone and oxycodone are some of the most abused opioids in the United States. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been widely used for their quantitation in forensic toxicology. The analytical methods typically use normal LC flow rates (~0.5 mL/min) and sample preparation usually involves solid phase extraction (SPE) for sensitive detection. Microflow LC uses significantly lower flow rates (15 to 50 µL/min). With the same sample amount and identical LC peak width, the reduction in LC flow rate results in a much-improved detection limit for concentration-dependent detection techniques such as electrospray ionization (ESI) mass spectrometry. Because of this sensitivity increase, we can achieve a similar analytical performance for sensitive measurements of urine opioids for forensic toxicology purposes with a simple "dilute-and-shoot" approach.

Our goal was to use a super-dilution approach to improve the dilute-and-shoot detection of opioids in urine by minimizing matrix effects, and to compensate the sensitivity decrease from super-dilution by using microflow LC. We anticipated savings in solvent consumption and the cost of waste disposal, better environmental conservation, and improved longevity of the LC-MS/MS system.

Methods

Sample Preparation

Urine samples were spiked with internal standards (IS) and then mixed with β-glucuronidase and incubated at 60 °C for hydrolysis. Methanol was added to the mixture and the supernatant was diluted. The tested dilution factors were 100, 250 and 500. The mixture was centrifuged at 17,000 *g* for 5 minutes, and 20 µL of supernatant was injected for microflow LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a TSQ Vantage™ triple stage quadrupole mass spectrometer coupled to an UltiMate™ 3000 RSLCnano LC system equipped with a microflow flow rate selector. The microflow LC plumbing was set up in "pre-concentration on a trapping column" mode (Figure 1). The temperature of the columns was maintained at 35 °C. The trapping column was a Thermo Scientific Hypersil GOLD PFP drop-in guard cartridge (10 × 1 mm, 5 µm particle size) in the guard holder, and the analytical column was a Hypersil GOLD™ PFP column (100 × 0.32 mm, 5 µm particle size). LC connections were made with Thermo Scientific Dionex nanoViper fingertight fittings. The LC gradients for sample loading and analytical elution are shown in Figure 2. The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. Data was acquired in selected-reaction monitoring (SRM) mode. Detailed source parameters and SRM settings are shown in Figure 3. For each analyte, two SRM transitions were monitored. One of them was used as the quantifier and the other as qualifier. The signal ratio between the qualifier and the quantifier was used to evaluate the validity of the results, and any ratio outside 20% (relative to the ratio) was considered an invalid data point.

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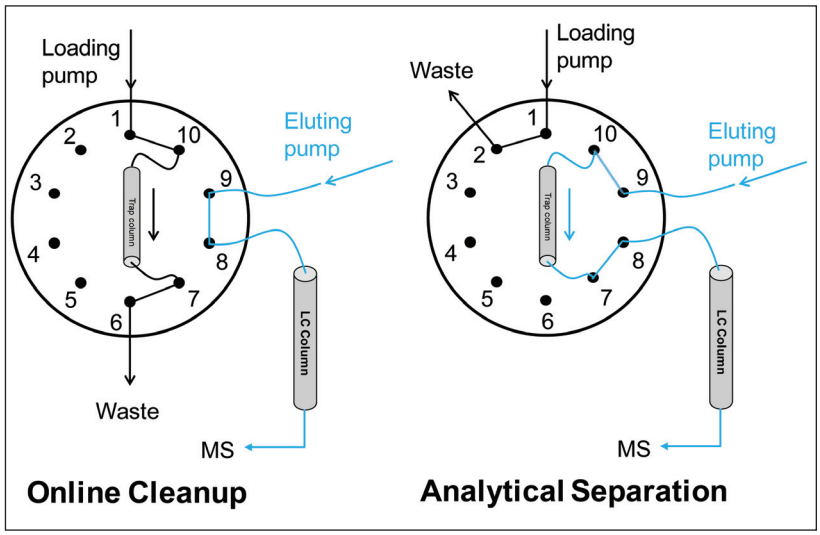

Figure 1. Microflow LC setup with pre-concentration trapping column

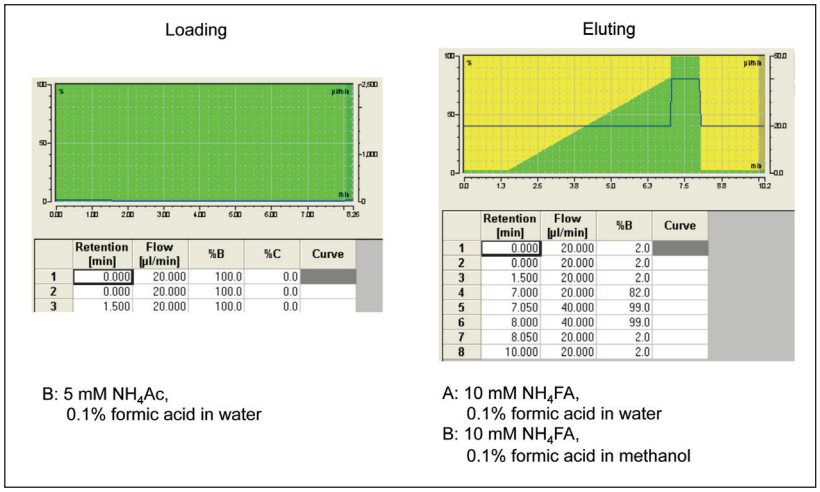

Figure 2. LC gradients of microflow LC with online clean-up

Polarity: positive
Spray Voltage (V): 4000
Vaporizer Temperature (°C): 150
Capillary Temperature (°C): 270
Sheath Gas (AU): 15
Aux Gas (AU): 2

Scan Mode: SRM
Scan Width (*m/z*): 0.02
Scan Time (Sec): 0.1
Q1 (FWHM, *m/z*): 0.7
Q3 (FWHM, *m/z*): 0.7
Collision Gas (Torr): 1.5

Analytes	Precursor (*m/z*)	Quantifier (*m/z*)	Qualifier (*m/z*)	Ion Ratio (%)	IR Window (%)
Morphine	286.1	152.2	201.1	90.0	18.0
Codeine	300.1	165.1	215.1	68.0	13.6
Hydromorphone	286.11	185.1	157.1	79.0	15.8
Hydrocodone	300.11	199.1	171.1	39.0	7.8
Oxymorphone	302.1	227	198.1	69.0	13.8
Oxycodone	316.1	298.1	256.1	22.5	4.5

Figure 3. MS source parameters and SRM transitions

Results and Discussion

Validation

The validation procedure includes tests for 1) recovery; 2) lower limit of quantitation (LLOQ), dynamic range, accuracy; 3) precision; and 4) carryover.

Recovery

First, we determined the optimal dilution factor for urine sample preparation. Twelve lots of blank human urine samples, six lots of donor urine samples, and two water samples were spiked with the IS, hydrolyzed, and diluted 100-, 250- and 500-fold with water. The SRM signals of the internal standards from the urine samples and the water samples were compared for absolute recovery. Table 1 shows the average recoveries (n=18) for the six opioids using different dilution factors. Clearly, the 500-fold dilution led to the highest recoveries for all six opioids.

We used the 500-fold dilution to determine the recoveries for unlabeled opioids spiked into 12 lots of blank urine samples. Two concentrations of opioids at 100 and 500 ng/mL were tested. The absolute recovery was determined by comparing the signals of unlabeled opioids in urine and water samples. The relative recovery was determined by comparing the analyte/IS ratio in urine and water samples. The recovery results are summarized in Table 2. There was minimum ion suppression for morphine, codeine, hydromorphone and hydrocodone. Although there was moderate ion suppression for oxymorphone and oxycodone even after 500-fold dilution, the relative recoveries against their IS were nearly 100% in both concentration levels after compensation from the IS.

Table 1. Dilution factor test results

Recovery (%, n=18)	500x	250x	100x
Morphine-d3	101.2	86.6	85.4
Codeine-d3	99.5	88.0	79.7
Hydromorphone-d6	85.9	73.1	63.7
Hydrocodone-d3	78.0	68.2	67.2
Oxymorphone-d3	59.9	45.1	43.2
Oxycodone-d3	68.2	52.3	42.3

Table 2. Summary of recoveries with 500-fold dilution

		100 ng/mL[a]		500 ng/mL[a]	
Analyte	Recovery (%)	Average (%, n=12[b])	Standard Deviation (%, n=12)	Average (%, n=12)	Standard Deviation (%, n=12)
Morphine	Absolute	76.4	6.8	78.6	5.4
	Relative	92.1	10.9	96.1	9.6
Codeine	Absolute	86.5	6.0	89.7	6.2
	Relative	88.7	10.6	95.6	8.2
Hydromorphone	Absolute	74.4	7.1	73.2	6.6
	Relative	92.8	8.1	89.9	7.0
Hydrocodone	Absolute	82.6	9.0	71.8	6.7
	Relative	101.9	17.1	83.6	13.4
Oxymorphone	Absolute	57.5	7.6	57.9	7.0
	Relative	103.7	17.8	103.0	15.1
Oxycodone	Absolute	63.4	9.9	68.7	8.1
	Relative	90.6	8.5	103.8	8.5

[a] Two levels of spiked opioids concentrations were tested.

[b] Twelve different individual urine lots were tested and compared to water samples (n=2).

Lower Limit of Quantitation (LLOQ), Dynamic Range, and Accuracy

Blank human urine samples were spiked with the six opioids and their IS. Concentrations of the opioids ranged from 20 to 5000 ng/mL. At each concentration level, three individually processed replicates were tested. The concentration of IS was 100 ng/mL for all samples. Linearity samples were analyzed in triplicate along with one set of calibrators, which were also prepared in blank human urine. The calibration curves for morphine and codeine (Figures 4 and 5) were constructed by plotting the analyte/IS peak area ratio vs. analyte concentration.

The linearity was determined to be 20 to 5000 pg/mL for all six opioids. The LLOQ for the six opioids were determined to be 20 ng/mL. At LLOQ, the accuracy (n=3) ranged from 99.2% to 115.5% for the six opioids and the precision (n=3) ranged from 3.9% to 8.8% (Table 3). Within the linear range, the accuracies (at higher than LLOQ levels) were within 11.2% for the six opioids (data not shown). Figures 4 and 5 show the calibration curves for morphine and codeine. Figure 6 shows the SRM chromatograms of the six opioids at their LLOQ in spiked human urine. The signal-to-noise ratios for all six opioids at their LLOQs were excellent.

Table 3. LLOQ, linear range and accuracy for the six opioids in urine

Analyte	LLOQ (ng/mL)	Linear range (ng/mL)	Accuracy at LLOQ (%, n=3)	Precision at LLOQ (%, n=3)
Morphine	20	20-5000	100.8	6.1
Codeine	20	20-5000	102.1	6.9
Hydromorphone	20	20-5000	115.5	8.8
Hydrocodone	20	20-5000	99.2	3.9
Oxymorphone	20	20-5000	102.3	6.2
Oxycodone	20	20-5000	107.4	4.4

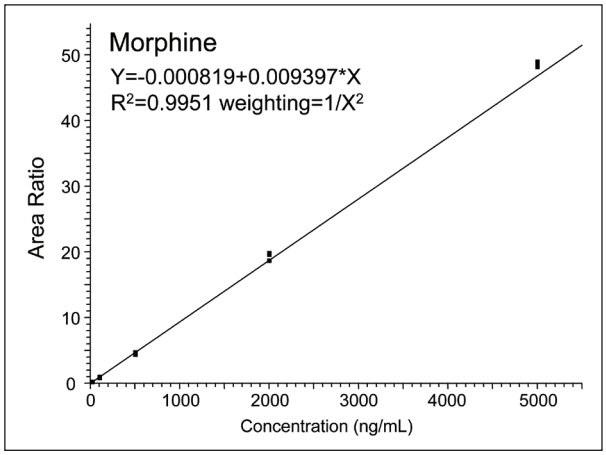

Figure 4. Calibration curve of morphine in human urine

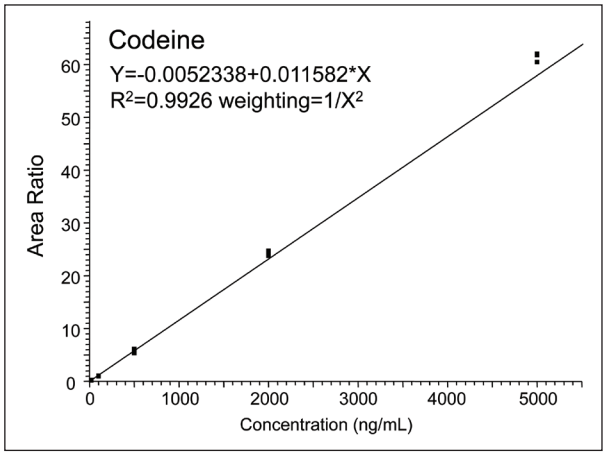

Figure 5. Calibration curve of codeine in human urine

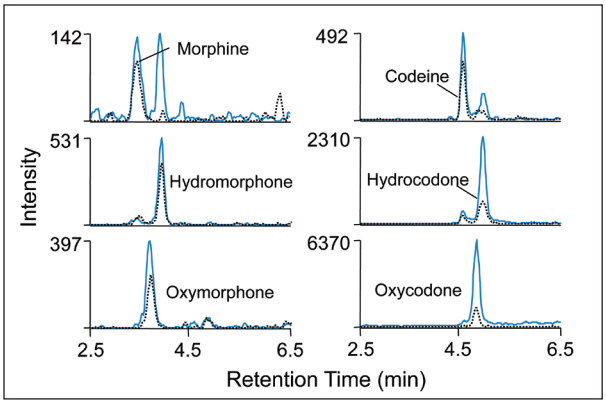

Figure 6. SRM chromatograms (quantifier: solid line; and qualifier: dotted line) of the six opioids at LLOQ in spiked human urine

Precision

Precision was assessed with spiked human urine at concentrations of 40 and 200 ng/mL. Inter- and intra-assay CV values at low and high quality-control concentrations varied between 5.0% and 12.9% (Table 4).

Table 4. Precision data

Precision (%)	Intra (n=5)	Inter (n=15)	Intra (n=5)	Inter (n=15)
Concentration (ng/mL)	40	40	200	200
Morphine	12.0	10.8	9.7	7.4
Codeine	6.8	6.4	9.3	8.0
Hydromorphone	7.0	7.7	5.9	5.0
Hydrocodone	8.3	8.2	12.9	10.0
Oxymorphone	14.1	11.4	7.9	6.4
Oxycodone	5.1	6.3	6.7	5.8

Carryover

No carryover was observed.

Solvent Usage

The method used only 5%–10% of the solvent amount used at a normal flow rate setting (0.5 mL/min). This dramatically lower solvent use will significantly lower both initial solvent cost and the cost of disposing of solvent waste.

Conclusion

We have used a novel approach for sensitive quantitation of six opioids in urine for forensic toxicology purposes. This approach used super-dilution to minimize frequently observed ion suppression in urine samples and used a microflow LC setup (Ultimate 3000 RSLCnano LC system and TSQ Vantage mass spectrometer) to compensate for sensitivity losses from super-dilution. This robust method was linear between 20 and 5000 ng/mL for the six opioids and highly accurate and precise. The method used only 5%–10% of the solvent amount used at a normal LC flow rates, significantly lowering both solvent purchase and waste disposal costs.

For forensic toxicology use only.

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Quantitation of Amphetamines in Urine for SAMHSA Mandated Workplace Drug Testing Using a Triple Stage Quadrupole LC-MS System

Kristine Van Natta, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra
- Hypersil Gold
- NIDA
- Methamphetamine
- Phentermine

Introduction

Federal employees and public transportation workers are required to pass a pre-employment drug screen known as the NIDA5, which refers to the five drugs of abuse that are required to be tested for by the National Institute of Drug Abuse (NIDA), or the Substance Abuse and Mental Health Services Administration (SAMHSA) panel. The assays are divided into 5 groups: opiates, amphetamines, cocaine (benzoylecgonine), cannabis (THCA) and PCP. In the past, these five groups have been screened by immunoassay and confirmed by gas chromatography-mass spectrometry (GC/MS). In October 2010, SAMHSA approved the use of liquid chromatography-mass spectrometry (LC/MS) for confirmation of workplace drug testing samples. Here we will focus on the amphetamine group which consists of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA or Ecstasy) and methylenedioxyethylamphetamine (MDEA).

Goal

To develop a specific and robust dilute-and-shoot quantitative method for the confirmation of amphetamine, methamphetamine, MDA, MDMA, MDEA in urine that meets SAMHSA cutoffs. Additionally, the method should be able to discriminate between the structural isomers methamphetamine and phentermine.

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with β-glucuronidase. While amphetamines do not require hydrolysis, other compounds in the SAMHSA panel such as the opiates and THC do require hydrolysis. Adding this step enables all SAMHSA panel compounds to be processed with one method. Methanol was added to the hydrolysis mixture and the resulting mixture was centrifuged. The supernatant was further diluted and subjected to LC-MS analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD aQ column (50 x 4.6 mm, 1.9 µm particle size). The mobile phase consisted of 5 mM ammonium formate with 0.1% formic acid in both water and methanol. The flow rate was 1.5 mL/min and the column was maintained at 30 °C. The total run time was 4.5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low (LQC), middle (MQC) and high (HQC) end of the calibration range. Intra-run variability and robustness were determined by analyzing six replicates of each QC level with a calibration curve on three different days. Matrix effects were investigated by comparing peak area of analytes prepared in multiple lots of urine to those of a sample prepared in water.

Results and Discussion

The limits of quantitation (LOQs) for all compounds meet the SAMHSA confirmation requirements. (Table 1). The method is linear up to 5,000 ng/mL with R2 values > 0.99 for all compounds. Figure 1 shows representative calibration curves for all compounds. Quality control results for the validation are shown in Table 2. Figure 2 shows an SRM chromatogram at LOQ. Peak areas of analytes in samples prepared from seven different lots of blank human urine compared to that of a sample prepared in water were all within 15% for amphetamine, methamphetamine, MDMA and MDEA. The peak areas were within 30% for MDA.

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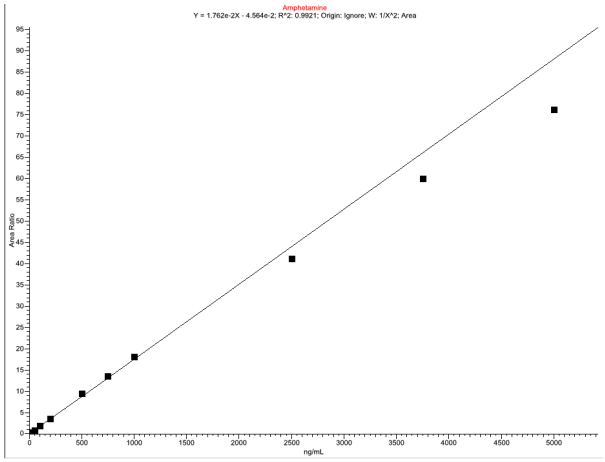
Table 1. Method summary for quantitation of amphetamines in urine

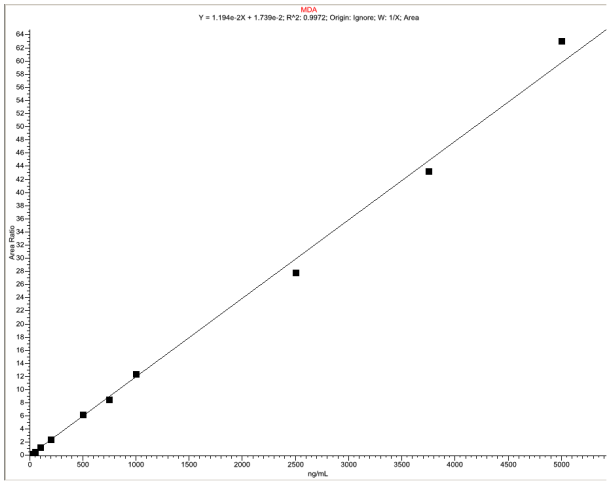
Compound	LOQ	ULOQ	SAMHSA Cutoff
Amphetamine	10 ng/mL	5000 ng/mL	250 ng/mL
Methamphetamine	5 ng/mL	5000 ng/mL	250 ng/mL
MDA	20 ng/mL	5000 ng/mL	250 ng/mL
MDMA	5 ng/mL	5000 ng/mL	250 ng/mL
MDEA	5 ng/mL	5000 ng/mL	250 ng/mL
Phentermine	Not quantitated, but chromatographically well-separated from isomeric methamphetamine.		
Total run time: 4.5 minutes			

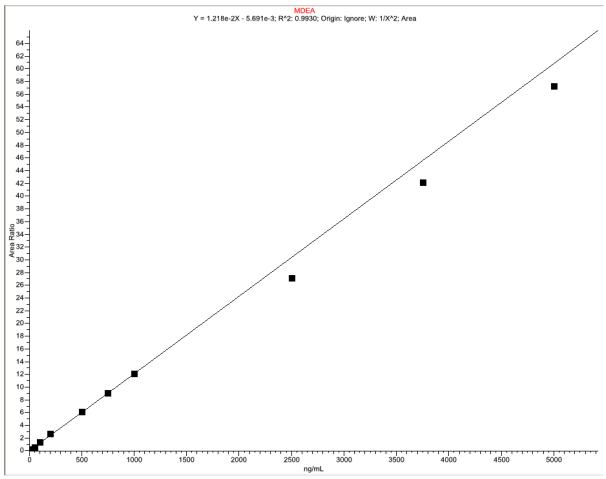
Table 2. %CV/%Bias for QCs analyzed during validation of amphetamines in urine

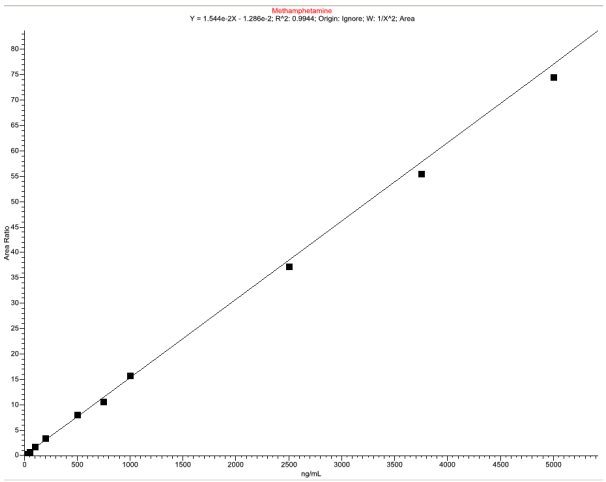
Compound	LQC (10 ng/mL)	MQC (100 ng/mL)	HQC (500 ng/mL)
Amphetamine	10.9/-2.24	4.45/6.39	2.56/0.431
Methamphetamine	7.03/0.420	3.02/7.78	4.26/1.67
MDA	NA	5.97/3.46	4.17/-0.196
MDMA	5.88/0.737	3.31/7.88	4.95/3.45
MDEA	4.51/3.35	2.96/8.20	4.34/2.54

NA: LQC concentration is below LOQ for MDA; data not reported.









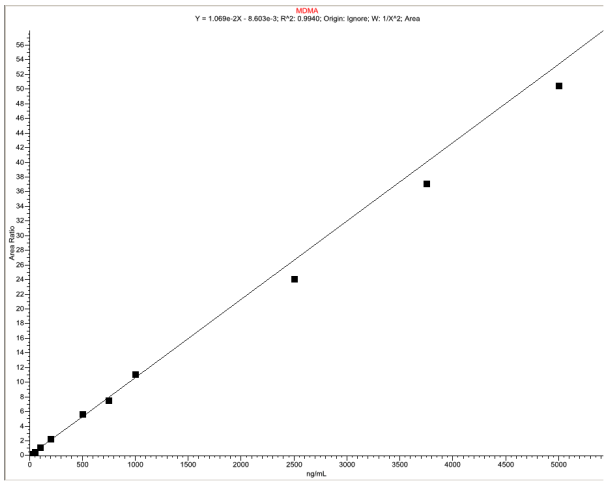


Figure 1. Representative calibration curves for amphetamine, methamphetamine, MDA, MDMA, MDEA in urine

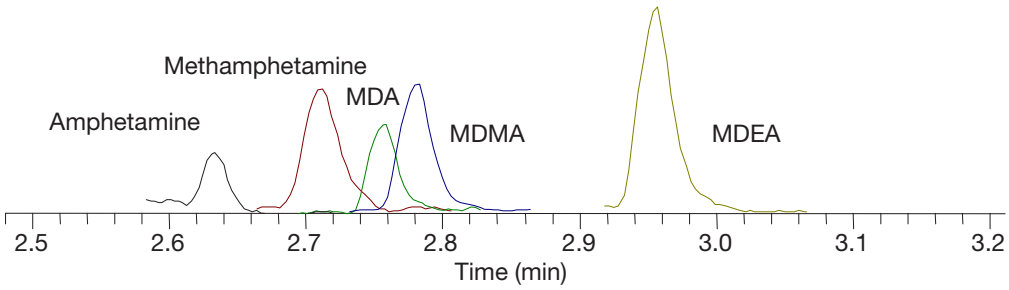


Figure 2. SRM chromatogram of amphetamine, methamphetamine, MDA, MDMA and MDEA in urine at their respective LOQs

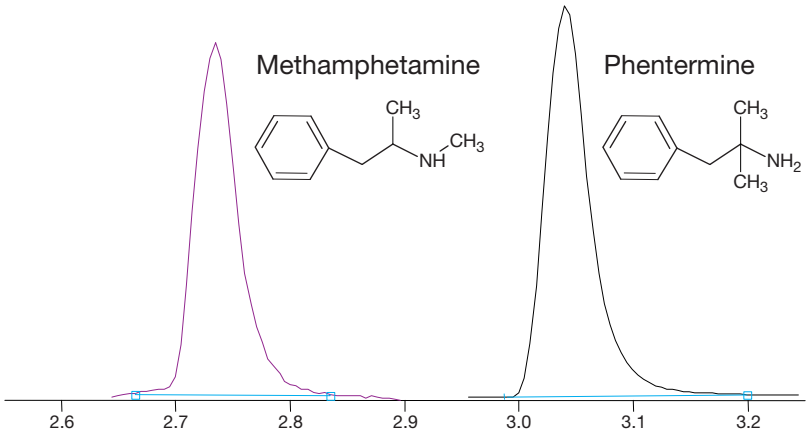


Figure 3. SRM chromatogram showing excellent resolution between structural isomers methamphetamine and phentermine

Methamphetamine and phentermine (an anti-obesity drug) are structural isomers with identical molecular masses and similar fragments. To avoid false positives, they must be separated chromatographically. As seen in Figure 3, these two compounds are well-resolved and will not interfere with each other.

Conclusion

A method with simple dilute-and-shoot sample preparation for the confirmation of amphetamines in urine was developed. This method is suitable for SAMHSA-mandated workplace drug testing, meeting cutoff and specificity requirements within a 4.5-minute run. The sample processing method also enables all SAMHSA panels to be processed at once.

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Quantitation of Synthetic Cannabinoids in Urine Using a Triple Stage Quadrupole LC-MS System in Forensic Toxicology

Kristine Van Natta, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra
- JWH-018
- JWH-073
- Spice
- K2
- Forensic Toxicology

Introduction

Synthetic cannabinoids are compounds made to mimic the effects of natural cannabinoids found in the cannabis plant (marijuana). They were first synthesized by pharmaceutical companies seeking to mimic the beneficial analgesic and anti-nausea effects of cannabis while trying to eliminate the psychoactive euphoric effects for which the plant is so abused. In the mid 1980's, these compounds began appearing in herbal incense, marketed as "legal highs" under the names "Spice" and "K2." Effects are similar to those of cannabis, but with reports of increased anxiety and paranoia. In early 2011, the U.S. Drug Enforcement Administration (DEA) regulated five of these compounds as Schedule I drugs.

Simple, robust and precise analytical methods are needed to quantitate these now illegal compounds in biological matrices for forensic purposes. Here we will focus on JWH-018 and JWH-073. Research has shown that parent compound is not excreted in urine. The reported metabolites seen in urine are the alkyl-hydroxy and alkyl-carboxy metabolites of each compound.

Goal

To develop a specific and robust dilute and shoot quantitative method for the analysis of the alkyl-hydroxy and alkyl-carboxy metabolites of JWH-018 and 073: JWH-018-OH, JWH-018-COOH, JWH-073-OH and JWH-073-COOH in urine.

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with β-glucuronidase. Fisher Chemical acetonitrile was added to the hydrolysis mixture and the resulting mixture was centrifuged. Supernatant was further diluted and subjected to liquid chromatography-mass spectrometry (LC-MS) analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD column (100 x 2.1 mm, 3 µm particle size). Mobile phase consisted of 5 mM ammonium formate in both water and methanol. The total run time was 15.5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe (Figure 1). Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low, middle and high end of the calibration range. Inter- and intra-run variability and robustness were determined by analyzing replicates of each QC level with a calibration curve on three different days.

Figure 1. TSQ Quantum Ultra triple stage quadrupole mass spectrometer with Accela HPLC system

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Results and Discussion

The method is linear from 2 to 1,000 ng/mL with R^2 values greater than 0.99 for all compounds (Figure 2). Table 1 shows QC precision and bias data for the validation runs.

A 15-minute run was required to chromatographically separate the analytes of interest from endogenous interferences. Figures 3 and 4 show this chromatographic resolution in a 2-ng/mL and 100-ng/mL standard, respectively. Figure 5 shows a SRM chromatogram from a self-confessed consumption sample.

Table 1. Inter-Assay %CV and % Bias for Quality Control Samples

	LQC	MQC	HQC
JWH-018-OH	10.4/-0.790	3.50/-2.21	7.81/2.51
JWH-018-COOH	8.07/11.6	3.82/6.38	6.37/6.29
JWH-073-OH	9.02/3.72	3.42/-0.359	5.99/0.847
JWH-073-COOH	11.8/14.0	3.75/9.46	4.78/7.34

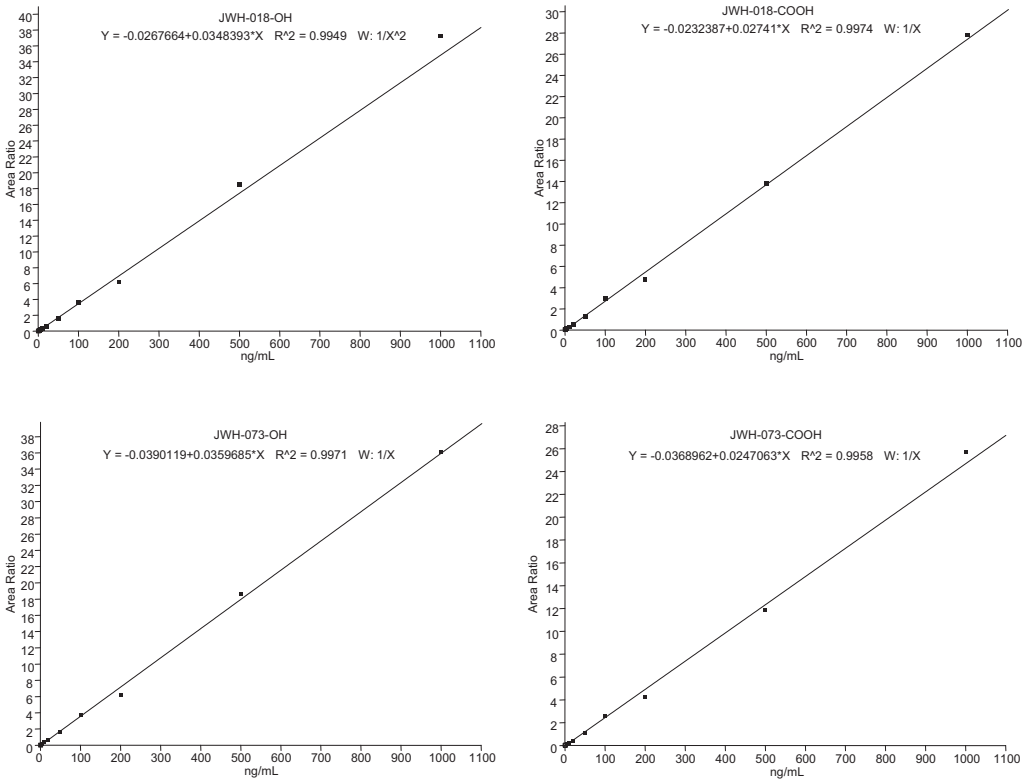

Figure 2. Representative calibration curves for JWH-018 and JWH-073 metabolites showing linearity from 2-1,000 ng/mL in urine

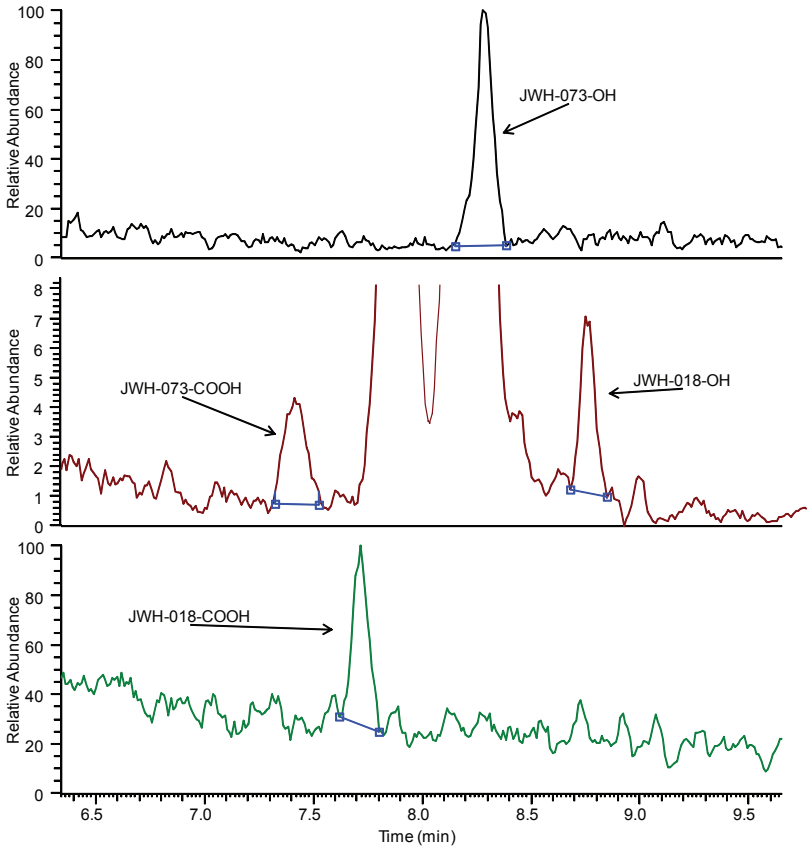

Figure 3. SRM chromatogram of a 2 ng/mL standard showing resolution of analytes from unknown endogenous interferences.

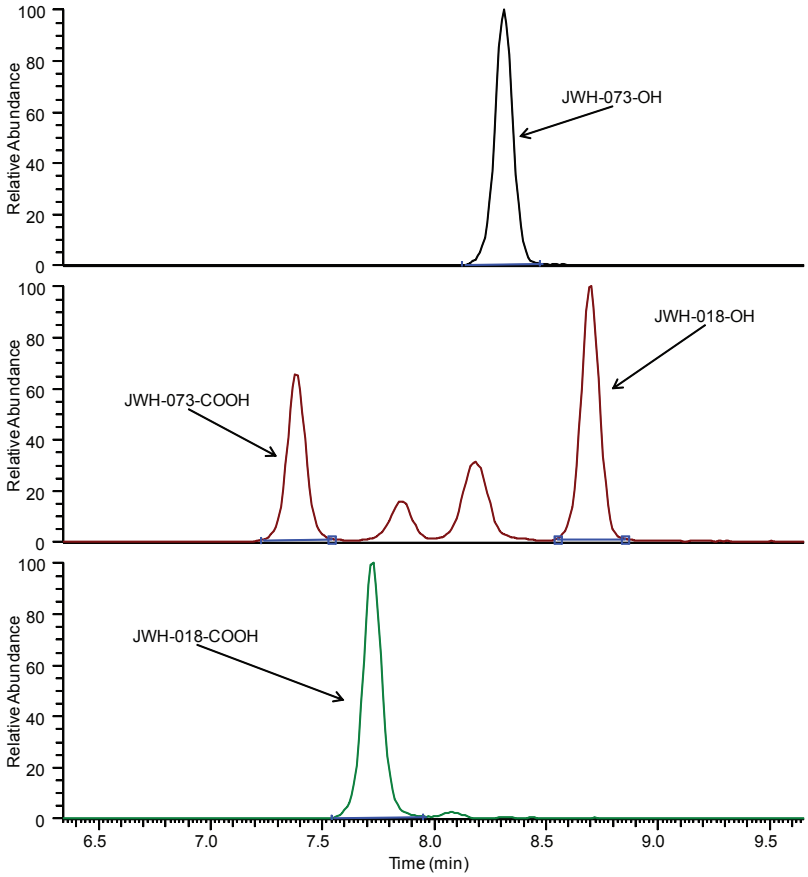

Figure 4. SRM chromatogram of a 100 ng/mL standard.

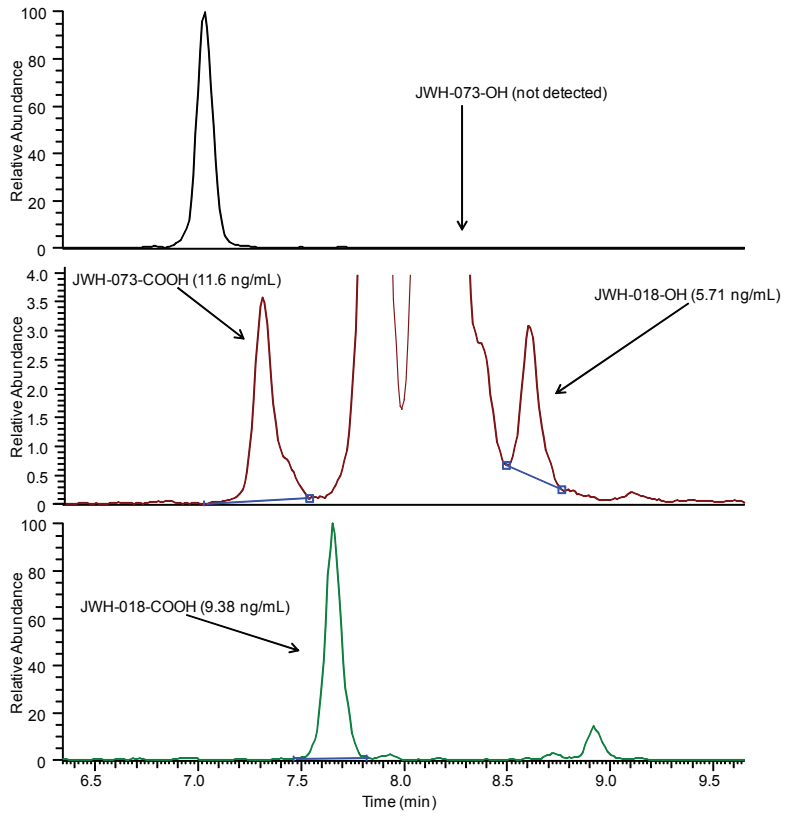

Figure 5. SRM chromatogram of self-confessed human in vivo sample. JWH-073-N-(4-hydroxybutyl), a compound validated in this assay, is not seen in this sample. The unidentified peak in the JWH-073-OH channel is JWH-073-N-(3-hydroxybutyl), a major metabolite not known at the time of this validation.

Conclusion

A simple dilute and shoot method for the analysis of synthetic cannabinoid metabolites in urine was developed for forensic toxicology use. Since analysis of these compounds is relatively new to forensic applications, cut-off values have not been established. The current method has an LOQ of 2 ng/mL for all compounds. Based on published research, using an SPE or liquid/liquid extraction processing method will lower the current LOQ to 0.2 ng/mL, if required.

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Antidepressants and Neuroleptics Quantitation Using Tandem Mass Spectrometry and Automated Online Sample Preparation

Hans-Rudolf Kuhn; Unilabs, Switzerland
Bénédicte Duretz; Thermo Fisher Scientific, Les Ulis, France

Key Words

- TurboFlow Technology
- TSQ Quantum Access MAX
- Triple Quadrupole
- Clinical Research

Introduction

Liquid chromatography-mass spectrometry (LC/MS) is a powerful technique applied in clinical research for the analysis of a broad number of analytes. Offline sample preparation techniques (solid phase extraction and liquid-liquid extraction) are widely used but are often time consuming and labor intensive. The Thermo Scientific Transcend system powered by TurboFlow™ technology provides an alternative approach simplifying sample preparation.

Goal

To develop a fast and efficient LC-MS/MS method using Thermo Scientific TurboFlow technology for the analysis of 18 antidepressants and neuroleptics.

Experimental

Sample Preparation

A 100 µL aliquot of serum or plasma sample was mixed with 300 µL of methanol containing internal standards (Venlafaxine-d6 and Sertraline-d3) at 100 ng/mL. The resulting mixture was thoroughly vortexed, allowed to stand for 10 minutes at room temperature and then centrifuged at 4 °C for 10 minutes.

Chromatography and Mass Spectrometry

High pressure LC (HPLC) was performed using the Transcend™ TLX system. Serum and plasma samples were extracted using a TurboFlow Cyclone P (0.5 x 50 mm) extraction column. Chromatographic separation was performed using a Thermo Scientific Hypersil GOLD column (50 x 3 mm, 3 µm particle size). Gradient elution was used. Total analysis time was 8 minutes.

The TurboFlow method conditions were as follows:

Eluent A:	0.1% Formic acid in water
Eluent B:	0.1% Formic acid in methanol
Eluent C:	Acetonitrile, isopropanol and acetone (45/45/10, v/v/v)
Eluent D:	Acetonitrile, water (90/10, v/v)

The analytical LC conditions were as follows:

Eluent A:	0.1% Formic acid in water
Eluent B:	0.1% Formic acid in methanol

The entire LC effluent from the sample injections was directed to the Thermo Scientific Ion Max source, utilizing heated electrospray ionization (HESI), on a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer in positive ion selected reaction monitoring (SRM) mode.

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Results and Discussion

For each analyte, linearity and quantitative results were obtained using SRM transitions. Quantitation of the 18 drugs was performed with a calibration range of 5 to 500 ng/mL for 5 compounds, 10 to 1000 ng/mL for 9 compounds, 2 to 200 ng/mL for 3 compounds, and 1 to 100 ng/mL for 1 compound. The R^2 value for each of the calibration curves was above 0.998, which indicates an excellent linear fit over the dynamic range. Figure 1 shows the chromatogram of the lowest calibration standard. Calibration curves for risperidone and clozapine are reported in Figure 2. Table 1 displays the calibration ranges and method precision for all analyzed drugs.

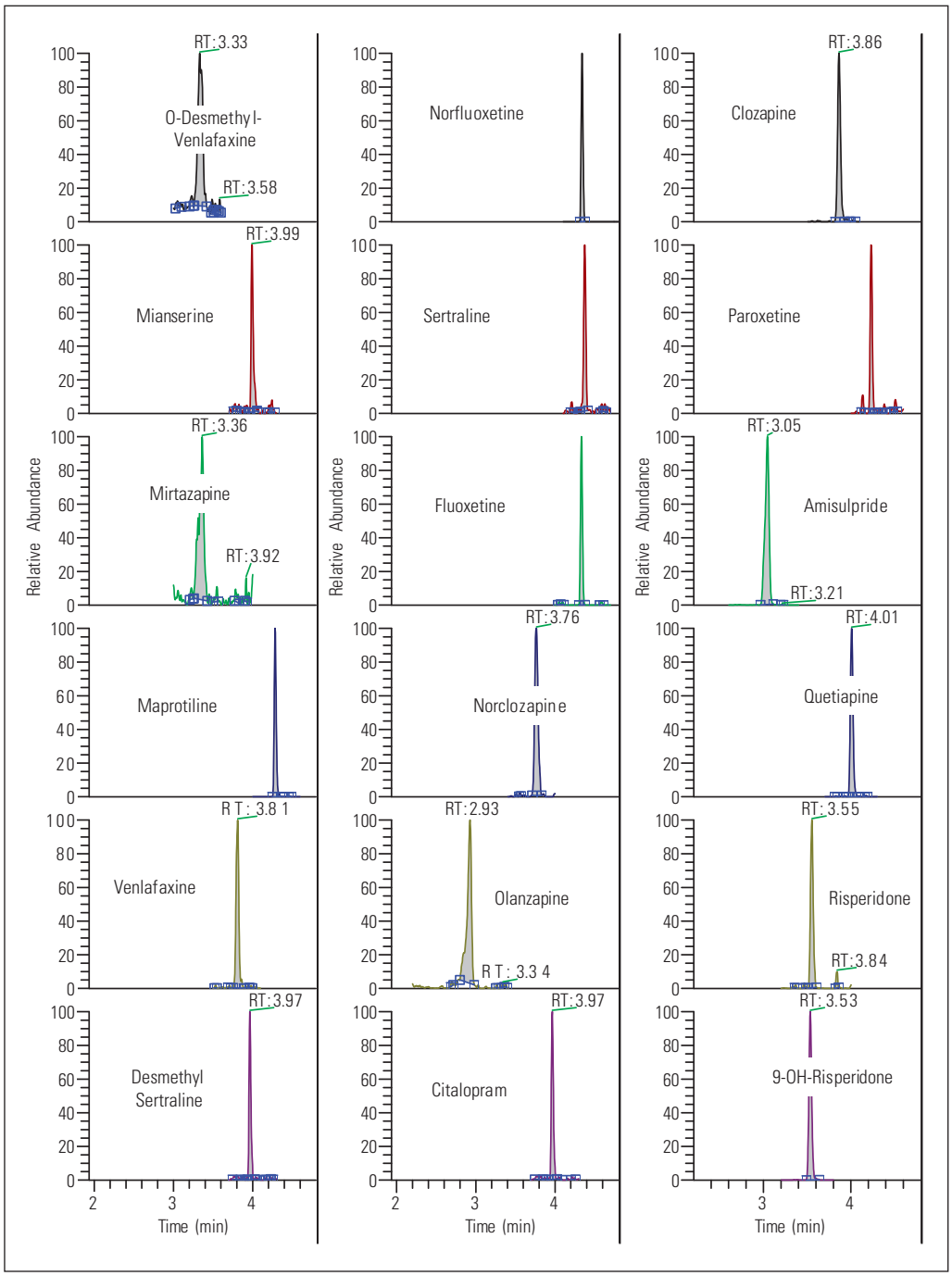

Figure 1. Representative chromatograms for the methods at the low end of the calibration curve

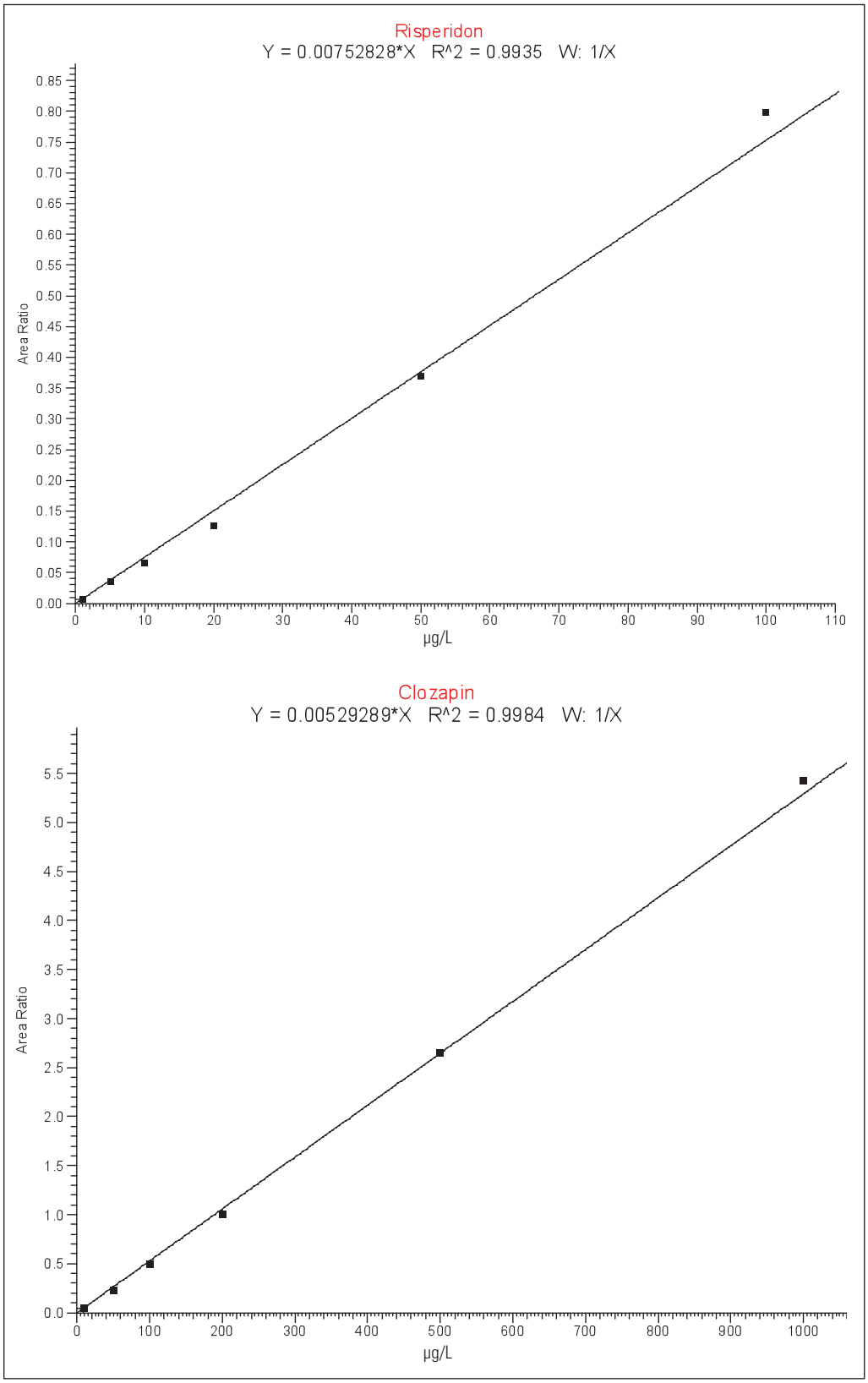

Figure 2. Calibration curves for risperidone and clozapine

Table 1. Calibration ranges and method precision for all the analytes

Analyte	Calibration range (ng/mL)	Within-day (%RSD)*	Between-days (%RSD)**
9-OH-Risperidone	5-500	7.1	5.5
Amisulpride	10-1000	3.9	3.4
Citalopram	5-500	4.9	5.1
Clozapine	10-1000	5.8	4.3
Desmethyl Sertraline	2-200	6.3	6.0
Fluoxetine	10-1000	3.4	3.4
Maprotiline	10-1000	4.2	4.1
Mianserine	5-500	6.2	5.1
Mirtazapine	2-200	5.9	4.4
Norclozapine	10-1000	5.9	3.6
Norfluoxetine	10-1000	6.5	5.0
O-Desmethyl-Venlafaxine	10-1000	4.3	4.8
Olanzapine	5-500	6.2	3.3
Paroxetine	5-500	6.2	5.3
Quetiapine	10-1000	5.2	3.5
Risperidone	1-100	5.8	5.4
Sertraline	2-200	4.5	3.4
Venlafaxine	10-1000	4.5	3.4

* Replicates analyzed each day = 10
** Days averaged = 10

Conclusion

A fast and analytically sensitive method for the detection of 18 antidepressants and neuroleptics is described. The Transcend TLX automated online sample preparation system allows minimal sample preparation and time saving in the absence of SPE sample preparation for clinical research laboratories.

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Demonstrating High-Performance Quantitative Analysis of Benzodiazepines using Multiplexed SIM with High-Resolution, Accurate Mass Detection on the Q Exactive LC/MS

Kevin J. McHale; Thermo Fisher Scientific, Somerset, NJ

Key Words

- Q Exactive
- Accela UHPLC
- Selected Ion Monitoring
- Drug Quantitation

Introduction

In today's modern forensic toxicology laboratories, there is a growing demand to have a mass spectrometer with the power and flexibility to perform experiments both for the identification of unknown compounds and for trace-level quantification of target analytes. Additionally, this platform must execute these analyses with minimal sample preparation, provide consistent results and be easily assimilated into the laboratory workflows. With the introduction of the Thermo Scientific Q Exactive high-performance benchtop quadrupole-Orbitrap mass spectrometer, the most stringent qualitative and quantitative objectives can be met. By using high-resolution, accurate mass (HRAM) detection with quadrupole selected ion monitoring (SIM), targeted quantification of benzodiazepines in urine can be accomplished with sensitivity that rivals triple stage quadrupole instruments in selected reaction monitoring (SRM) mode.

Goal

To demonstrate the feasibility of high sensitivity liquid chromatography-mass spectrometry (LC/MS) quantification of benzodiazepines in urine by combining multiplexed SIM with high-resolution, accurate mass detection on the Q Exactive™ high-performance benchtop quadrupole-Orbitrap mass spectrometer.

Experimental

Sample Preparation

Eight benzodiazepines were spiked into blank human urine containing acetonitrile at 10% (v/v) from 0.0125 to 250 ng/mL prior to LC/MS.

UHPLC

Ultra high performance LC (UHPLC) analyses were performed using a Thermo Scientific Accela 1250 liquid chromatography system with an Open Accela™ autosampler. Gradient elution with a Thermo Scientific Hypersil GOLD PFP column (50 x 2.1 mm; 1.9 µm particle size) was used at a flow rate of 500 µL/min. The injection volume was 5 µL.

Mass Spectrometry

MS measurements were accomplished on a Q Exactive mass spectrometer with a heated electrospray ionization (HESI) source in positive ion mode. Quadrupole isolation was set to 1.5 *m/z* with subsequent detection at a mass resolution of 140,000 FWHM via external mass calibration.

Results and Discussion

SIM is a well-established technique for targeted LC/MS quantitation using single quadrupole mass spectrometers. However, its utility is limited owing to the low specificity of unit mass resolution on single quads. The Q Exactive mass spectrometer, which employs Orbitrap-based high-resolution, accurate mass detection, overcomes this limitation. Additionally, the duty cycle on the Q Exactive MS is enhanced by measuring multiple SIM ions simultaneously in the Orbitrap mass analyzer. The process of multiplexed SIM is illustrated in Figure 1. Four different ions are selected by the quadrupole and stored in the C-trap while the Orbitrap analyzer measures the ions from the previous cycle. This process is repeated by passing the four SIM ions from the C-trap to the Orbitrap analyzer for the next mass measurement. The Q Exactive mass spectrometer has the capability to multiplex between two and ten SIM ions.

Table 1 lists the eight benzodiazepines quantified by HRAM LC/MS with their multiplexed SIM time windows, the measured mass errors using external mass calibration, and the lower limits of quantitation (LLOQs) in urine on the Q Exactive mass spectrometer. Two key points to highlight in Table 1 are that (1) mass errors on the Q Exactive system are significantly less than 5 ppm without the need of an internal calibration mass, and (2) the LLOQs of the eight benzodiazepines analyzed in urine are in the pg/mL range.

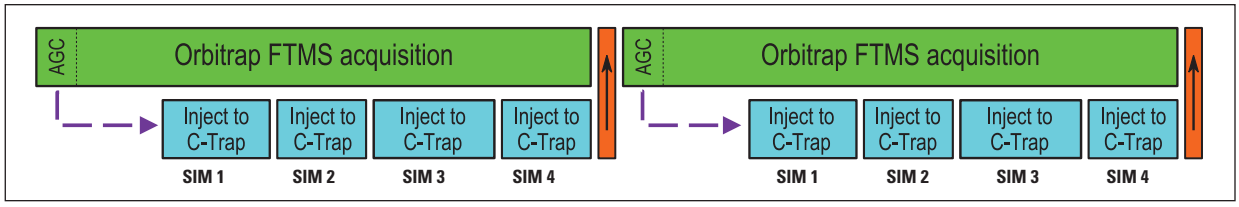

Figure 1. Schematic of multiplexed SIM on the Q Exactive mass spectrometer

Table 1. List of benzodiazepines quantified by HRAM LC/MS on the Q Exactive mass spectrometer

Compound	SIM Time Window (min)	Exact *m/z*	Measured *m/z*	Error (ppm)	LLOQ (ng /mL)
Oxazepam	0.00-3.45	287.05818	287.05829	+0.4	0.0625
Lorazepam	0.00-3.65	321.01921	321.01926	+0.2	0.1250
Nitrazepam	0.00-3.65	282.08732	282.08746	+0.5	0.0625
Clonazepam	0.00-3.85	316.04835	316.04828	-0.2	0.0625
Temazepam	3.45-6.00	301.07383	301.07410	+0.9	0.0250
Flunitrazepam	3.65-6.00	314.09355	314.09296	-1.9	0.0625
Alprazolam	3.65-6.00	309.09015	309.09024	+0.3	0.0125
Diazepam	3.85-6.00	285.07892	285.07901	+0.3	0.0125

Figure 2 presents an example LC/MS analysis of benzodiazepines at 0.125 ng/mL in urine using multiplexed SIM on the Q Exactive mass spectrometer. By acquiring these data at a mass resolution of 140,000 FWHM, little or no chemical noise is observed for the ± 5 ppm extracted ion chromatograms of the benzodiazepines in urine. The selectivity afforded by the Q Exactive mass spectrometer at a resolution of 140,000 FWHM is illustrated in the SIM spectrum for oxazepam (Figure 3). In addition to the oxazepam ion at *m/z* 287.05829, there are at least 12 other ions observed within a 0.25 *m/z* range. Yet, the oxazepam ion is easily separated from the other chemical interference ions with the high resolving power of the Q Exactive mass spectrometer.

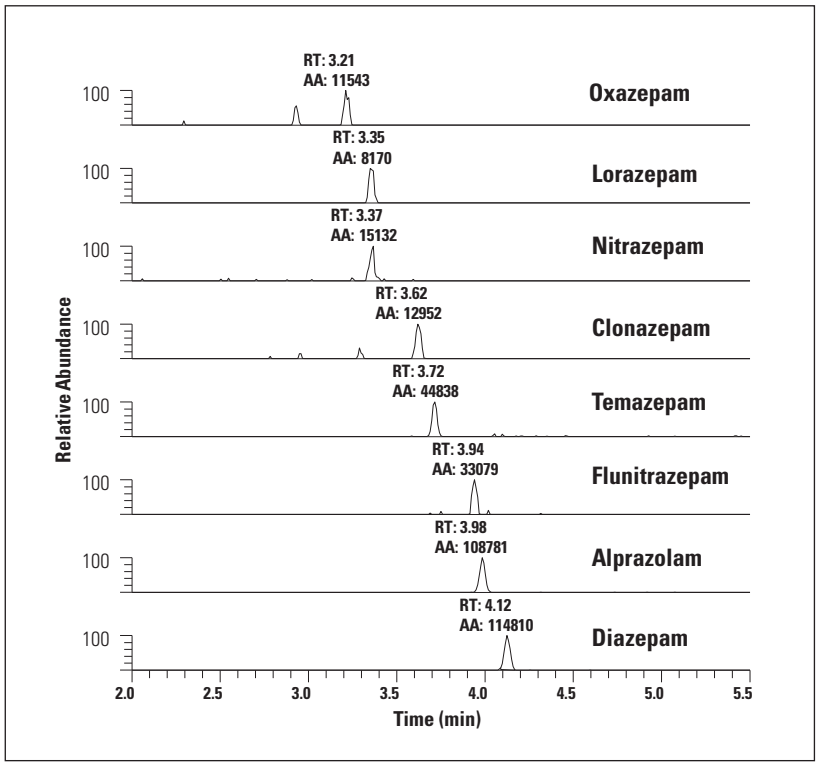

Figure 2. Extracted ion chromatograms (5 ppm) for 0.125 ng/mL benzodiazepines in urine

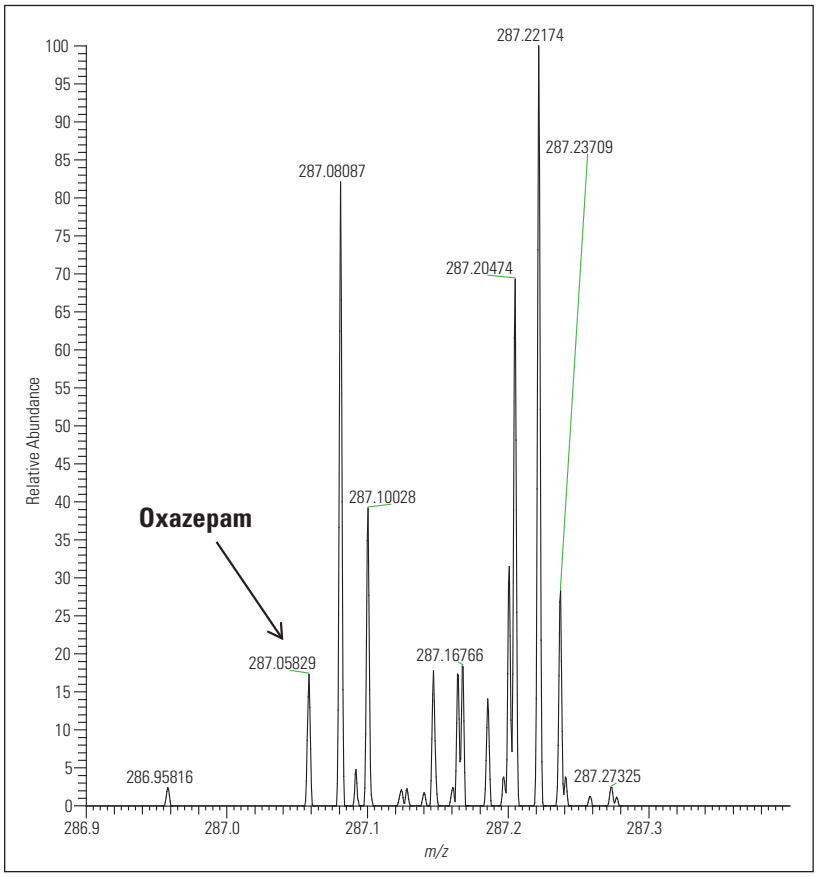

Figure 3. SIM spectrum of oxazepam in urine at mass resolution of 140,000 FWHM

Figure 4 and Table 2 demonstrate the overall quantitative performance of the Q Exactive mass spectrometer for diazepam in urine. The calibration curve for diazepam in Figure 4 shows a linear dynamic range of over four decades (0.0125 – 250 ng/mL), including the inset from 0.0125 to 0.25 ng/mL, with

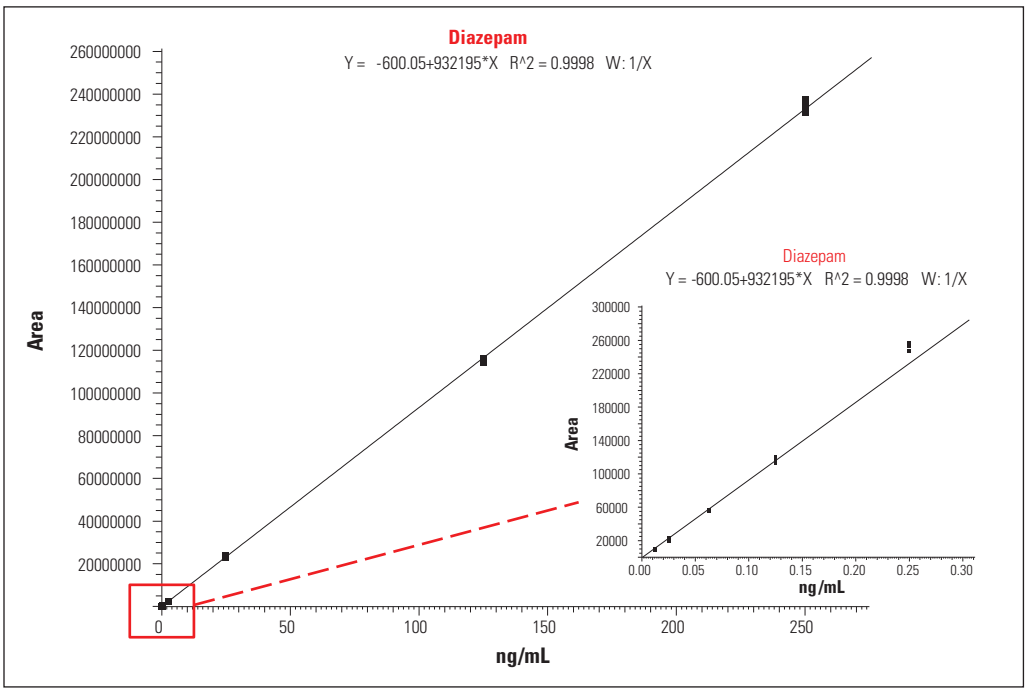

Figure 4. Calibration curve for diazepam in urine from 0.0125 – 250 ng/mL

an R^2 regression value of 0.9998 using 1/x weighting. Table 2 presents the statistical results for the HRAM quantification of diazepam. The quantitative accuracy and precision values obtained by the Q Exactive mass spectrometer using multiplexed SIM are comparable to those observed on triple stage quadrupole mass spectrometers in SRM mode.

Table 2. Statistical results for HRAM LC/MS quantitation of diazepam in urine

Specified Amount (ng /mL)	Mean Calculated Amt. (ng/mL)	%Accuracy	%CV
0.0125	0.0113	90.1	6.0
0.0250	0.0236	94.3	7.9
0.0625	0.0610	97.6	1.4
0.1250	0.127	101.9	2.9
0.250	0.273	109.1	1.7
2.50	2.65	105.9	1.0
25.0	25.5	102.0	1.7
125.0	123.5	98.8	0.8
250.0	250.8	100.3	1.2

Conclusion

The Q Exactive HRAM LC/MS system is a powerful and flexible instrument that can provide both sample identification and quantitative information for forensic toxicology with a single sample analysis. By using the method of multiplexed SIM, eight benzodiazepines in urine were quantified with LLOQs at the pg/mL level and with linear dynamic ranges of 3 to 4 orders of magnitude.

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THC-COOH Quantification in Urine Using Dilute and Shoot LC-MS/MS Method for Forensic Toxicology

Samuele Scurati, Thermo Fisher Scientific, Rodano, Italy
Maura Brambilla, Ludovico D'Amato, Paolo Brambilla, University Department of Laboratory Medicine, Hospital of Desio, Desio, Italy

Key Words

- TSQ Quantum Access MAX
- Accela Pump
- Cannabinoids
- Forensic Toxicology

Introduction

Cannabis sativa is a widely used drug of abuse. Tetrahydrocannabinol (THC) is the major psychoactive chemical compound in the cannabis plant. After smoke inhalation, THC is absorbed and distributed in blood. Subsequently, it is rapidly metabolized to THC-COOH, conjugated with glucuronic acid, and excreted through urine. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered a useful tool to establish the consumption of cannabis by the assessment of THC-COOH in urine for forensic toxicology purposes.

Goal

To develop a reliable and fast analytical method for the quantitative determination of THC-COOH in urine using a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer.

Experimental

Sample Preparation

A urine sample was hydrolyzed with 10M NaOH and heated at 60 °C for 15 minutes. The pH was restored with Fisher Chemical acetic acid. Hydrolyzed samples as well as calibrators were diluted 1:10 in Fisher Chemical water/acetonitrile (1:1). Then, 10 µL were directly injected. Quantitative analysis was performed on the basis of calibration curves prepared in urine, ranging from 7.8 to 1000 ng/mL. Calibrators were injected in duplicate.

UHPLC conditions

Liquid chromatography separation was performed using a Thermo Scientific Accela autosampler and pump. The sample was injected directly on a Thermo Scientific Hypersil GOLD column (50 × 2.1 mm, 1.9 µm). A gradient LC method used mobile phases A (0.1% aqueous formic acid) and B (Fisher Chemical Optima LC/MS acetonitrile) at a flow rate of 300 µL/min. The run time was 6 minutes.

Mass Spectrometry

MS analysis was carried out on a TSQ Quantum Access MAX™ triple stage quadrupole mass spectrometer equipped with a Thermo Scientific Ion Max source with a heated electrospray ionization (HESI) probe. The MS conditions were as follows:

Scan type:	SRM
Divert valve:	2 - 4 min to source
Selected ions for quantification:	*m/z* 343 → 299 + 245 for THC-COOH in negative mode

Results and Discussion

Figures 1 and 2 show the ion chromatograms of the lowest and highest calibration points. Excellent linearity ($r^2 = 0.99$) fits for the calibration curve were observed over the range of 7.8-1000 ng/mL urine, with a Coefficient of Variation (%CV) at the lower end of 6.5%. The limit of quantitation (LOQ) was established as 7.8 ng/mL in urine.

Figure 4 reports an ion chromatogram of a real urine sample positive for cannabinoids (225 ng/mL urine), analyzed as described.

To examine the difference between hydrolyzed and non-hydrolyzed urine, we analyzed the same urine sample without the hydrolysis step. When urines were not hydrolyzed, the portion excreted as free THC-COOH was detected at 3.06 minutes, while THC-COOH-glucuronide was detected at 2.58 minutes (Figure 5). The precursor ion *m/z* 343 was generated as result of an in-source fragmentation and a consequent loss of glucuronic acid.

Because THC-COOH is mainly excreted as glucuronic acid conjugate, it is always necessary to perform urine hydrolysis before the LC-MS analysis to obtain an accurate quantification of THC-COOH.

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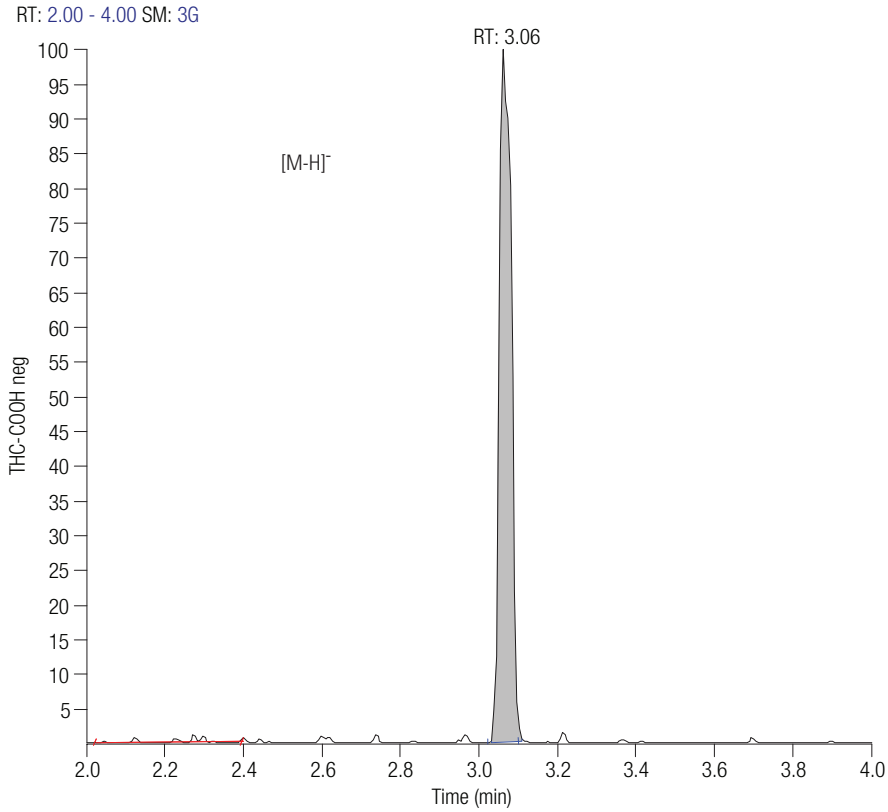

Figure 1. Ion chromatogram of 7.8 ng/mL urine calibration standard

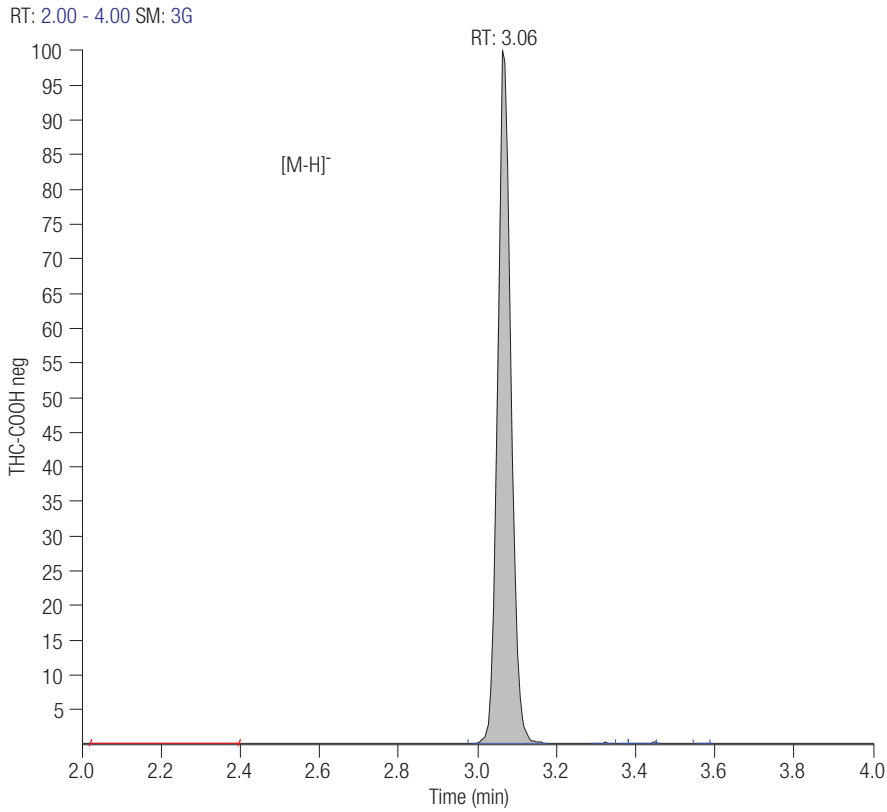

Figure 2. Ion chromatogram of 1000 ng/mL urine calibration standard

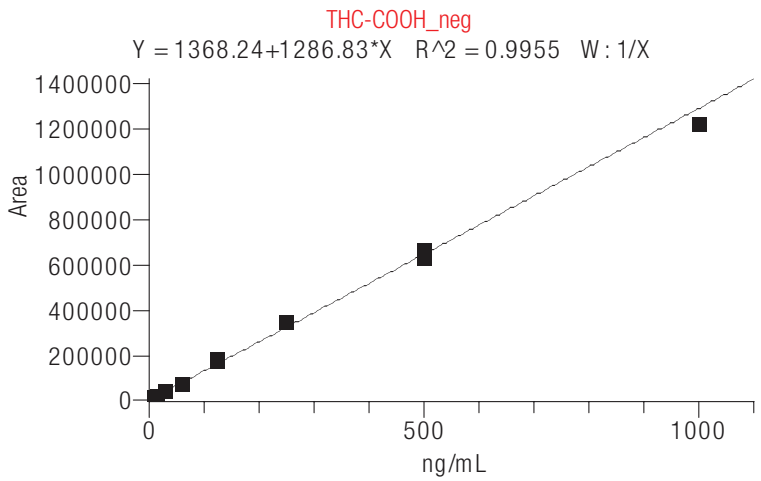

Figure 3. Calibration curve of THC-COOH in negative ionization mode

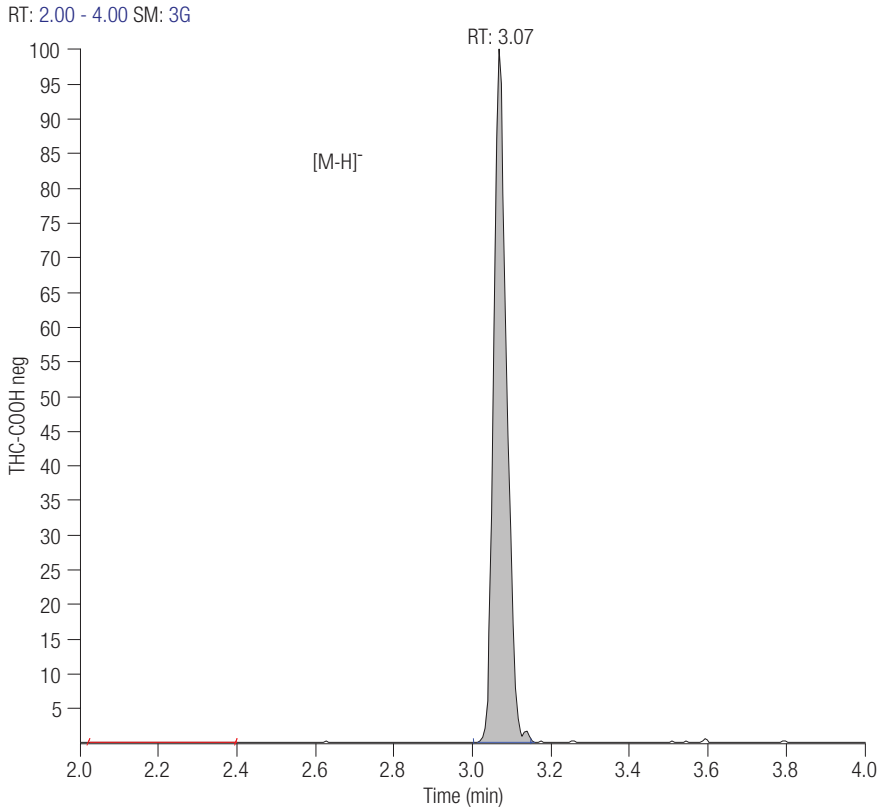

Figure 4. Ion chromatogram of urine sample containing 225 ng/mL. Sample was hydrolyzed and diluted 1:10 before the analysis.

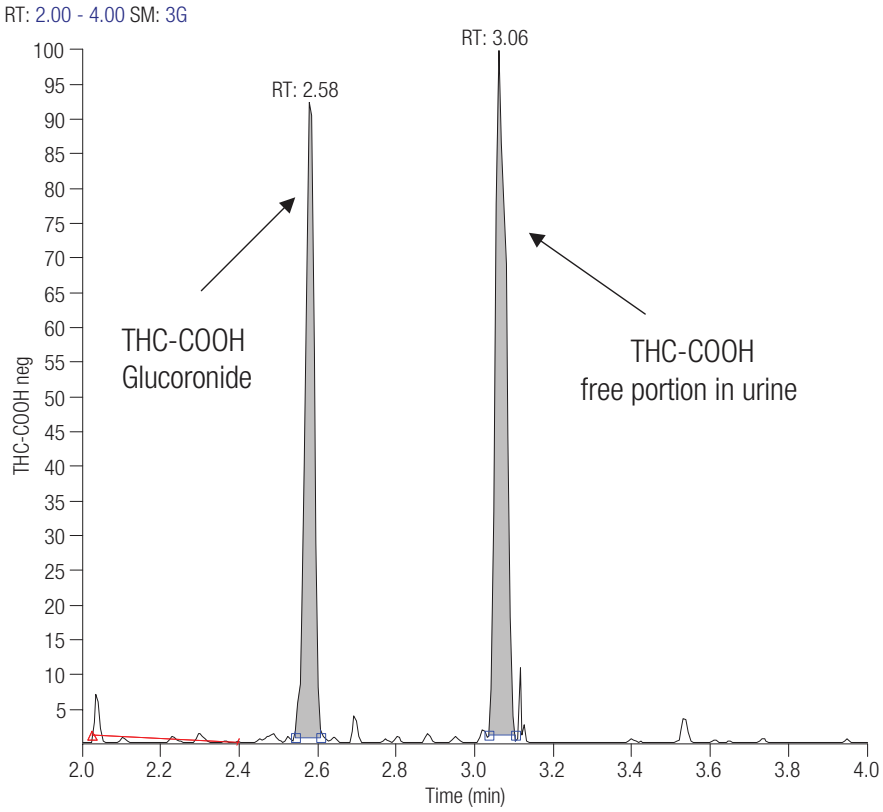

Figure 5. Ion chromatograms of urine sample containing 225 ng/mL. Sample was diluted 1:10 before the analysis; no hydrolysis was performed.

Conclusion

A robust 6-minute method for the quantification of THC-COOH with a dynamic range of 7.8-1000 ng/mL urine has been developed using the TSQ Quantum Access MAX mass spectrometer for forensic toxicology purposes.

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Quantitation of 14 Benzodiazepines and Benzodiazepine Metabolites in Urine Using a Triple Stage Quadrupole LC-MS System

Kristine Van Natta, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra
- Forensic Toxicology

Introduction

Benzodiazepines have a broad range of therapeutic use and are widely prescribed as safe drugs for the treatment of insomnia, anxiety and seizures and for their amnesic effects prior to medical procedures. They are also abused for their psychoactive effects, in suicide and in drug-facilitated sexual assault. Simple, robust and precise analytical methods are needed to quantitate these compounds in biological matrices for forensic purposes.

Goal

To develop a specific and robust dilute and shoot quantitative method for the analysis of 14 benzodiazepines and metabolites in urine. These compounds include: 2-hydroxyethylflurazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, α-hydroxyalprazolam, α-hydroxytriazolam, alprazolam, desalkylflurazepam, diazepam, lorazepam, midazolam, nordiazepam, oxazepam and temazepam.

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with β-glucuronidase. Deuterated analog internal standards were used for all compounds except α-hydroxytriazolam and lorazepam. Isotopic contribution from the di-chlorinated parent interfered with the d4 internal standards. Deuterated α-hydroxyalprazolam and oxazepam, respectively, were used instead. After hydrolysis, methanol was added to the hydrolysis mixture and the resulting mixture was centrifuged. Supernatant was further diluted and subject to LC-MS analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD aQ column (50 x 4.6 mm, 1.9 µm particle size). The total run time was 6.5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

The timed selected reaction monitoring (T-SRM) was used. T-SRM allows the instrument to scan only for those compounds that are expected to be eluting at a certain time. The data for a particular target compound is acquired only in a short window around the known retention time, not throughout the entire run. Using T-SRM significantly reduces the number of SRM transitions that are monitored in parallel at a certain retention time. At a constant acquisition rate (cycle time) a significantly longer scan time (dwell time) is available for each transition resulting in higher sensitivity and lower quantitation limits, improved RSDs and more data points per chromatographic peak.

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low, middle and high end of the calibration range. Intra-run variability and robustness were determined by analyzing six replicates of each QC level with a calibration curve. Matrix effects were investigated by preparing samples in 8 different lots of human urine at twice the limit of quantitation (LOQ) of the method and monitoring peak area recovery compared to samples prepared in water.

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Results and Discussion

The method is linear from 25 to 10,000 ng/mL with R^2 values > 0.99 for all 14 compounds (Figure 1). All calibrators back calculate to within 15% of nominal (20% for LOQ). All quality controls quantitated to within 15% of nominal for the middle and high controls and within 20% for the low control. The %CV was less than 10% for all QC levels.

No matrix effects were observed during validation. All samples showed recoveries within 20% of nominal. Table 1 shows the matrix effect results.

Figure 2 shows an SRM chromatogram at LOQ.

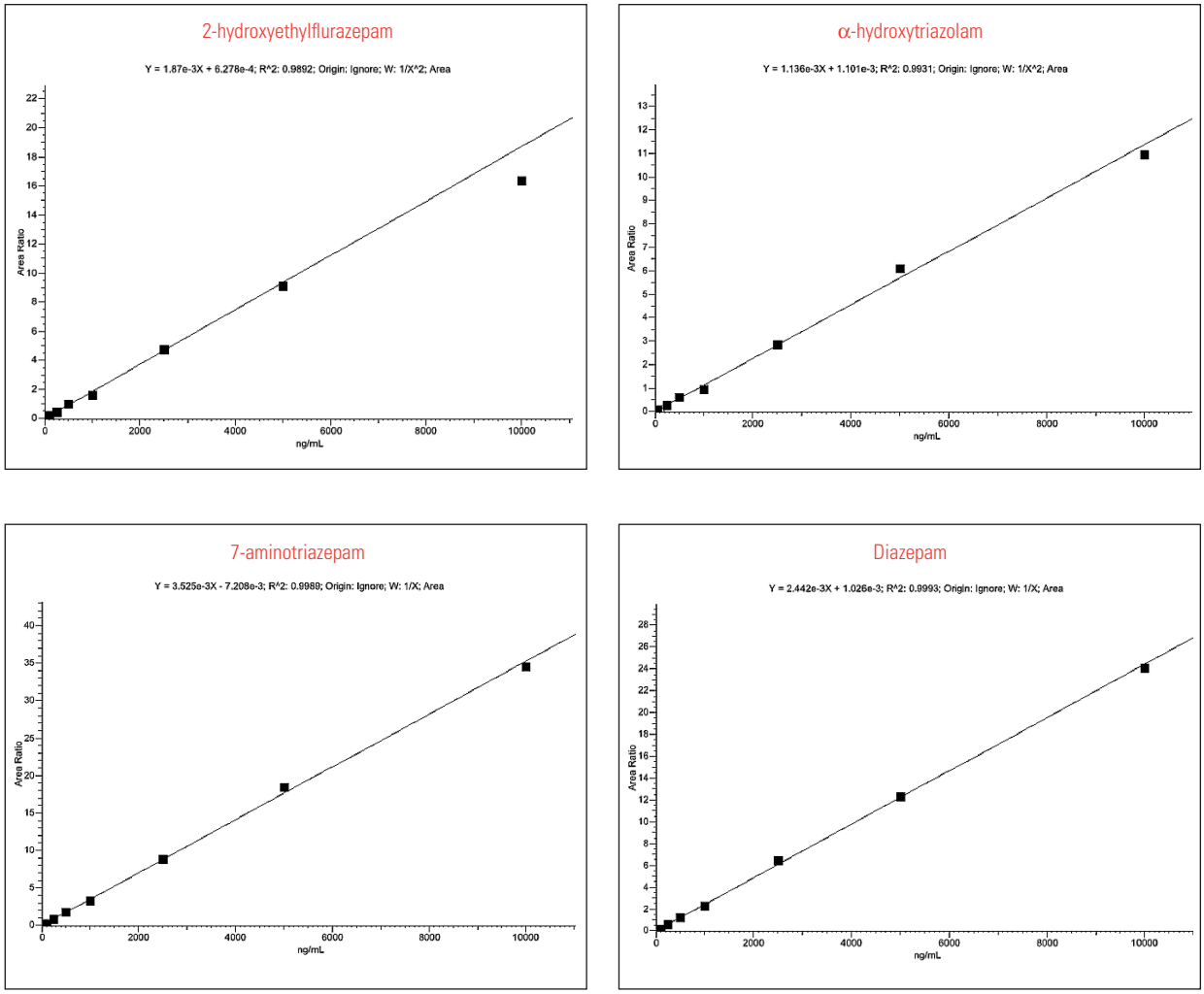

Figure 1. Representative calibration curves for some benzodiazepines showing linearity from 25-10,000 ng/mL in urine

Table 1. Percent recovery of 14 benzodiazepines in eight lots of urine

Compound	Lot A	Lot B	Lot C	Lot D	Lot E	Lot F	Lot G	Lot H
2-hydroxyethyl-flurazepam	83.6	94.7	113	106	131	107	101	102
7-amino-clonazepam	90.9	92.4	93.1	90.0	95.5	98.5	92.0	92.2
7-aminoflunitrzepam	97.1	98.0	100	101	97.6	108	94.9	96.5
7-aminonitrazepam	88.9	99.6	94.9	101	94.0	101	96.5	89.3
α-hydroxyalprazolam	107	104	90.9	112	105	106	113	99.3
α-hydroxytriazolam	95.5	107	101	96.9	87.5	90.7	109	107
alprazolam	108	101	107	110	107	98.9	92.7	95.5
desalkylflurazepam	108	89.3	104	97.6	103	98.9	105	103
diazepam	105	102	113	106	105	111	89.3	103
lorazepam	104	93.1	94.9	95.8	91.1	94.4	108	107
midazolam	113	111	110	101	104	107	105	95.6
nordiazepam	112	99.3	112	109	98.4	109	95.6	102
oxazepam	96.4	91.5	96.7	96.9	92.0	99.3	95.1	96.0
temazepam	105	98.2	99.1	95.5	101	99.1	98.2	101

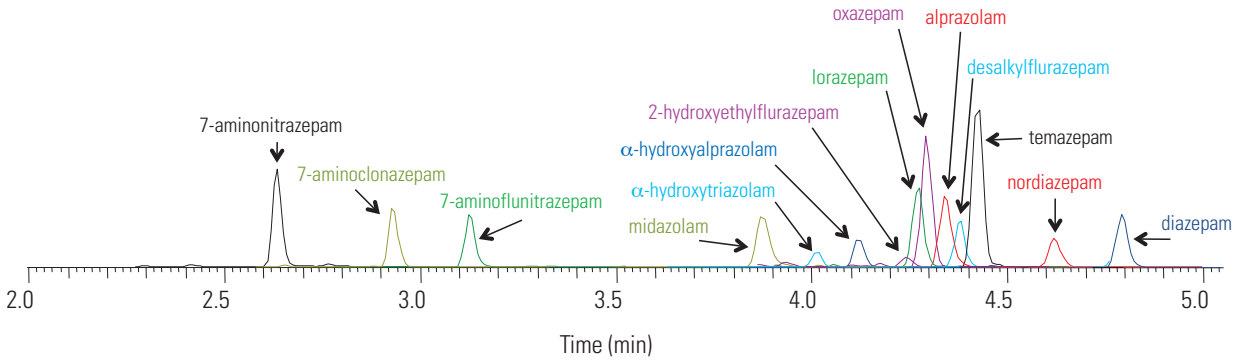

Figure 2. SRM chromatogram of 14 benzodiazepines and metabolites in urine at a concentration of 25 ng/mL

Conclusion

A robust dilute and shoot method with simple and easy sample preparation for the analysis of 14 benzodiazepines in 6.5 minutes was developed for forensic toxicology use. The data window and total run time make this method amenable to multiplexing with the Thermo Scientific Aria Transcend system. Multiplexing with the Transcend™ system would result in a run time of 3.5 minutes per sample.

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Quantitation of Six Opiates in Urine Using a Triple Stage Quadrupole LC-MS System

Kristine Van Natta, James Byrd, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra
- Transcend LX-2 system
- Forensic Toxicology

Introduction

The natural opiates morphine and codeine are widely prescribed drugs for their analgesic, antitussive and antidiarrheal effects. However, they are also widely abused for their psychoactive effects and are often diverted from lawful prescriptions to unlawful recreational use. Simple, robust and precise analytical methods are needed to quantify these compounds in biological matrices for forensic purposes.

Goal

To develop a specific and robust dilute and shoot quantitative method for the analysis of primary natural opiates and their metabolites in urine. These compounds include: morphine, codeine, oxymorphone, oxycodone, hydromorphone and hydrocodone.

Methods

Sample Preparation

Urine was spiked with deuterated analog internal standards and hydrolyzed with ß-glucuronidase. Methanol was added to the hydrolysis mixture and the resulting mixture was centrifuged. Supernatant was further diluted and subject to LC-MS analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD aQ column (50 x 4.6 mm, 1.9 µm particle size). The total run time was 5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low (LQC), a middle (MQC) and high (HQC) end of the calibration range. Intra-run variability and robustness were determined by analyzing six replicates of each QC level with a calibration curve. Matrix effects were investigated by spiking seven different lots of human urine with analytes at 50 ng/mL and calculating peak area recovery.

Results and Discussion

The method is linear from 10 to 6,000 ng/mL with R^2 values > 0.99 for all six compounds. Figure 1 shows calibration curves for the six compounds. All calibrators back calculate to within 15% of nominal (20% for LOQ). All quality controls quantitated to within 15% of nominal for the middle and high controls and within 20% for the low control. %CV was less than 10% for all QC levels, except for codeine LQC which was 17.2%. Table 1 shows quality control statistics for the validation runs.

No matrix effects were observed during validation. All samples showed recoveries within 20% of nominal. Table 2 shows matrix effects testing results.

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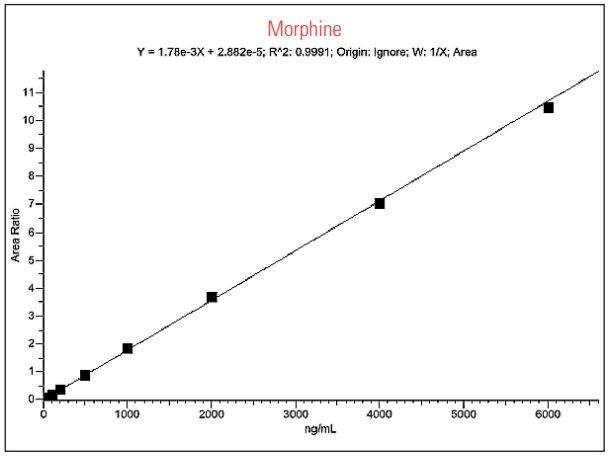

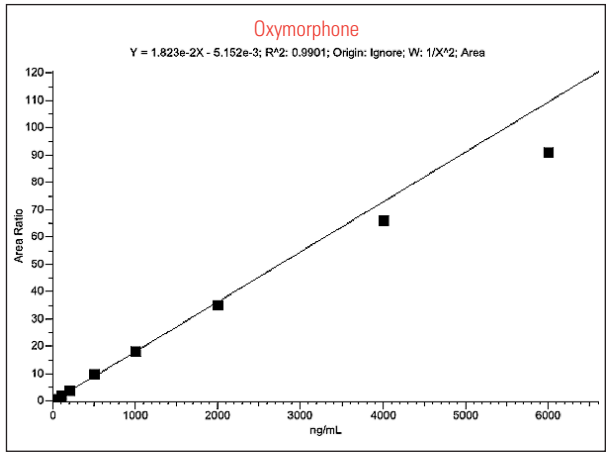

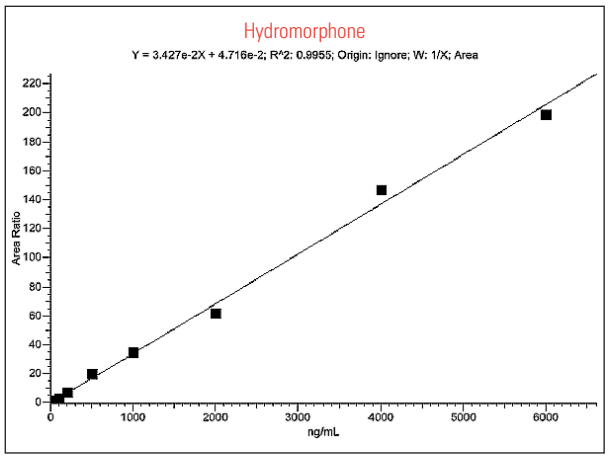

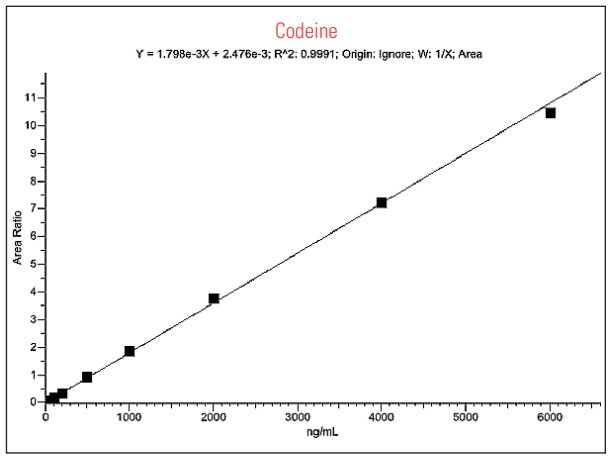

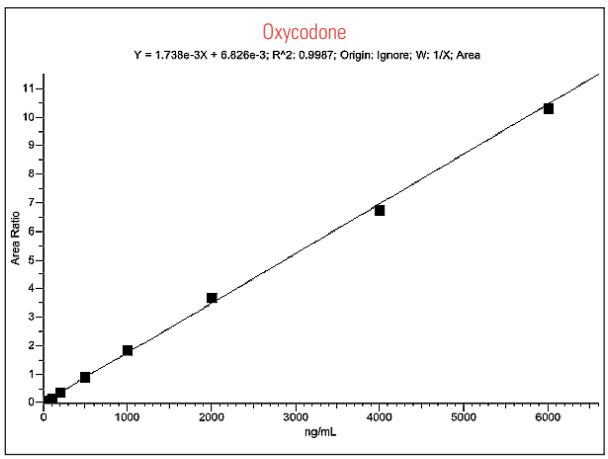

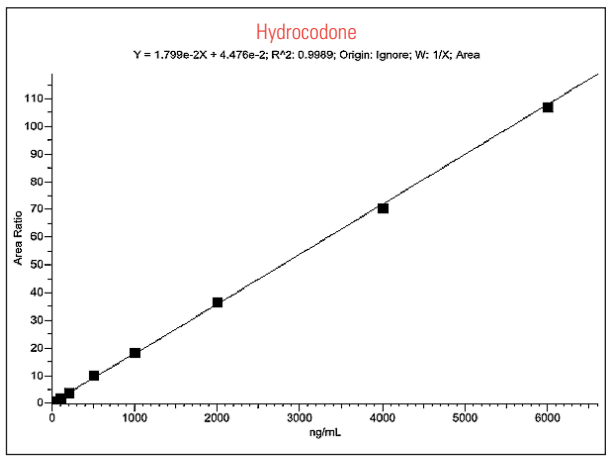

Figure 1. Representative calibration curves for opiates in urine

Table 1. Intra-assay quality control %Bias and %CV

	LQC		MQC		HQC	
	%Bias	%CV	%Bias	%CV	%Bias	%CV
Morphine	-9.42	8.72	2.92	3.29	4.50	2.24
Oxymorphone	12.2	3.45	7.50	2.35	0.00	4.16
Hydromorphone	-1.92	9.79	0.0833	6.63	-4.17	4.93
Codeine	-7.92	17.2	1.50	3.21	3.25	3.46
Oxycodone	-8.08	8.99	8.17	2.24	5.17	2.44
Hydrocodone	-2.42	8.84	7.25	3.60	5.58	4.07

Table 2. Percent recovery of six synthetic opioids in seven lots of human urine

	% Recovery						
Compound	**Lot 1**	**Lot 2**	**Lot 3**	**Lot 4**	**Lot 5**	**Lot 6**	**Lot 7**
Morphine	92.0	98.9	98.9	91.6	96.4	103	94.2
Oxymorphone	105	109	110	116	115	107	113
Hydromorphone	117	93.5	81.5	89.5	101	98.9	92.7
Codeine	113	113	104	98.9	112	108	103
Oxycodone	85.8	97.8	100	103	101	84.4	89.8
Hydrocodone	103	95.6	99.6	99.3	86.5	119	118

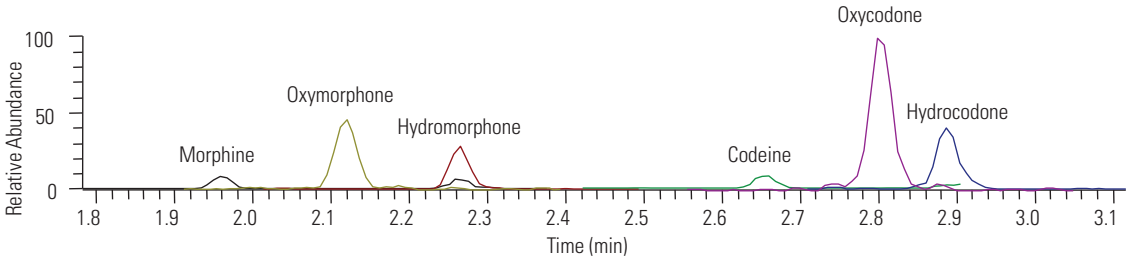

Figure 2. Representative chromatogram of six opiates in urine at LOQ of 10 ng/mL

Conclusion

A robust method with simple and easy sample preparation was developed for forensic toxicology laboratories. The data window and total run time make this method amenable to multiplexing with the Thermo Scientific Transcend system. Multiplexing with the Transcend™ LX-2 LC system would result in a run time of 2.5 minutes per sample. With an LX-4 LC system, the run time could be further reduced to 1.53 minutes per sample.

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Quantitation of Six Synthetic Opioids in Urine Using a Triple Stage Quadrupole LC-MS System

Kristine Van Natta, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra
- Transcend LX-2 system
- Forensic Toxicology

Introduction

Synthetic opioids have analgesic, antitussive and anti-addictive effects. However, they are also abused for their psychoactive effects and are often diverted from lawful prescriptions to unlawful recreational use. Simple, robust and precise analytical methods are needed to quantify these compounds in biological matrices for forensic purposes.

Goal

To develop a specific and robust dilute and shoot quantitative method for the analysis of six synthetic opioids and their primary metabolites in urine. These compounds include: methadone, EDDP, merperidine, normeperidine, propoxyphene and norpropoxyphene.

Methods

Sample Preparation

Urine was mixed with methanol containing deuterated analog internal standards. The supernatant was diluted with water prior to liquid chromatography-mass spectrometry (LC-MS) analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD aQ column (50 x 4.6 mm, 1.9 µm particle size). The total run time was 5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low (LQC), a middle (MQC) and high (HQC) end of the calibration range. Intra- and Inter- run variability and robustness were determined by analyzing five replicates of each QC level with a calibration curve on three different days. Matrix effects were investigated by spiking seven different lots of human urine with analytes at 50 ng/mL and calculating peak area recovery.

Results and Discussion

The method is linear from 20 to 5,000 ng/mL with R^2 values > 0.99 for all six compounds. Figure 1 shows the representative calibration curves. All IRCs passed within 20% of the standards average. All calibrators back calculate to within 15% of nominal, 20% for the limit of quantitation (LOQ). All quality controls quantitated to within 15% of nominal for the middle and high controls and within 20% for the low control. Inter-assay %CV was less than 10% for all QC levels. Table 1 shows quality control statistics for the validation runs.

No matrix effects were observed during validation. All samples showed recoveries within 20% of nominal. Internal standard variation was less than 5% between the different lots. Table 2 shows matrix effects testing results.

Figure 2 shows a reconstructed SRM chromatogram at LOQ.

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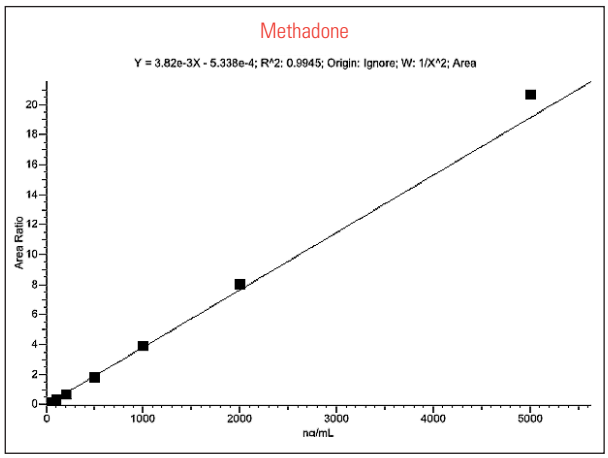

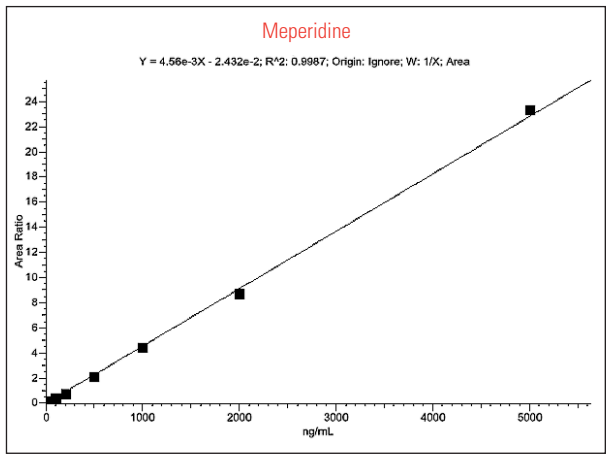

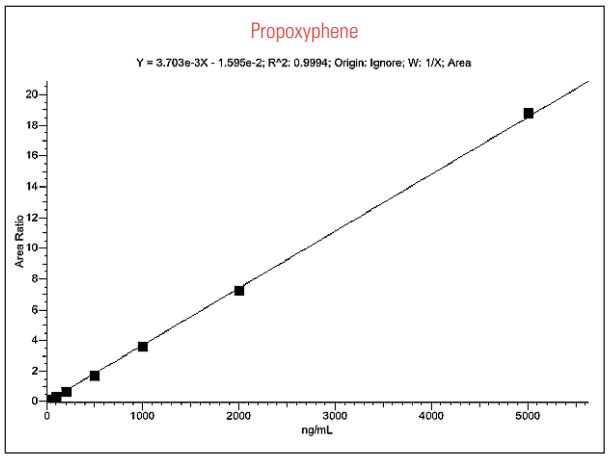

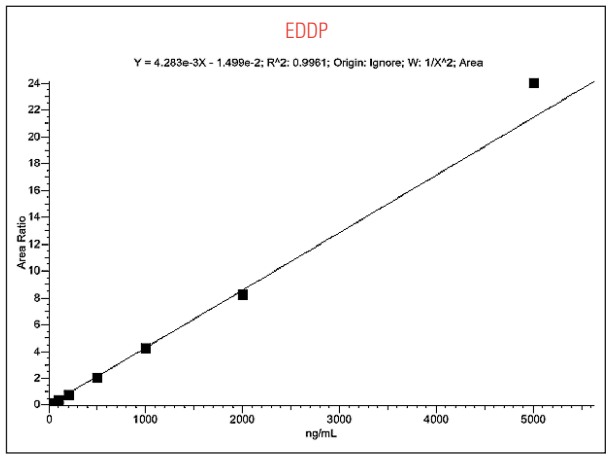

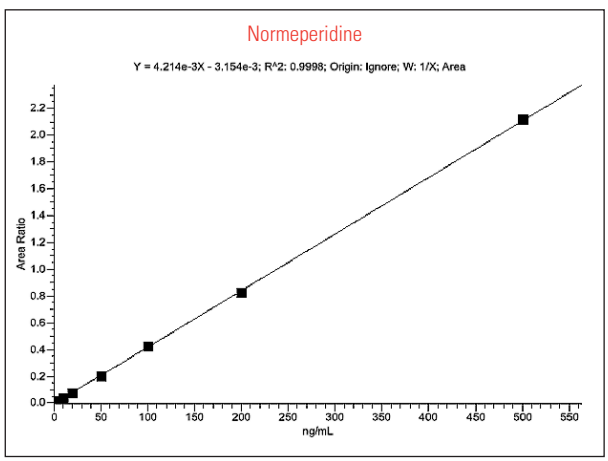

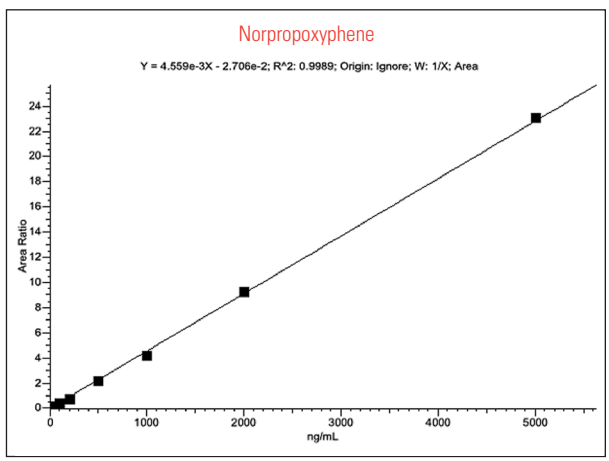

Figure 1. Representative calibration curves for methadone, EDDP, merperidine, normeperidine, propoxyphene and norpropoxyphene

Table 1. Inter-assay quality control statistics for validation runs

	%Bias/%CV					
	Methadone	**EDDP**	**Meperidine**	**Normeperidine**	**Propoxyphene**	**Norpropoxyphene**
LQC	4.16/4.71	2.67/6.04	0.493/5.93	7.04/9.17	5.45/3.42	3.28/8.64
MQC	7.32/2.47	-5.72/4.48	3.55/5.31	-0.747/7.61	4.36/5.28	0.933/3.88
HQC	10.9/2.69	-0.587/2.28	5.67/4.13	3.93/5.92	1.81/5.96	-3.77/8.18

Table 2. Percent recovery of six synthetic opioids in eight lots of human urine

	% Recovery							
Compound	**Lot A**	**Lot B**	**Lot C**	**Lot D**	**Lot E**	**Lot F**	**Lot G**	**Lot H**
Methadone	105	92.5	97.3	103	98.5	98.5	99.3	98.4
EDDP	94.9	97.1	95.1	96.0	105	92.4	94.9	98.2
Meperidine	104	97.6	105	98.4	110	104	98.7	102
Normeperidine	117	115	111	94.7	105	111	108	118
Propoxyphene	98.7	97.3	96.4	99.8	98.7	89.1	95.5	102
Norpropoxyphene	97.8	92.2	87.3	101	94.5	96.4	97.1	104

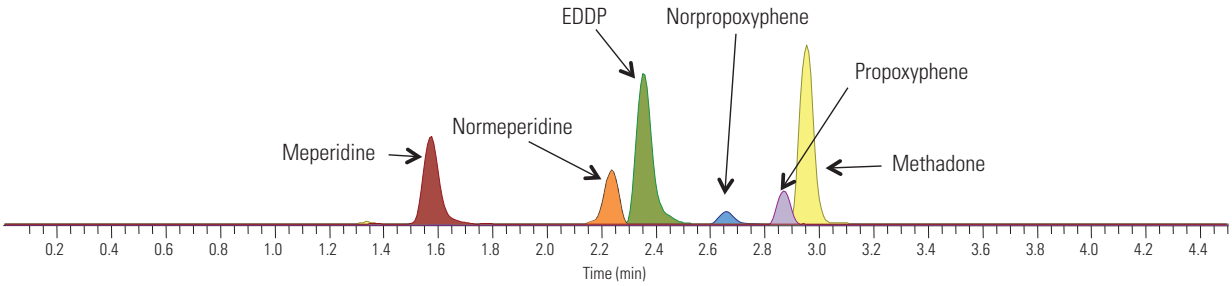

Figure 2. SRM chromatogram of six synthetic opioids and metabolites in urine at 20 ng/mL

Conclusion

A robust method with simple and easy sample preparation was developed and validated for forensic toxicology laboratories. The data window and total run time make this method amenable to multiplexing with the Thermo Scientific Transcend LX-2 LC system. Multiplexing with the Transcend™ LX-2 LC system would result in a run time of 2.5 minutes per sample.

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Software Driven Quantitative LC-MS Analysis of Opioids in Urine for Forensic Laboratories

Kristine Van Natta, Xiang He, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TraceFinder Software
- TSQ Quantum Ultra
- Drugs of Abuse

Introduction

Thermo Scientific TraceFinder software provides an integrated workflow approach for routine forensic screening and quantitation from method development and data acquisition to data processing and on through reporting. The TraceFinder™ software supports all Thermo Scientific quantitative liquid chromatography-mass spectrometry (LC-MS) systems with fully integrated support for Thermo Scientific Transcend multiplexing systems. The software also provides integrated levels of security from a lab manager to a routine user.

Goal

To demonstrate the software driven quantitative analysis of six opioids in urine using the Thermo Scientific TSQ Quantum Ultra mass spectrometer and TraceFinder software.

Experimental

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with β-glucuronidase. Fisher Chemical Optima® LC/MS Methanol was added to the hydrolysis mixture and the resulting mixture was centrifuged. The supernatant was further diluted and subjected to LC-MS analysis.

LC-MS/MS conditions

LC-MS analysis was performed on a TSQ Quantum Ultra™ mass spectrometer equipped with a heated electrospray ionization (HESI) probe coupled with a Transcend™ TLX system operating in LX mode. Two selected reaction monitoring (SRM) transitions were monitored for each compound. High pressure liquid chromatography (HPLC) was carried out on a Thermo Scientific Hypersil GOLD aQ column (50 × 4.6 mm, 1.9 µm particle size) at 30 °C. The MS source conditions were as follows:

Spray Voltage	3500 V
Vaporizer Temp	350 °C
Sheath Gas	80 (arbitrary units)
Ion Sweep Gas	0 (arbitrary units)
Aux Gas	5 (arbitrary units)
Capillary Temp	250 °C

Software

TraceFinder software was used for method development and routine analysis during validation.

Main Tabs in TraceFinder

Figure 1 shows the four main tabs in TraceFinder software: Acquisition, Data Review, Method Development and Configuration.

Compound Data Store

Figure 2 shows the Compound Data Store (CDS) for this opioid application. Entries of the analytes in this CDS contain the quantifier ion, qualifier ion and retention times for easy addition to a Master Method.

Master Method

The Master Method contains all of the information needed for an assay including that for instrument acquisition, data processing and reporting. The five main categories of information are: General (including assay type, injection volume, and instrument method), Compound (including acquisition list selected from the CDS, detection parameters, calibration and control levels), Flags, Groups and Reports. Selected tabs in the General and Compounds sections are shown in Figure 3. Many flagging parameters are available to customize data review and reports. Some of these parameters are shown in Figure 4.

Instrument Method

Instrument methods including autosampler, HPLC, and mass spectrometer parameters can be directly edited within TraceFinder 1.1 through a Thermo Scientific Xcalibur software interface.

Batch

Creating a batch involves assigning a project, linking to the master method, building a run-sequence and finally submitting the batch. Multiplexing channels are also controlled in the batch creation as seen in Figure 5.

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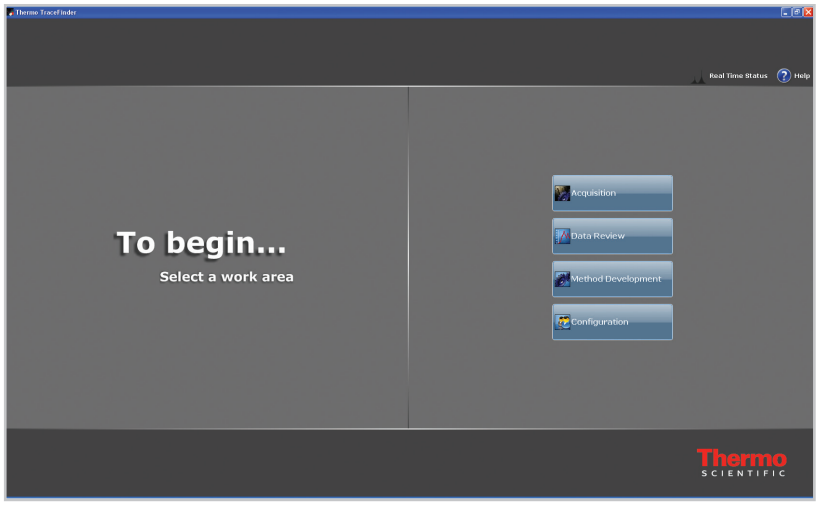


Figure 1. TraceFinder 1.1 welcome screen

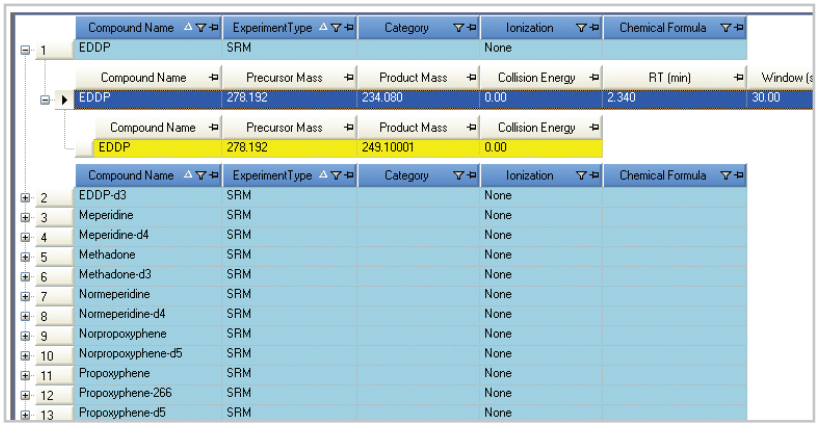


Figure 2. CDS showing quantifier and qualifier ions

Data Acquisition and Real Time Status

During acquisition, the data may be viewed in real time per the parameters set up in the Master Method. The status of the acquisition, pressure profile, event log, devices, multiplexing status (Figure 6) and sample queue are also monitored in the Real Time Status view.

Data Review

As soon as data are acquired, they are automatically processed per parameters set in the Master Method. Any sample parameters out of range are automatically flagged in the data review. Figure 7 shows the review pane for one compound.

Reporting

TraceFinder 1.1 software comes with over 50 report templates with additional custom reports available. Figures 8 and 9 show examples of standard reports.

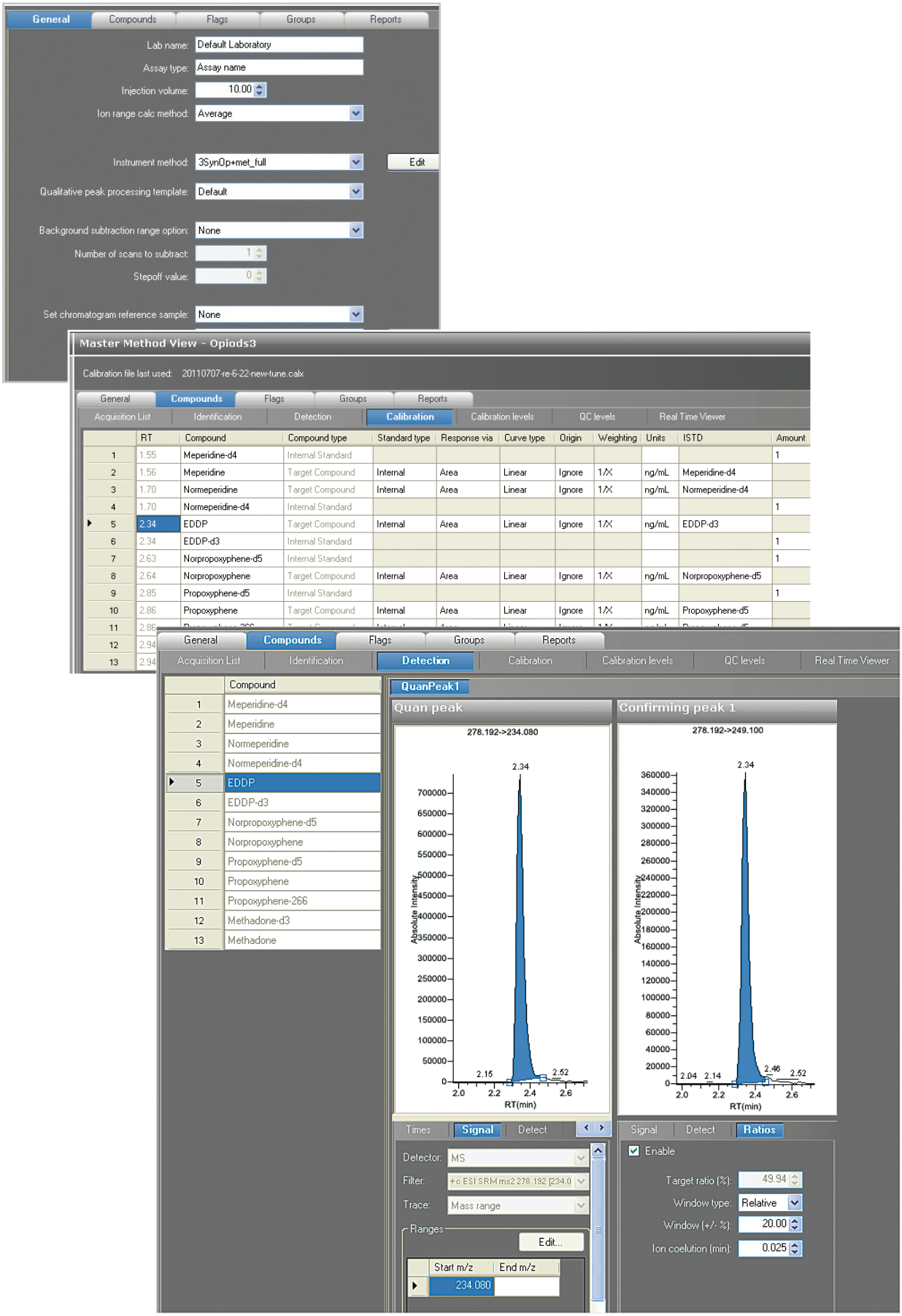


Figure 3. Master Method creation process showing general parameters, peak detection settings including retention times, mass filters, ion ratio settings and calibration curve settings

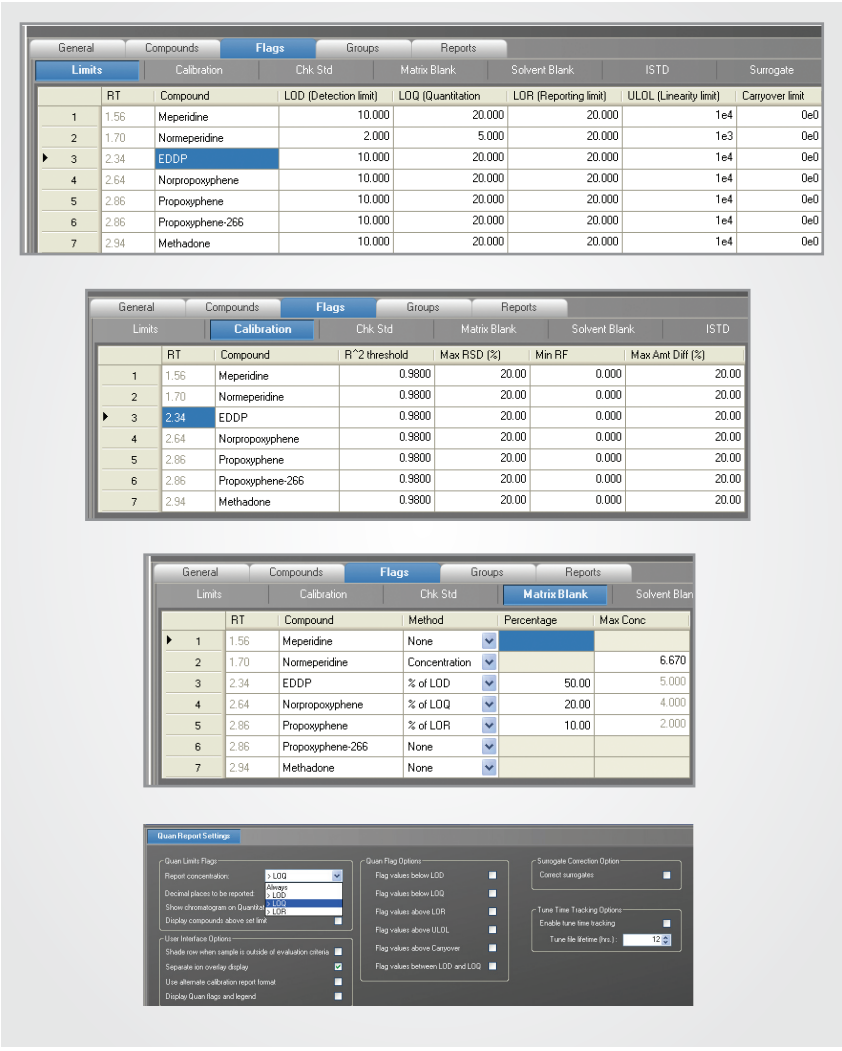


Figure 4. Many flagging parameters can be set for samples, standards, controls and blanks. The user can later select which flags to use for reporting (selected tabs).

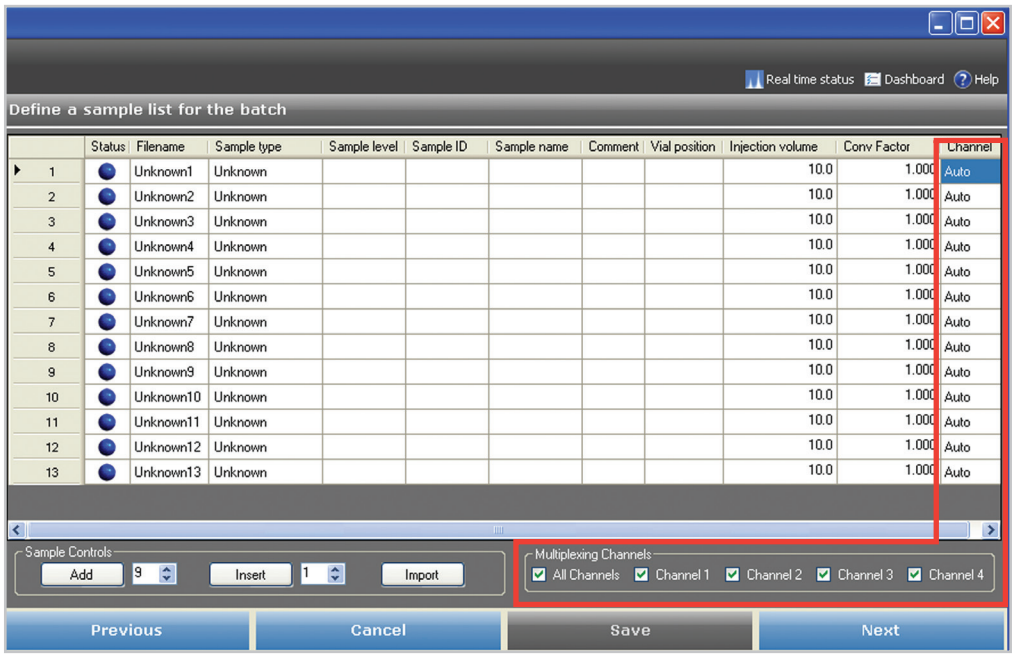


Figure 5. A sample batch ready for acquisition including assignment of multiplexing channels

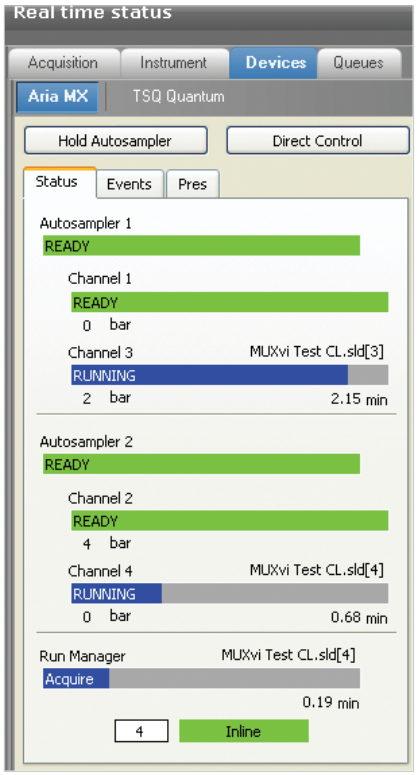


Figure 6. Real Time Status view displaying multiplexing status

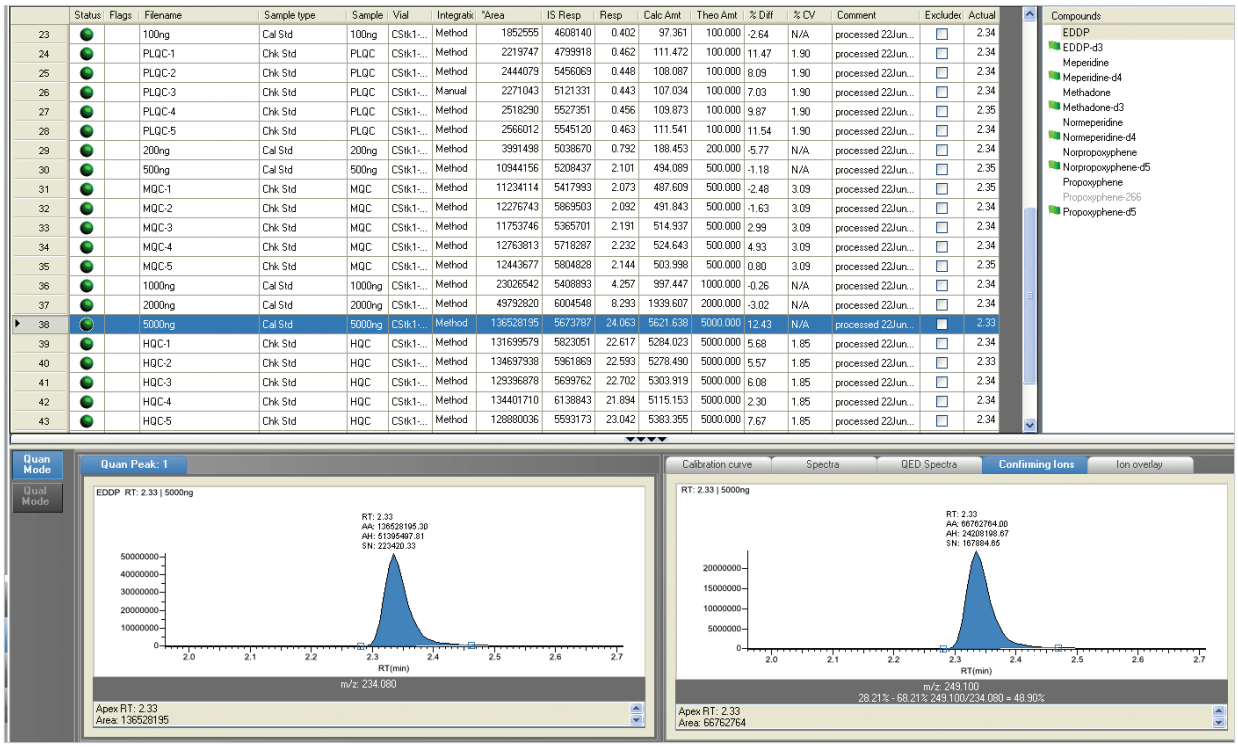


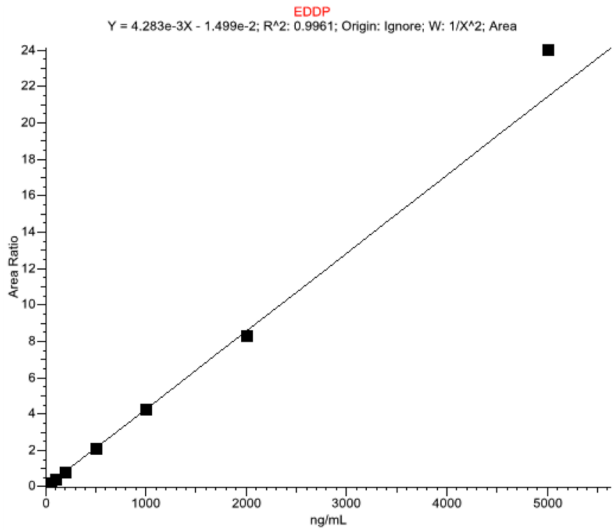
Figure 7. Data Review Confirming Ion window for EDDP, one of the six synthetic opioids, showing injection results, quantifier ion chromatogram and qualifier ion chromatogram

Compound Calibration Report

Lab Name: Clinical Marketing
Instrument: TSQ Quantum Ultra
User: Thermo Scientific
Batch: 20110707

Method: 20110707_Opiods3

Cali File: 20110707.calx



Linear Pass Level	Std Amount	Std Area	IS Amount	IS Area	Response ratio	Calc Amt	Units	%CV	%RSD
20ng	20.000	367529	200	5101141	0.072	20.321	ng/mL	N/A	N/A
50ng	50.000	1045386	200	5315652	0.197	49.415	ng/mL	N/A	N/A
100ng	100.000	1852555	200	4608140	0.402	97.361	ng/mL	N/A	N/A
200ng	200.000	3991498	200	5038670	0.792	188.453	ng/mL	N/A	N/A
500ng	500.000	10944156	200	5208437	2.101	494.089	ng/mL	N/A	N/A
1000ng	1000.000	23026542	200	5408893	4.257	997.447	ng/mL	N/A	N/A
2000ng	2000.000	49792820	200	6004548	8.293	1939.607	ng/mL	N/A	N/A
5000ng	5000.000	136528195	200	5673787	24.063	5621.638	ng/mL	N/A	N/A

Figure 8. Compound Calibration Report for EDDP

	Status	Compound Name	Compound Type	Quan Peak m/z	Total Response	Quan Peak Response	Quan Peak RT	Theoretical amount	Concentration	Confirming 1 Mass	Confirming 1 Response	Confirming 1 Ion Ratio Flag	Confirming 1 Ion Ratio	Confirming 1 Range
▶		Meperidine-d4	Internal Standard	224.200	3213110	3213110	1.56	200.000	200.000	178.200	2201547	False	68.52 %	56.04 % - 84.06 %
	✔	Meperidine	Target Compound	220.110	6823552	6823552	1.57	500.000	471.029	174.130	4565657	False	66.91 %	53.69 % - 80.54 %
	✔	Normeperidine	Target Compound	160.120	490038	490038	1.72	50.000	50.311	188.200	66808	False	13.63 %	5.99 % - 17.98 %
		Normeperidine-d4	Internal Standard	164.200	2346250	2346250	1.71	200.000	200.000	192.200	247015	False	10.53 %	5.00 % - 14.99 %
	✔	EDDP	Target Compound	234.080	11234114	11234114	2.35	500.000	490.069	249.100	5551494	False	49.42 %	39.95 % - 59.93 %
		EDDP-d3	Internal Standard	234.100	5417993	5417993	2.34	200.000	200.000	249.120	2628125	False	48.51 %	33.46 % - 55.76 %
		Norpropoxyphene-d5	Internal Standard	100.100	479874	479874	2.64	200.000	200.000	147.100	63747	False	13.28 %	9.79 % - 18.18 %
	✔	Norpropoxyphene	Target Compound	100.100	1083361	1083361	2.64	500.000	501.453	143.100	259174	False	23.92 %	18.53 % - 30.89 %
		Propoxyphene-d5	Internal Standard	271.300	1193078	1193078	2.85	200.000	200.000	58.220	824192	False	69.08 %	56.55 % - 84.83 %
	✔	Propoxyphene	Target Compound	58.190	2152782	2152782	2.87	500.000	491.610	266.170	1402361	False	65.14 %	50.41 % - 75.62 %
		Methadone-d3	Internal Standard	268.140	5655433	5655433	2.95	200.000	200.000	105.020	1407969	False	24.90 %	18.30 % - 30.50 %
	✔	Methadone	Target Compound	265.130	11460288	11460288	2.95	500.000	530.605	105.050	3388653	False	29.57 %	22.35 % - 37.25 %

Figure 9. Sample Report showing ion ratio confirmation

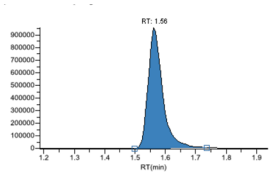
High Density Sample Report 1 Long

Lab Name: Clinical Marketing
Instrument: TSQ Quantum Ultra
User: Thermo Scientific
Batch: 20110707

Method: 20110707_Opiods3

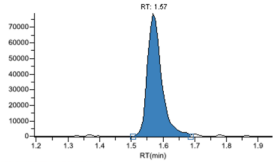
Cali File: 20110707.calx

Vial Pos	Sample ID	File Name	Level	Sample Name	File Date	Comment
CStk1-03:2		20ng	20ng		7/8/2011 4:23:59 PM	processed 22Jun2011

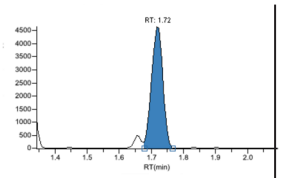


Meperidine-d4
Quan *m/z*: 224.20
Total Area: 3159582
Peak Area: 3159582
RT: 1.56min (1.55)

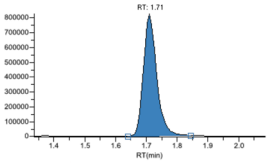
Amount: 1.000



Meperidine
Quan *m/z*: 220.11
Total Area: 246644
Peak Area: 246644
RT: 1.57 min (1.56)
TAmount: 20.000 ng/mL
Amount: 22.452 ng/mL

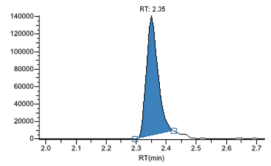


Normeperidine
Quan *m/z*: 160.12
Total Area: 11446
Peak Area: 11446
RT: 1.72min (1.70)
TAmount: 2.000 ng/mL
Amount: 1.844 ng/mL

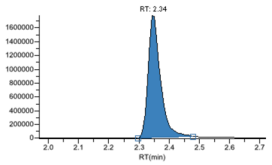


Normeperidine-d4
Quan *m/z*: 164.20
Total Area: 2478539
Peak Area: 2478539
RT: 1.71min (1.70)

Amount: 1.000

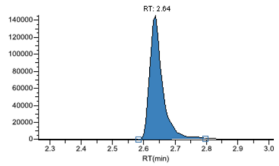


EDDP
Quan *m/z*: 234.08
Total Area: 367529
Peak Area: 367529
RT: 2.35 min (2.34)
TAmount: 20.000 ng/mL
Amount: 20.321 ng/mL



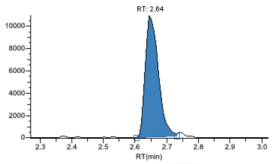
EDDP-d3
Quan *m/z*: 234.10
Total Area: 5101141
Peak Area: 5101141
RT: 2.34 min (2.34)

Amount: 1.000

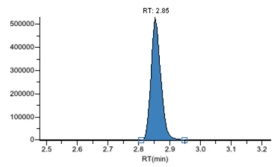


Norpropoxyphene-d5
Quan *m/z*: 100.10
Total Area: 412479
Peak Area: 412479
RT: 2.64min (2.63)

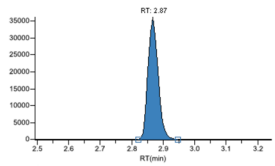
Amount: 1.000



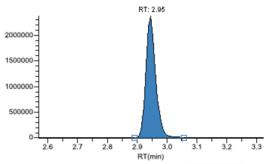
Norpropoxyphene
Quan *m/z*: 100.10
Total Area: 31463
Peak Area: 31463
RT: 2.64 min (2.64)
TAmount: 20.000 ng/mL
Amount: 22.669 ng/mL



Propoxyphene-d5
Quan *m/z*: 271.30
TotalArea: 1188454
Peak Area: 1188454
RT: 2.85min (2.85)
Amount: 1.000

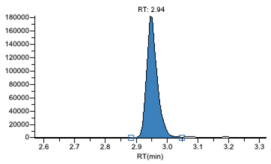


Propoxyphene
Quan *m/z*: 58.19
Total Area: 80927
Peak Area: 80927
RT: 2.87 min (2.86)
TAmount: 20.000 ng/mL
Amount: 22.697 ng/mL



Methadone-d3
Quan *m/z*: 268.14
Total Area: 5496688
Peak Area: 5496688
RT: 2.95min (2.94)

Amount: 1.000



Methadone
Quan *m/z*: 265.13
Total Area: 436828
Peak Area: 436828
RT: 2.94min (2.94)
TAmount: 20.000 ng/mL
Amount: 20.943 ng/mL

Figure 10. SRM chromatograms of six synthetic opiates in urine at 20 ng/mL

Results and Discussion

The method was linear from 20 to 5000 ng/mL for five of the six compounds. Normeperidine was linear from 2 to 1000 ng/mL. Standard accuracy ranged between 87.3% and 115%. Matrix effects were investigated by analyzing QCs prepared from six different lots of blank human urine. All samples showed recoveries within 20% at 50 ng/mL. The assay performance is summarized in Table 1. Figure 10 shows the SRM chromatograms of all six synthetic opiates at the limit of quantitation (LOQ).

Table 1. Assay performance for six synthetic opiates in urine

	% Recovery										
	Lot 1	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	R^2	Linear Range	LOQ
Methadone	105.0%	92.5%	97.3%	103.0%	98.5%	98.5%	99.3%	98.4%	0.9945	20-5000 ng/mL	20
EDDP	94.9%	97.1%	95.1%	96.0%	105.0%	92.4%	94.9%	98.2%	0.9951	20-5000 ng/mL	20
Meperidine	104.0%	97.6%	105.0%	98.4%	110.0%	104.0%	98.7%	102.0%	0.9935	20-5000 ng/mL	20
Normeperidine	117.0%	115.0%	111.0%	94.7%	105.0%	111.0%	108.0%	118.0%	0.9998	2-1000 ng/mL	2
Propoxyphene	98.7%	97.3%	96.4%	99.8%	98.7%	89.1%	95.5%	102.0%	0.9994	20-5000 ng/mL	20
Norpropoxyphene-dehydrate	97.8%	92.2%	87.3%	101.0%	94.5%	96.4%	97.1%	104.0%	0.9989	20-5000 ng/mL	20

Conclusion

TraceFinder 1.1 software was effectively used to perform routine analysis of the synthetic opiates in urine. The software enabled easy method setup, batch creation and submission, and real time monitoring. The data review functionality was useful for quick review and verification of the data. The generated reports had all the necessary information for record keeping for forensic laboratories.

In addition to these offices, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

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Application Note: 536

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Targeted Screening of Drugs of Abuse and Toxic Compounds with LC-MS/MS Using Triple Stage Quadrupole Technology

C. Gechtman, A. Masarin, A.O. Ospedale Niguarda Cà Granda, Milano, Italy
S. Scurati, Thermo Fisher Scientific, Milano, Italy
B. Duretz, P. Regulus, Thermo Fisher Scientific, Courtaboeuf, France

For Forensic Toxicology Use Only.

Key Words

- TSQ Quantum Access MAX
- TraceFinder Software
- QED
- Forensic Toxicology

Introduction

Screening of biological samples for drugs of abuse and other toxic compounds is one of the main issues in forensic toxicology. The challenge is to provide rapid and accurate results despite the large number of targeted molecules and the complexity of biological matrices.

Here we present the workflow and results obtained by using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) timed selected reaction monitoring (T-SRM) method utilizing a triple stage quadrupole mass spectrometer. In a T-SRM experiment, the method is set to look for specific transitions only during the expected retention-time window. This increases the number of SRM transitions that can be monitored in a single experiment. It also increases the dwell time and duty cycle for monitoring individual compounds per experiment. Then, quantitation-enhanced data dependent (QED) MS/MS scan functions are used to trigger data dependent full scan MS/MS spectra from SRM transitions. When a particular SRM transition reaches a predefined intensity threshold, the instrument automatically triggers QED-MS/MS, using the reverse energy ramp (RER) scan function to increase the product ion sensitivity (Figure 1). Dynamic exclusion settings allow the maximum number of MS/MS collected for each compound to be specified, thus giving the ability to collect MS^2 spectra of coeluting molecules.

Goal

To evaluate a triple stage quadrupole mass spectrometer for targeted screening in human urine utilizing a LC-QED-MS/MS method for forensic toxicology laboratories. This screening technique is asked to be fast and reliable enabling high throughput screening.

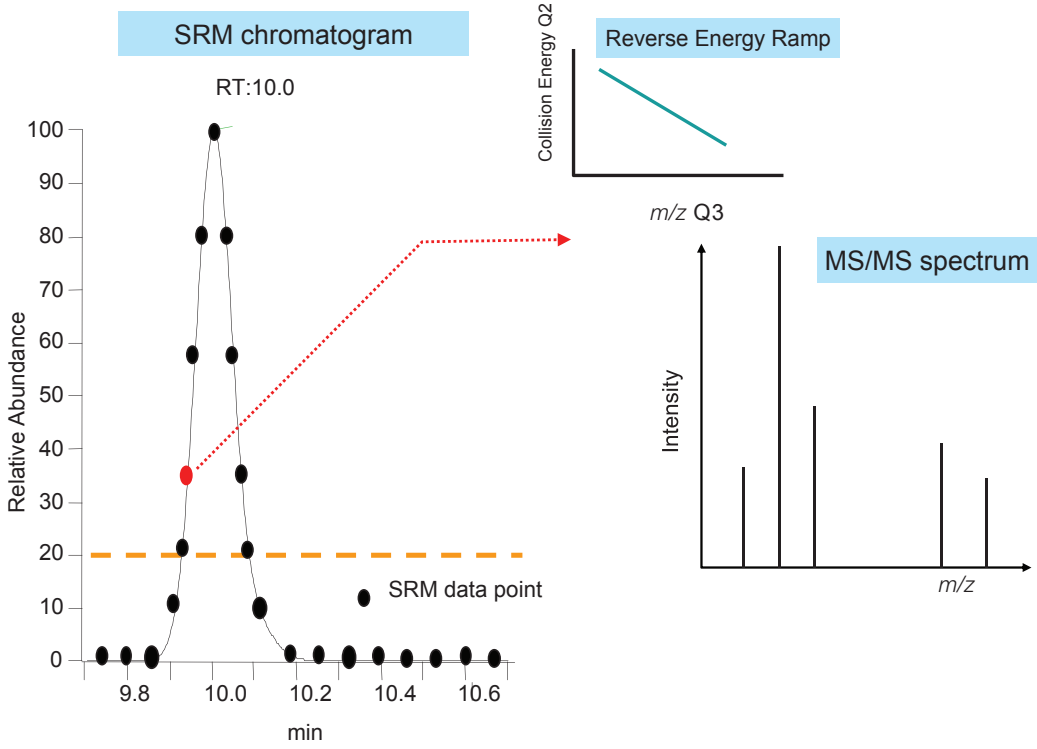


Figure 1: QED detection mode: when a monitored SRM transition reaches a targeted threshold, a full MS^2 spectrum is acquired using a Reverse Energy Ramp scan.

Thermo SCIENTIFIC

Experimental Conditions

Sample Preparation

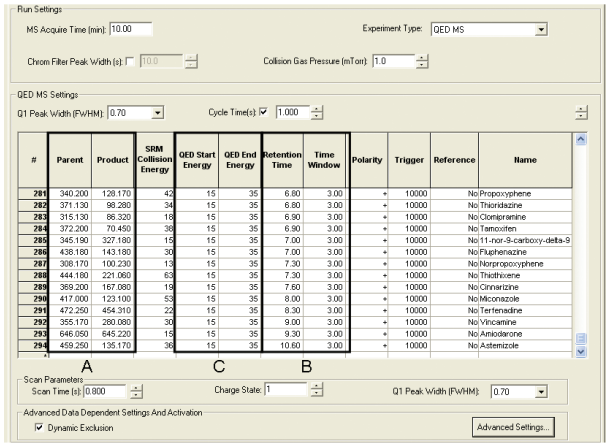
Urine was stored at -20 °C; for the analysis. After thawing, the urine was diluted 10 times with water. For the analysis, 10 µL of urine was directly injected into the LC-MS/MS.

Chromatography and Mass Spectrometry

A Thermo Scientific Hypersil GOLD PFP analytical column (50 x 2.1 mm, 5 µm) was used for separation of the compounds. A 15-minute gradient was set up using 10 mM ammonium formate and 0.1% formic acid in water for the mobile phase A and acetonitrile containing 0.1% formic acid for the mobile phase B.

The mass spectrometer was a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole with an Ion Max ion source. The instrument acquired SRM (Figure 2A) transitions of 294 compounds (drugs, toxic compounds, and metabolites) using T-SRM (Figure 2B). When an SRM transition reached 10,000 counts, QED detection was activated to collect full MS/MS spectra applying a ramp of collision energy from 15 to 35 eV (Figure 2C).

Data generated were processed with Thermo Scientific TraceFinder software for automated target screening. TraceFinder™ software can identify compounds based on their respective retention time, SRM transition, and full MS/MS spectra. The library contains 294 spectra of toxic and illicit compounds, and the corresponding SRM transitions are reported in the method.



#	Parent	Product	SRM Collision Energy	QED Start Energy	QED End Energy	Retention Time	Time Window	Polarity	Trigger	Reference	Name
281	340.200	128.170	42	15	35	6.80	3.00	+	10000	No	Propoxyphene
282	371.130	98.280	34	15	35	6.80	3.00	+	10000	No	Thioridazine
283	315.130	86.320	18	15	35	6.90	3.00	+	10000	No	Clomipramine
284	372.200	70.450	36	15	35	6.90	3.00	+	10000	No	Temoxifen
285	345.190	327.180	15	15	35	7.00	3.00	+	10000	No	11-nor-9-carboxy-delta-9
286	438.180	143.180	30	15	35	7.00	3.00	+	10000	No	Fluphenazine
287	308.170	100.230	13	15	35	7.30	3.00	+	10000	No	Norpropoxyphene
288	444.180	221.060	63	15	35	7.30	3.00	+	10000	No	Thiothixene
289	369.200	167.080	19	15	35	7.60	3.00	+	10000	No	Cinnarizine
290	417.000	123.100	53	15	35	8.00	3.00	+	10000	No	Miconazole
291	472.250	454.310	22	15	35	8.30	3.00	+	10000	No	Terfenadine
292	355.170	280.080	30	15	35	9.00	3.00	+	10000	No	Vincamine
293	646.050	645.220	15	15	35	9.30	3.00	+	10000	No	Amiodarone
294	459.250	136.170	36	15	35	10.60	3.00	+	10000	No	Astemizole

Figure 2: Method parameters used for LC-MS/MS screening of 294 compounds
Panel A: SRM transitions monitored
Panel B: Time segment used for Timed SRM
Panel C: When QED is activated an energy ramp from 15 to 35 eV is applied

Results and Discussion

The analysis time was 15 minutes. Figure 3A shows an example of an ion chromatogram of one of the monitored SRMs. Using QED-RER, the corresponding full MS^2 was recorded also (Figure 3B).

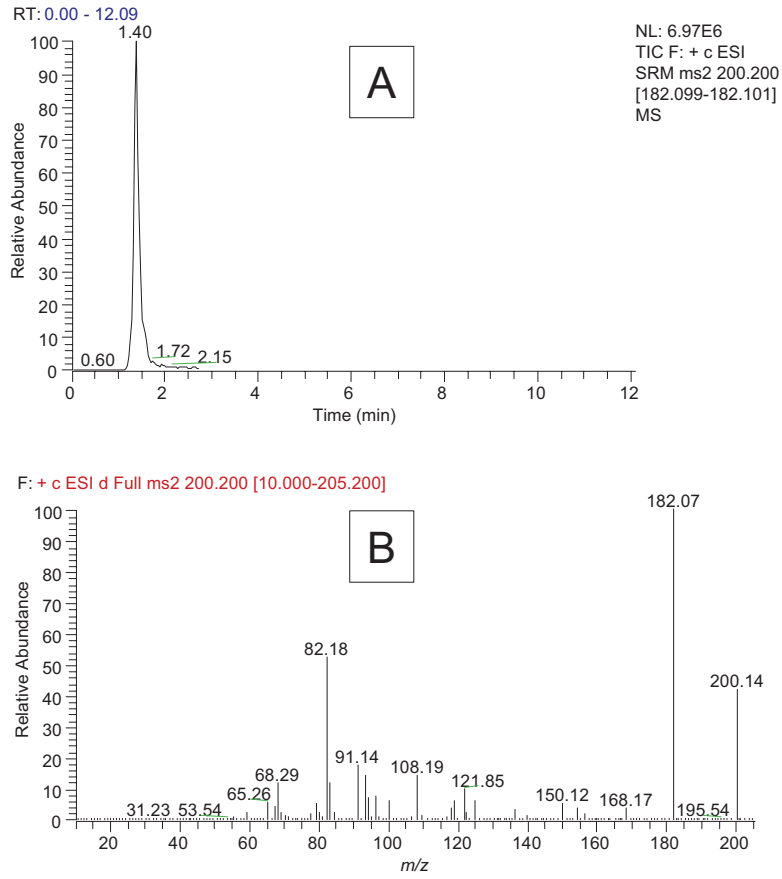


Figure 3: Example of ion chromatogram of transition 200 → 182 (A) and corresponding full MS^2 spectra collected (B)

Analyses were then processed with TraceFinder software using the Target Screening option (Figure 4), which allows the identification of target compounds present in the sample. Data obtained are highly specific and reliable because the identification of compounds is based on three parameters: retention time of the molecule, SRM transition, and MS/MS spectra.

Figure 5 shows an example of a summary report generated by TraceFinder software after the analysis of a urine sample that tested positive for cocaine. In addition to cocaine, *in vivo* metabolites such as benzoylecgonine, ecgonine methyl ester, and cocaethylene were also identified. The same sample was found positive for methadone – its metabolite, EDDP, was also identified.

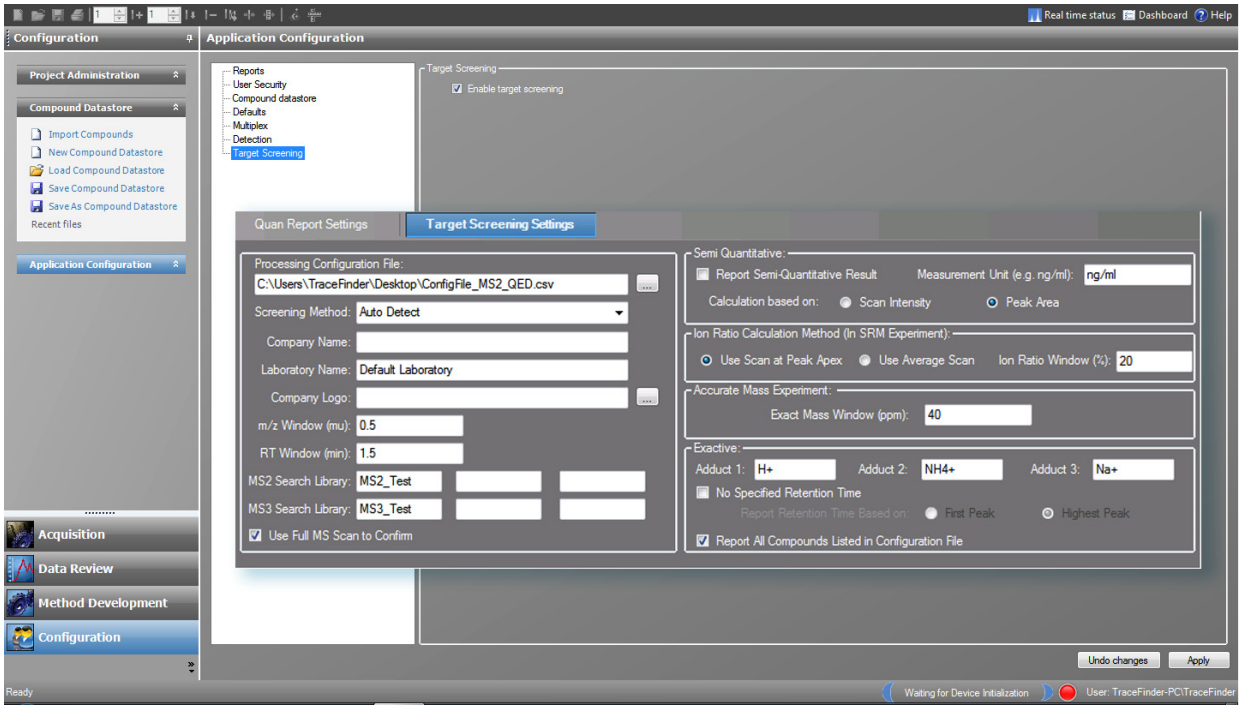
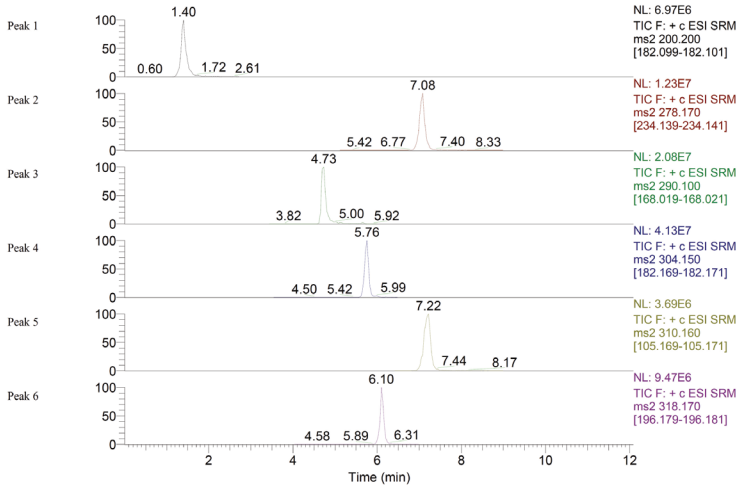


Figure 4. Selection of the Target Screening option in the configuration panel of TraceFinder software and settings used



Peak Number	Compound Name	Code	SI	RSI	m/z	Expected RT	Actual RT	Intensity	Library Name
1	Ecgonine-Methyl-Ester	m	758	784	200.200	1.20	1.40	6974863	MSSEARCH_08
2	EDDP	p	767	774	278.167	6.60	7.08	12298266	MSSEARCH_08
3	Benzoylecgonine	p	827	832	290.200	3.60	4.73	20833310	MSSEARCH_08
4	Cocaine	p	799	817	304.145	5.00	5.76	41325020	MSSEARCH_08
5	Methadone	p	770	777	310.163	6.70	7.22	3686206	MSSEARCH_08
6	Cocaethylene	p	742	758	318.166	5.50	6.10	9467052	MSSEARCH_08

Figure 5: TraceFinder Target Screening Short Report showing ion chromatograms and a list of compounds detected in urine positive for cocaine and methadone

Figure 6 shows an extract of the long report generated by TraceFinder software, showing the comparison between experimental spectra and library spectra for each compound. All of the spectra showed a high matching score confirming the presence of cocaine, methadone, and their metabolites in the urine sample.

Conclusion

The TSQ Quantum Access MAX™ with T-SRM and QED-RER acquisition mode was used to screen toxic compounds and their metabolites in urine. This screening approach provides rapid sample preparation, ease-of-use, sensitivity, specificity, and a low cost per sample analysis for forensic toxicology laboratories.

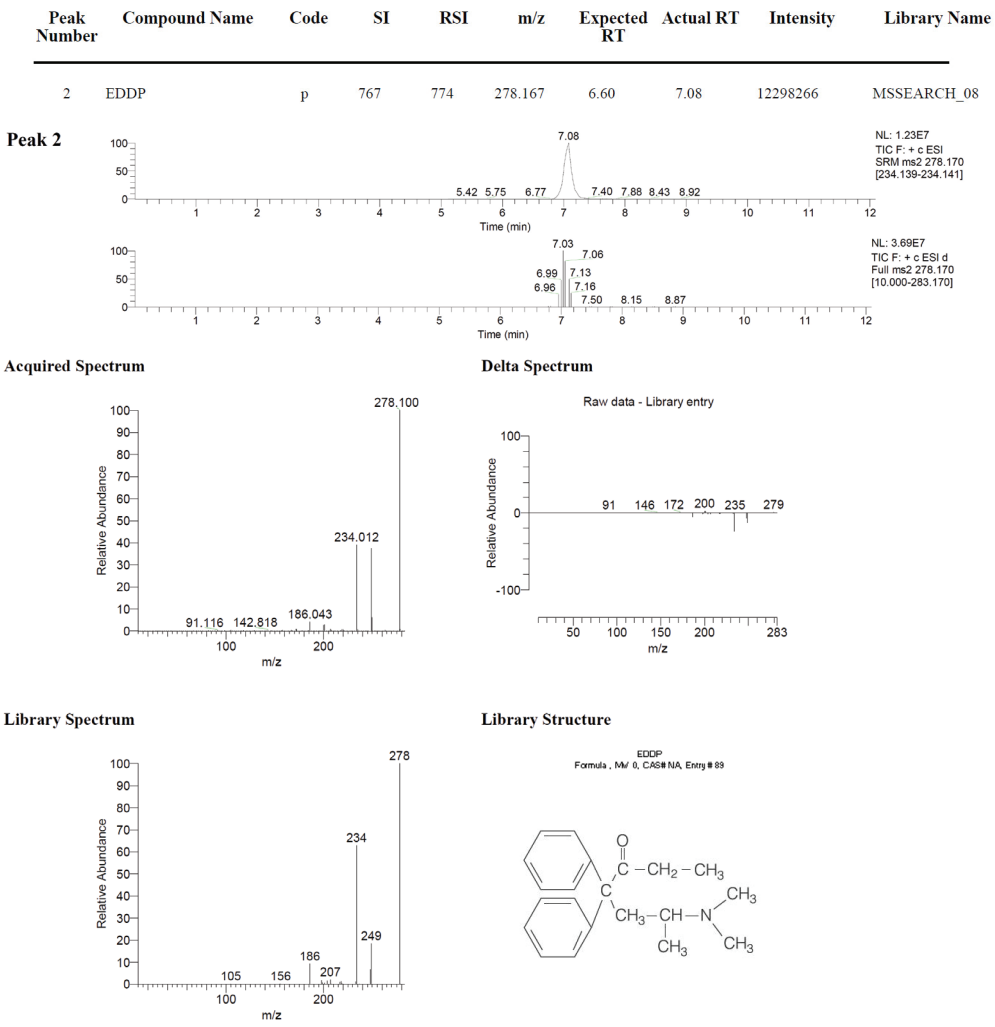


Figure 6. Extract of a TraceFinder Target Screening Long Report showing ion chromatograms and MS/MS spectra of EDDP detected in urine

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Quantitative LC-MS Analysis of 14 Benzodiazepines in Urine Using TraceFinder 1.1 Software and High Resolution Accurate Mass

Xiang He, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TraceFinder Software
- Exactive
- Clinical Research

Introduction

Thermo Scientific TraceFinder 1.1 software is developed for quantitative analysis for clinical research laboratories. The software is designed for routine data acquisition, quantitation, qualitative screening and reporting on all Thermo Scientific liquid chromatography mass spectrometry (LC-MS) systems, including high resolution accurate mass (HRAM) instruments, with fully integrated support for the Thermo Scientific Transcend multiplexing system.

TraceFinder™ 1.1 quantitative software simplifies routine analysis for the operator by executing a stepwise workflow from batch creation to reporting. For clinical research laboratories employing multiple types of LC-MS systems, TraceFinder 1.1 software eliminates the need to learn and maintain multiple software programs.

TraceFinder 1.1 software provides many easy approaches to execute workflow routines for operators and lab managers. The work presented here demonstrates the workflow used by lab managers during method development and includes processing method creation using the compound data store (CDS). The operator's workflow includes batch submission, real time monitoring, data review and report generation.

Goal

To demonstrate a new, easy-to-use workflow-driven quantitative method for 14 benzodiazepines in urine using the Thermo Scientific Exactive high performance benchtop mass spectrometer and TraceFinder 1.1 routine quantitative software.

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with beta-glucuronidase. Acetonitrile was added to the hydrolyzed sample and the resulting mixture was centrifuged. Supernatant was further diluted and subjected to LC-MS analysis.

LC-MS/MS conditions

LC-MS analysis was performed on an Exactive™ mass spectrometer with a heated electrospray ionization (HESI) source coupled with a Transcend™ TLX system used in LX mode. Full scan mass spectrometry analysis was done with resolution of 100,000 (FWHM at *m/z* 200) with a mass isolation window of 3 ppm. Exact mass was used for compound identification. High performance liquid chromatography (HPLC) was carried out on a Thermo Scientific Hypersil GOLD PFP column (100 × 2.1 mm, 5 µm particle size) at room temperature.

The MS conditions were as follows:

Ionization	HESI-II
Polarity	Positive
Vaporizer temp (°C)	350
Capillary temp (°C)	350
Spray voltage (V)	3500
Sheath gas (AU)	40
Auxillary gas (AU)	10
Data acquisition mode	Full scan
AGC target	1.00E+06
Lock mass *(m/z)*	279.2591
Scan range *(m/z)*	135-600
Max injection time (ms)	100
Resolution	100,000

Software

Method development, data acquisition, data processing and report generation were all executed in TraceFinder 1.1 routine quantitation software.

Results and Discussion

Streamlined Workflow:

The entire workflow in TraceFinder 1.1 software is easy to set up and is summarized in Figure 1.

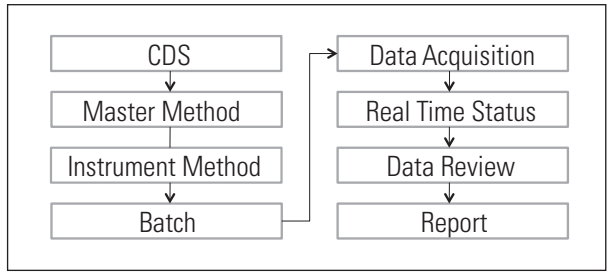


Figure 1. TraceFinder 1.1 workflow for quantitative analysis

Main Tabs in TraceFinder 1.1

Figure 2 shows the four main tabs: Configuration, Method Development, Data Review and Acquisition.

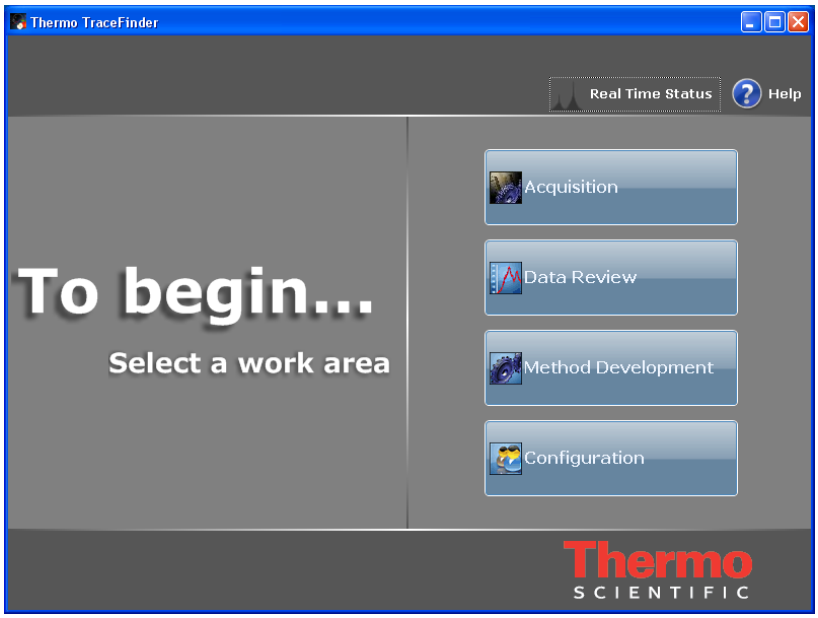
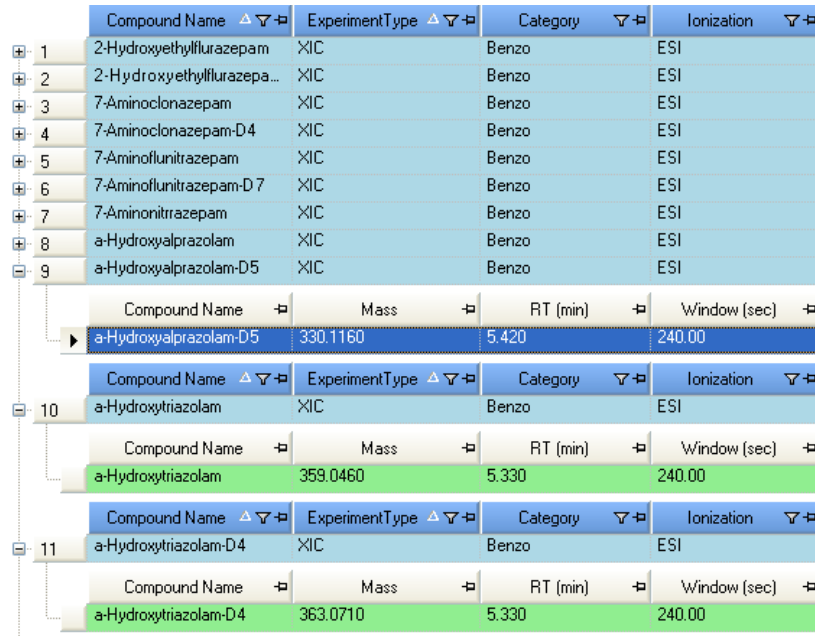


Figure 2. TraceFinder 1.1 welcome screen

Compound Data Store (CDS)

Figure 3 shows the CDS for this benzodiazepines application. Entries in this CDS are built based on the accurate masses. CDS can be later updated with retention times of analytes.



	Compound Name	ExperimentType	Category	Ionization
1	2-Hydroxyethylflurazepam	XIC	Benzo	ESI
2	2-Hydroxyethylflurazepa...	XIC	Benzo	ESI
3	7-Aminoclonazepam	XIC	Benzo	ESI
4	7-Aminoclonazepam-D4	XIC	Benzo	ESI
5	7-Aminoflunitrazepam	XIC	Benzo	ESI
6	7-Aminoflunitrazepam-D7	XIC	Benzo	ESI
7	7-Aminonitrrazepam	XIC	Benzo	ESI
8	a-Hydroxyalprazolam	XIC	Benzo	ESI
9	a-Hydroxyalprazolam-D5	XIC	Benzo	ESI

Compound Name	Mass	RT (min)	Window (sec)
a-Hydroxyalprazolam-D5	330.1160	5.420	240.00

	Compound Name	ExperimentType	Category	Ionization
10	a-Hydroxytriazolam	XIC	Benzo	ESI

Compound Name	Mass	RT (min)	Window (sec)
a-Hydroxytriazolam	359.0460	5.330	240.00

	Compound Name	ExperimentType	Category	Ionization
11	a-Hydroxytriazolam-D4	XIC	Benzo	ESI

Compound Name	Mass	RT (min)	Window (sec)
a-Hydroxytriazolam-D4	363.0710	5.330	240.00

Figure 3. Compound Data Store for benzodiazepines application

Master Method

The "Master Method" contains information on data acquisition (including instrument method), data processing, and analysis. In detail, it contains settings for 5 main categories: General (including method type, injection volume, instrument method, etc), Compound (acquisition list selected from CDS, detection, calibration, etc), Flags, Groups and Reports. Selected tabs in "General" and "Compound" are shown in Figure 4. To complete the master method setup, settings in "Flags", and "Reports" can also be customized. TraceFinder software provides 50 predefined report templates.

Instrument Method

The instrument method is comprised of individual LC, autosampler and MS portions. The software allows for optimization of chromatography and customizable autosampler programming.

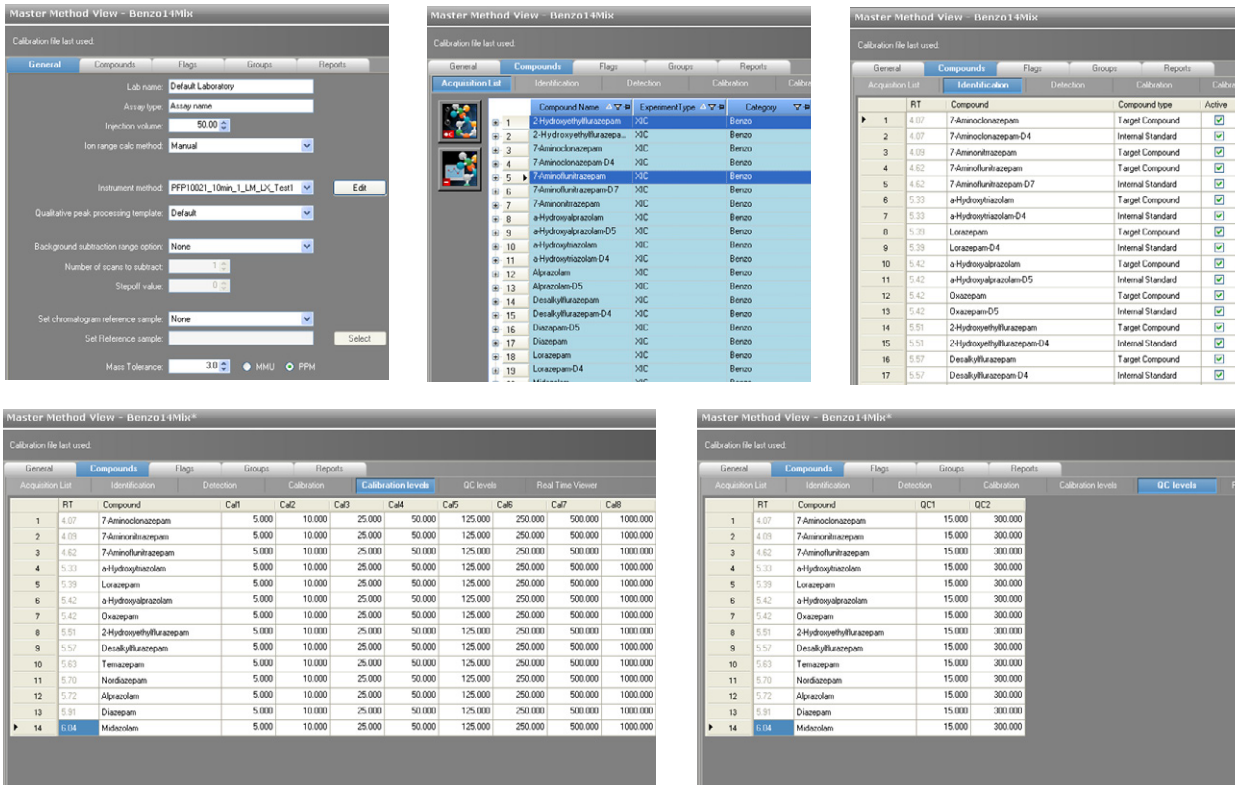


Figure 4. Master Method creation process (selected tabs)

Batch

After creation of the master method, a new sample batch can be created for data acquisition. Creating a batch involves assigning a project, linking to the master method, building a run-sequence and submitting. Figure 5 shows an exemplary batch view containing six calibrators and two levels of "Check Standards" (or QCs, n=5).

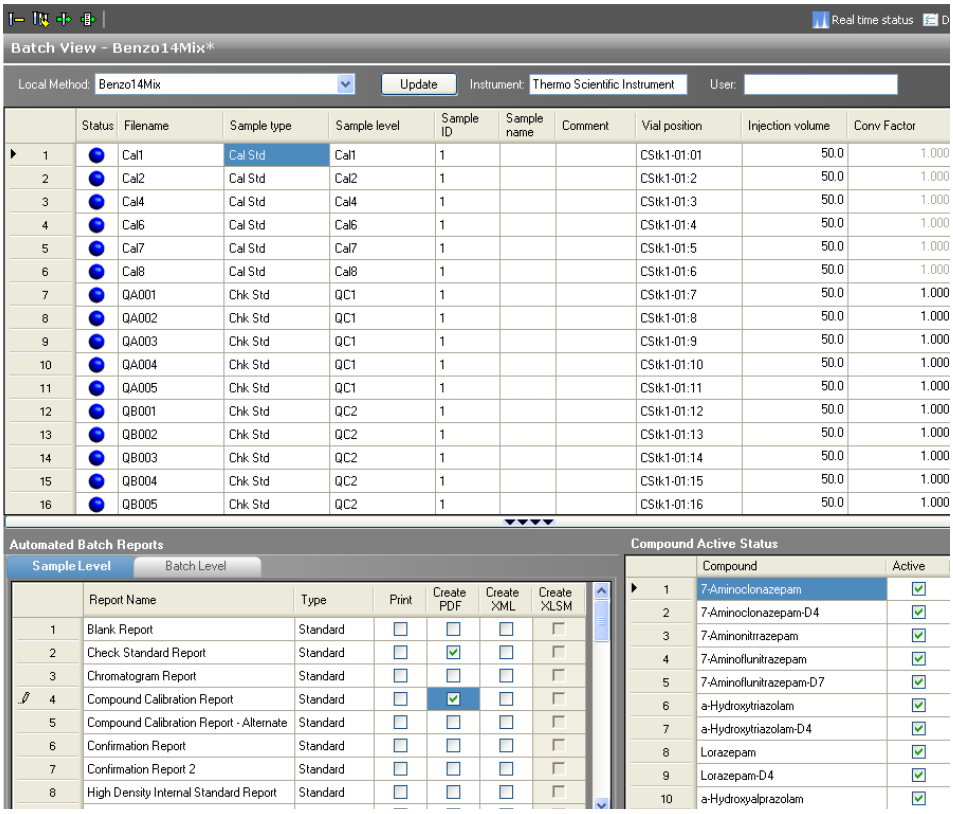


Figure 5. Acquisition Batch view

Data Acquisition and Real Time Status

After batch submission, data will be acquired and real time chromatograms can be shown in customizable ways (Figure 6). Status of acquisition (pressure profile, event log), device status, and sample queue can all be monitored in Real Time Status. TraceFinder software allows for multiple batches submission prioritization.

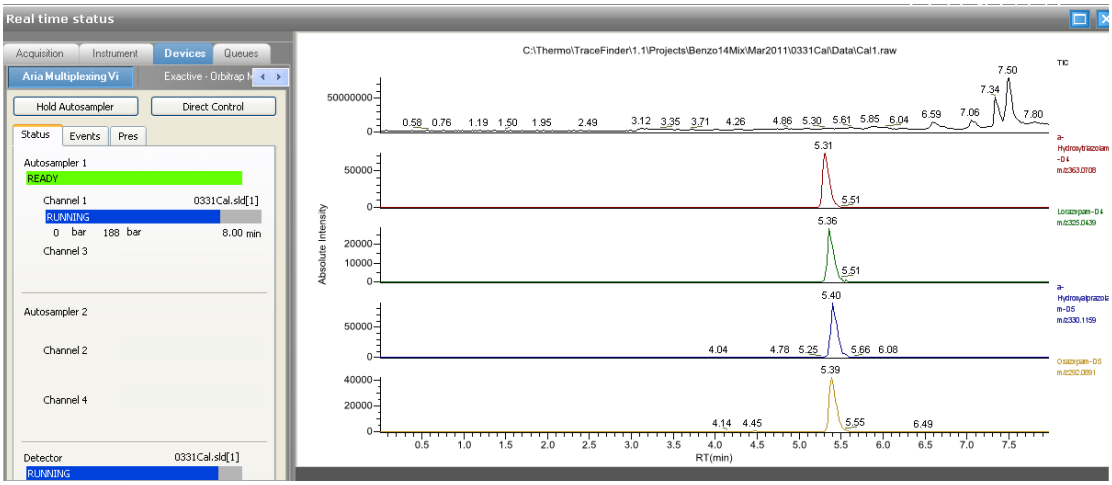


Figure 6. Real Time Status view

Data Review

Data Review (Figure 7) allows for flagging for any items that require attention (retention time drift, limit of quantitation, ion ratio discrepancy, etc.).

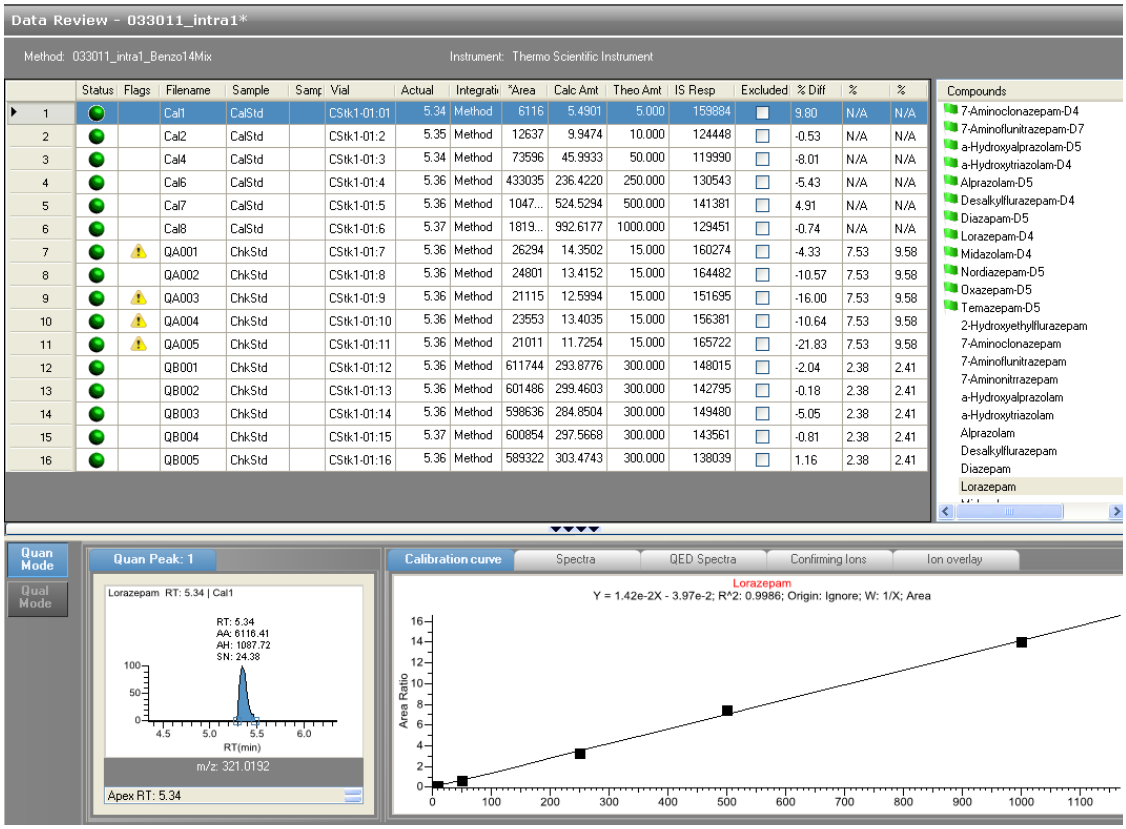


Figure 7. Data Review view for lorazepam, one of the 14 benzodiazepines

Reporting

Figures 8 and 9 are two examples (compound calibration and check standard/quality control) of the Report View.

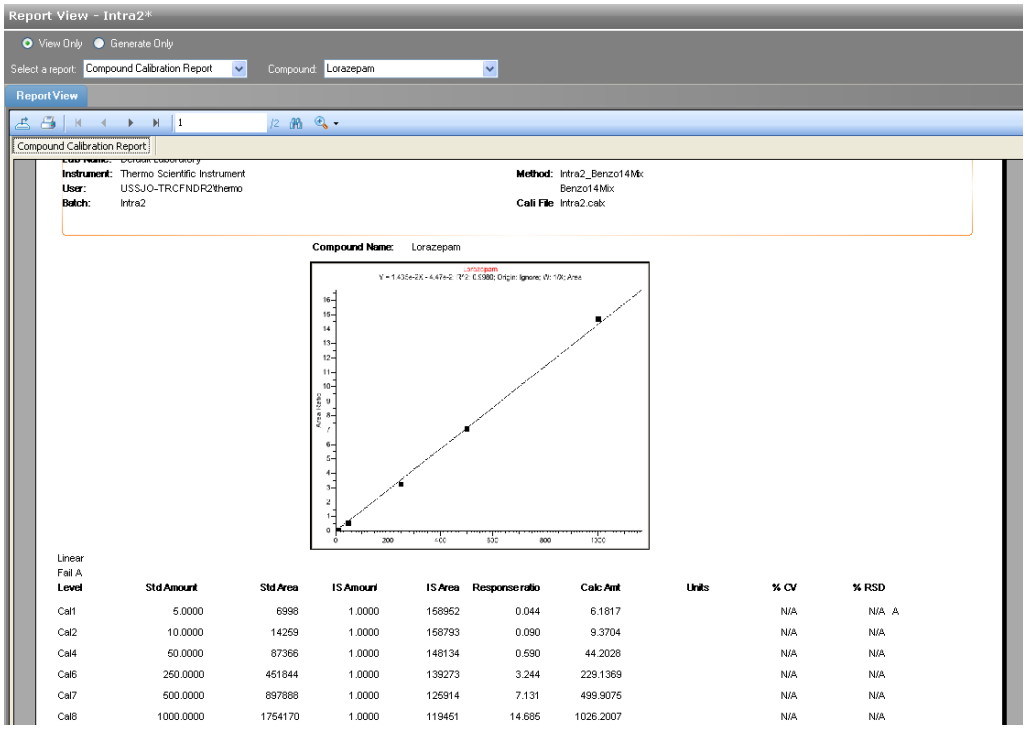


Figure 8. Compound Calibration Report for lorazepam

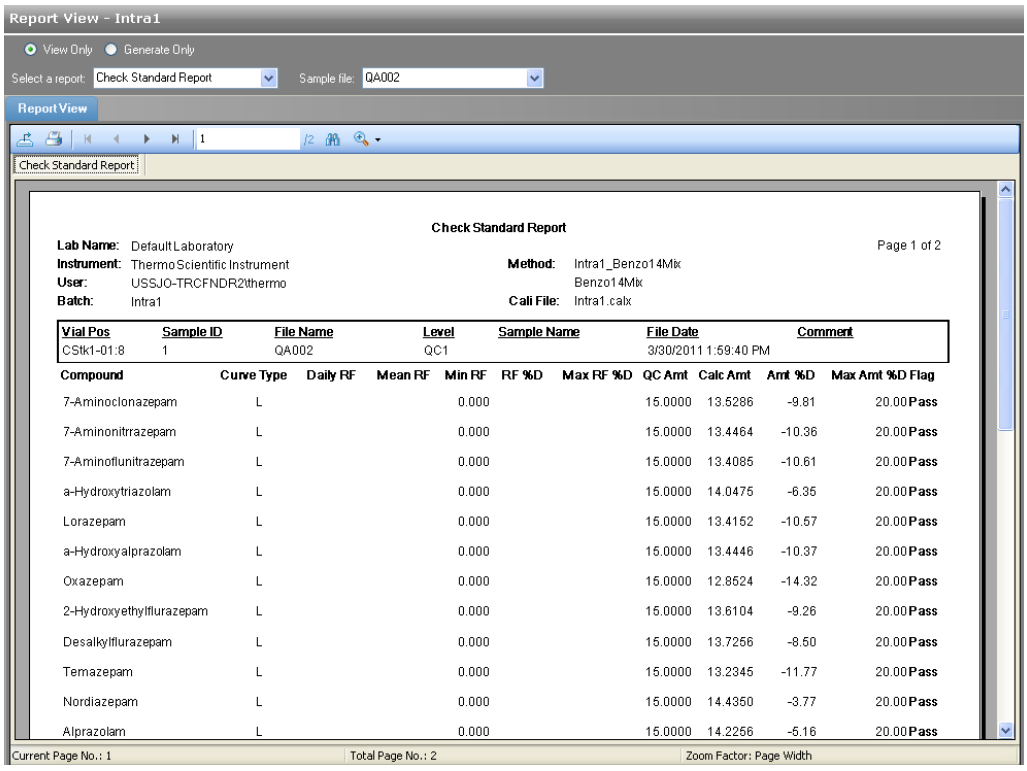


Figure 9. Check Standard (QC) Report for one QC sample

Method Performance

Sample preparation for urine analysis of benzodiazepines was previously done with solid phase extraction (SPE). Here we tested a simple urine dilution strategy. The absolute recovery of deuterated benzodiazepine internal standards was tested with several lots of human urine. It was determined that the absolute recoveries of the internal standards ranged from 83.0% to 100.5% at 100 ng/mL from all lots of urine tested (data not shown).

This method was linear from 5 to 1000 ng/mL for all 14 benzodiazepines with an accuracy of 85.4%-106.0%. Inter- (n=15) and intra-batch (n=5) coefficients of variation (CV) at two different concentration levels ranged from 0.5% to 11.7%. The method has a lower limit of quantitation (LLOQ) of 5 ng/mL for all 14 benzodiazepines tested. The method performance is summarized in Table 1. Figure 10 shows the extracted ion chromatograms (XICs) with 3 ppm mass isolation window of all 14 benzodiazepines at their LLOQ (5 ng/mL).

Table 1. Method performance for 14 benzodiazepines in urine

Name	m/z	QC level 1: 15 ng/mL		QC level 2: 300 ng/mL		Linear Range (ng/mL)	LLOQ (ng/mL)
		% Precision	% Accuracy	% Precision	% Accuracy		
7-Aminonitrazepam	252.1131	2.9	88.7	2.9	106.0	5 - 1000	5
Nordiazepam	271.0633	5.7	89.6	2.9	100.9	5 - 1000	5
7-Aminoflunitrazepam	284.1194	3.4	91.2	4.0	100.9	5 - 1000	5
Diazepam	285.0789	8.8	96.0	2.6	99.7	5 - 1000	5
7-Aminoclonazepam	286.0742	2.0	89.1	2.1	99.4	5 - 1000	5
Oxazepam	287.0582	5.0	85.6	3.5	98.4	5 - 1000	5
Desalkylflurazepam	289.0539	5.5	88.5	2.9	98.6	5 - 1000	5
Temazepam	301.0738	3.4	89.1	2.7	97.6	5 - 1000	5
Alprazolam	309.0902	3.1	90.0	3.2	101.5	5 - 1000	5
Lorazepam	321.0192	7.6	85.4	3.4	95.3	5 - 1000	5
α-Hydroxyalprazolam	325.0851	3.0	87.0	1.8	97.3	5 - 1000	5
Midazolam	326.0855	3.6	91.3	2.6	101.2	5 - 1000	5
2-Hydroxyethylflurazepam	333.0801	3.7	89.0	2.5	99.7	5 - 1000	5
α-Hydroxytriazolam	359.0461	5.9	86.9	2.8	97.5	5 - 1000	5

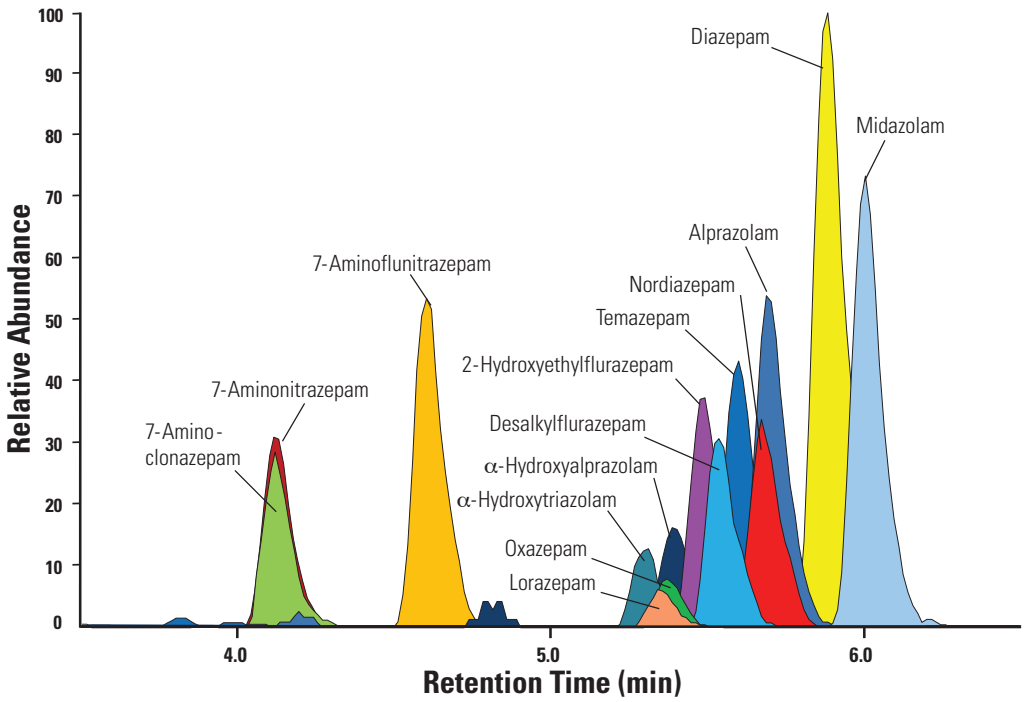


Figure 10. Extracted ion chromatograms of 14 benzodiazepines in urine at their LLOQ (5 ng/mL, mass isolation window=3 ppm)

Conclusion

We have developed a fast and sensitive LC-MS method for 14 benzodiazepines in urine using a benchtop Exactive mass spectrometer with TraceFinder 1.1 software. TraceFinder 1.1 software is easy to use and effective in performing quick routine quantitative analysis of benzodiazepines in urine. The software enables easy method development, batch creation, submission and real time monitoring for clinical research laboratories. The data review functionality was very useful in quick review and verification of the calibration accuracy and linearity. The report templates make selecting and generating reports with all the necessary information easy and quick.

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Screening of 20 Benzodiazepines and Four Metabolites in Whole Blood using UHPLC-MS/MS

Carine Roussel[1], Marie Spiandore[1], Marianne Lacmari[1], Bénédicte Duretz[2]
[1]Institut National de Police Scientifique, Toulouse, France; [2]Thermo Fisher Scientific, Les Ulis, France

Key Words

- Psychoactive drugs
- TSQ Quantum Ultra
- T-SRM method
- Accela UHPLC System
- Forensic Toxicology

Introduction

Benzodiazepines have a broad range of therapeutic use and are widely prescribed as safe drugs with relatively few side effects for the treatment of insomnia, anxiety and epilepsy. However, they are also abused in cases of crime, suicide, and drug-facilitated sexual assault. These molecules are active at very low concentrations and some of them have very short half lives. For this reason, the analytical methods must show extensive specificity and sensitivity for forensic purposes. We have developed and validated a method for 20 benzodiazepines and four metabolites in whole blood using liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled with ultrahigh pressure liquid chromatography (UHPLC) pumps.

Goal

To present a rapid and quantitative forensic screening approach for the analysis of benzodiazepines in blood matrix using UHPLC conditions.

Experimental

Sample Preparation

Extraction was performed using a liquid-liquid extraction (LLE) procedure. After the extraction, the sample was evaporated to dryness and reconstituted with 100 µL of a mixture containing acetonitrile/5 mM ammonium formate pH3 (30/70).

HPLC Conditions

Chromatographic analyses were performed using the Thermo Scientific Accela UHPLC system. The chromatographic conditions were as follows:

Column:	Thermo Scientific Hypersil GOLD 1.9 µm, 50 x 2.1 mm
Flow rate:	0.6 mL/min
Mobile phase A:	Water containing 5 mM ammonium formate, pH3
Mobile phase B:	Acetonitrile containing 0.1% formic acid

A gradient was performed starting from 95% of A to 95% of B in 6 minutes. The injection volume was 10 µL.

MS Conditions

Mass Spectrometer:	Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer
Source:	Heated electrospray ionization (HESI) mode
Ion Polarity:	Positive mode
Spray Voltage:	3000 V
Sheath/Auxiliary gas:	Nitrogen
Sheath gas pressure:	50 (arbitrary units)
Auxiliary gas pressure:	40 (arbitrary units)
Capillary temperature:	300 °C
Scan Type:	Selected reaction monitoring (SRM)
Q1, Q3 resolution:	Unit (0.7 Da FWHM)

Two SRM transitions were monitored for each component to provide ion ratio confirmations (IRC).

Results and Discussion

We validated a timed SRM (T-SRM) method for screening and quantifying 20 benzodiazepines and four metabolites. The run time was less than eight minutes, although most compounds eluted before four minutes. The T-SRM method allows the acquisition of an SRM transition only during a specified time window, not the entire run time. T-SRM divides the task into smaller batches by programming the instrument to look for each SRM only when it is expected to enter the instrument from an upstream LC system. Each time period is then optimized for the retention time of each compound. More time per transition results in better signal-to-noise (S/N) ratios or more scans per peak, allowing better quantitative data.

Standard spiking solutions of the analytes in porcine whole blood at concentrations of 5, 10, 50, 100, 300 and 500 ng/mL were prepared. All benzodiazepine calibration curves were evaluated using linear regression. Excellent linearity with a correlation coefficient of $R^2 > 0.99$ was obtained for each molecule. Seventeen were linear on the entire concentration range from 5 to 500 ng/mL. Six were linear from 10 to 500 ng/mL, and 3 were validated under linear conditions from 5 to 300 ng/mL. In all cases, the concentration range covered the therapeutic ranges.

Intra-method variability was calculated by processing five replicates of four calibration levels: the LOQ (limit of quantitation), two intermediate concentrations, and the maximum concentration. (%CV = coefficient of variance).

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Inter-method variability was determined by processing five replicates of four calibration levels in four different batches run on four different days. All values were below 15% and therefore within the guidelines set for a validated LC-MS/MS method.

Extraction efficiency also was evaluated and calculated at three concentration levels: 10 ng/mL, 100 ng/mL and 300 ng/mL. Values were between 50% and 100%, except for 7 amino-clonazepam which was around 30%.

The lower limit of quantitation (LLOQ) and the limit of detection (LOD) of the compounds were determined based on the calibration curve of S/N ratio versus concentration and the definitions of LOQ and LOD using S/N = 10 and 3. LLOQs were between 0.1 and 3 ng/mL for all molecules. Figure 1 shows the chromatogram obtained from a real sample acquired using the developed UHPLC-MS/MS method.

Conclusion

A rapid UHPLC-MS/MS method for quantifying benzodiazepines in whole blood samples was developed for forensic toxicology. The precision of the analysis meets current consensus guidelines. A T-SRM method was used to increase the acquisition time per compound and achieve better signal-to-noise ratios for the analytes.

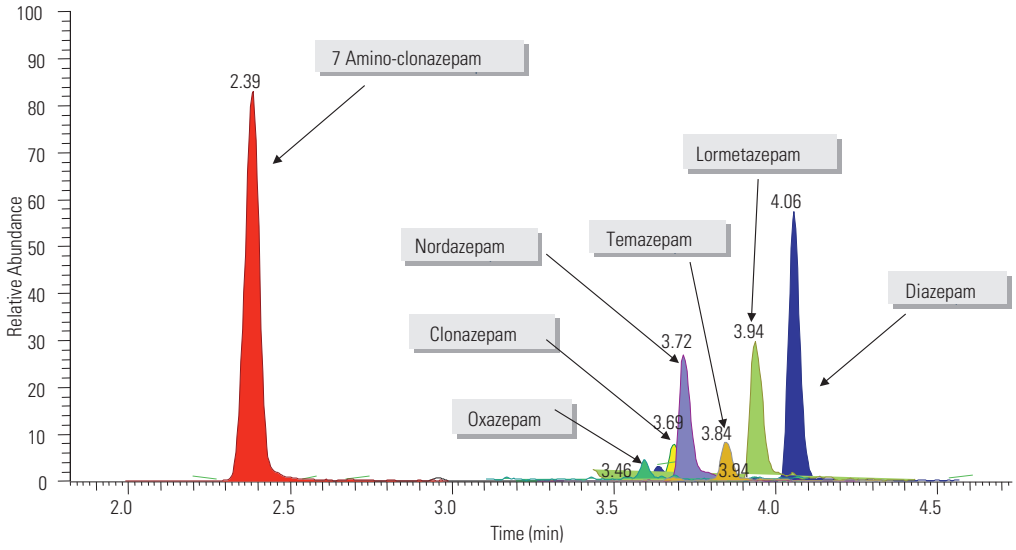

Figure 1. Chromatogram obtained from a real sample acquired using the T-SRM UHPLC-MS/MS method

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A Fully Automated LC-MS Screening System using Automated Online Sample Preparation for Forensic Toxicology

Mueller, D.M.[1]; Duretz, B.[2]; Espourteille, F.A.[3]; Rentsch, K.M.[1]
[1]Institute for Clinical Chemistry, University Hospital, Zurich, Switzerland; [2]Thermo Fisher Scientific, Les Ulis, France; [3]Thermo Fisher Scientific, Franklin, USA

Key Words

- Transcend TLX-1 system
- TurboFlow Technology
- LXQ Linear Ion Trap
- Illicit Drugs
- Forensic Toxicology

Introduction

Liquid chromatography-mass spectrometry (LC-MS) is a powerful tool widely used for forensic targeted drug screening. However, the quality of the results is highly affected by the sample preparation. Offline solid phase extraction (SPE) and liquid-liquid extraction (LLE) are widely used, but these methods are often time-consuming and costly. To provide a fast and sensitive approach, an automated online sample preparation method using Thermo Scientific Transcend TLX-1 system powered by TurboFlow™ technology for the forensic toxicological screening of more than 400 acidic, neutral, and basic drugs in urine with LC/MS^n has been developed.

Goal

To evaluate the performance of an automated online sample preparation method for an LC/MS^n screening approach.

Experimental

Sample preparation was performed by an online sample extraction method utilizing Thermo Scientific TurboFlow technology. Two TurboFlow columns (Cyclone, C18XL) were connected in series and used for sample extraction. Urine samples were run both natively and after enzymatic hydrolysis. The eluent was then transferred to the LC column (Thermo Scientific Betasil Phenyl-Hexyl, 100 x 3 mm, 3 µm) for separation.

Table 1. Tramadol and O-desmethylvenlafaxine information

	O-Desmethylvenlafaxine	Tramadol
Precursor mass	264.3	264.3
MS^2 Fragment	246.3	246.3
Retention Time	10.6 min	10.3 min

A 30-minute gradient from 1% to 98% organic was employed for separation of the analyte with flow rates of 300 µL/min. All samples were then analyzed on a Thermo Scientific LXQ linear ion trap mass spectrometer with the atmospheric pressure chemical ionization (APCI) source. A data-dependent polarity switching method was used for data acquisition. MS^2 and MS^3 spectra were acquired. Since polarity switching was used, a single injection of a sample containing unknown compounds was sufficient to detect both substances ionizing in negative and positive mode. The data was automatically processed, post-acquisition, by Thermo Scientific ToxID automated screening software.

Results and Discussion

The method using online extraction has been fully validated. A minor matrix effect (suppression < 5%) was observed for over 98% of the compounds. A recovery of more than 90% was seen in 90% of the substances. The limit of identification (LOI) was below 10 ng/mL for 60% of the substances and 90% could be identified at a concentration of 100 ng/mL. The 400-compound library contains both MS^2 and MS^3 spectra. MS^3 spectra bring an additional level of specificity, although in most cases, the analytes can be easily identified by using only the MS^2 spectra. However, some analytes may have the same molecular weight, very similar MS^2 spectra, and a very close retention time. For these reasons, MS^3 data have to be used for the identification. One example is the isobaric compounds O-desmethylvenlafaxine and tramadol. The two analytes have the same molecular weight, very close retention times (see details in Table 1), and the same MS^2 spectra (Figure 1). Therefore, by running only MS^2 experiments, it is impossible to properly differentiate the two analytes. When MS^3 spectra are recorded, tramadol does not fragment ions while O-desmethylvenlafaxine gives a specific spectrum (Figure 1). Therefore, the analytes can be properly identified. Total run time of the analysis is 30 minutes. An example of a chromatogram obtained from a sample is presented in Figure 2.

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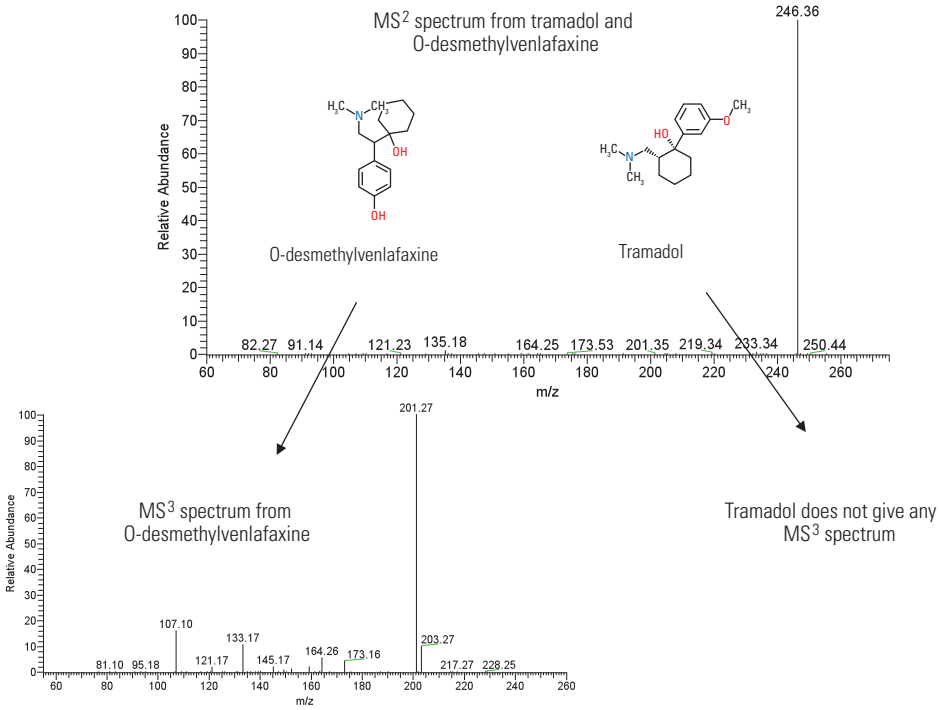

Figure 1. Fragmentation of tramadol and O-desmethylvenlafaxine in MS^2 and MS^3

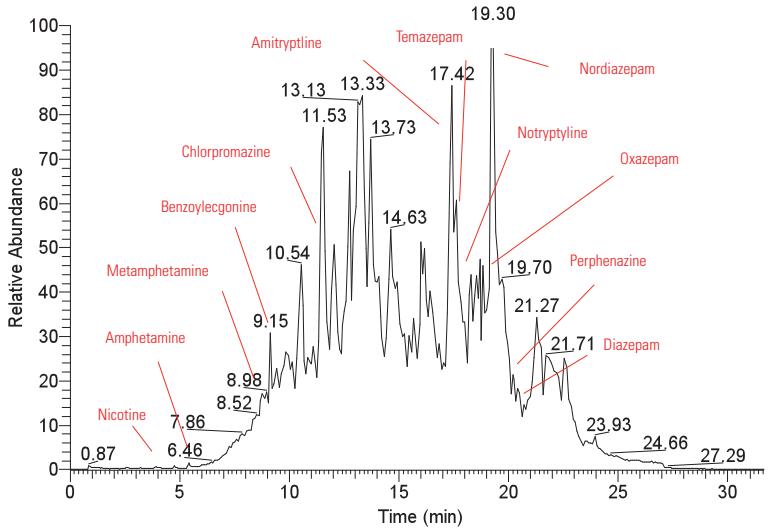

Figure 2. Full scan MS chromatogram of a sample containing 12 different analytes

Conclusion

The automated online TurboFlow method with the LXQ™ linear ion trap mass spectrometer allows a fast and specific approach for the identification of a broad range of compounds in positive and negative mode in a single run. The sample preparation time is 15 minutes with this method as compared to 2 hours with an offline approach. The LOIs are below 100 ng/mL for more than 90% of the analytes. MS^3 spectra acquisition brings an additional level of specificity for forensic toxicology laboratories.

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Screening for Drugs and Toxic Compounds: Comparison between LC-MS/MS, HPLC-DAD, and Immunoassay

C. Gechtman[1], A. Masarin[1], S. Scurati[2]; [1]A.O. Ospedale Niguarda Cà Granda, Milano, Italy; [2]Thermo Fisher Scientific, Milano, Italy

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Key Words

- ToxID software
- LXQ Linear Ion Trap
- Drugs of Abuse
- Toxicology Screening

Introduction

Screening of biological samples for drugs of abuse and other toxic compounds is a critical feature of forensic toxicology laboratories. The main challenge is to provide rapid and accurate results despite the large number of target molecules and the complexity of biological matrices. The classical approach is based on immunoassay or high pressure liquid chromatography-diode-array detection (HPLC-DAD). However, the advent of newer and more effective liquid chromatography-tandem mass spectrometry (LC-MS/MS) technologies can lead to a significant improvement in non-targeted screening.

Goal

Evaluate the Thermo Scientific ToxSpec Analyzer, LC-MS solution for forensic toxicology screening, for non-targeted screening of several compounds in human urine. LC-MS technology is used to increase the confidence of identification and to simplify the workflow in a forensic toxicology laboratory when compared with the classical screening approaches.

Experimental

Sample Preparation

Urine was stored at -20 °C for the analysis. After thawing, the sample was diluted 1:10 with water. For the analysis, 20 µL of diluted urine were directly injected.

Chromatography and Mass Spectrometry

The ToxSpec™ Analyzer was used for the analysis. Briefly, for the LC separation a Thermo Scientific Hypersil GOLD PFP analytical column (50 x 2.1, 5 µm) was used, with mobile phase A (10 mM ammonium formate in 0.1% formic acid) and B (ACN containing 0.1% formic acid). The gradient was from 95% A to 95% B in about 5 minutes with a flow rate of 200 µL/min. For the MS analysis, a Thermo Scientific LXQ linear ion trap mass spectrometer equipped with an electrospray ionization (ESI) source utilizing polarity switching was employed. A data dependent scan collected MS/MS spectra of all the compounds eluted. Data generated were processed with Thermo Scientific ToxID automated screening software. ToxID™ software identifies compounds on the basis of retention time, precursor ion, and MS/MS spectrum. Samples screened by LC-MS/MS were previously analyzed also with immunoassay or HPLC-DAD, allowing a comparison between methods.

Results and Discussion

The ToxSpec Analyzer is able to process a sample in about 15 minutes, which allows the performance of routine screening analysis. Data obtained are highly specific and reliable because the identification of compounds is based on three peculiar characteristics of the molecules: retention time, precursor ion, and MS/MS spectrum. Figure 1 shows a report generated by ToxID software after the analysis of a urine sample that tested positive for LSD.

The comparison of results obtained by analyzing the same urine samples with different screening approaches has given interesting results (see Table 1). The ToxSpec Analyzer confirmed, for the most part (Urine 1-4), the results obtained with HPLC-DAD or an immunoassay, but also identified additional compounds, such as metabolites or other minor components that were not recognized with other screening approaches.

Surprisingly, in Urine 5, the results are clearly not in agreement. Particularly, the immunoassay identified amphetamines, while the ToxSpec Analyzer method identified ranitidine and metoclopramide, two therapeutics drugs often used in combination. To better understand the difference between the techniques, we compared the MS/MS spectra of the molecules detected in Urine 5 with those present in the library.

Table 1. Comparison of results obtained analyzing the same urine samples using different screening techniques.

Sample	HPLC-DAD or Immunoassay	ToxSpec Analyzer	Results Comparison
Urine 1	Cocaine	Cocaine, Benzoylecgonine, Cocaethylene, Nicotine	√
Urine 2	Ketamine	Ketamine, Norketamine	√
Urine 3	Quetiapine	Lidocaine, Quetiapine	√
Urine 4	LSD	OH-LSD	√
Urine 5	Amphetamines	Ranitidine, Metoclopramide	X

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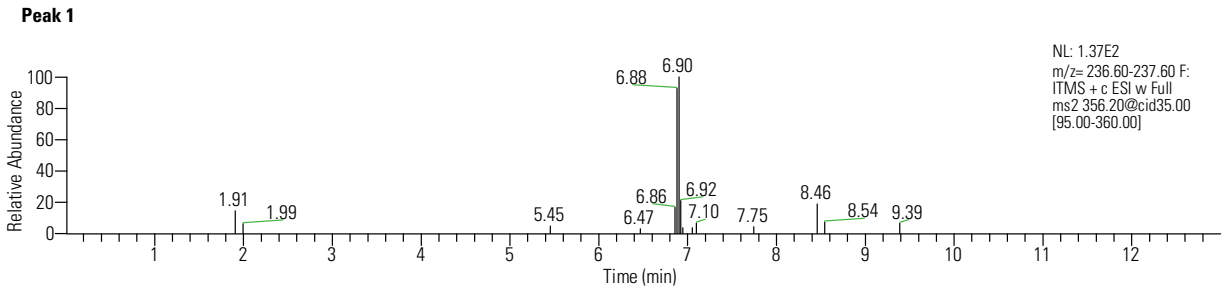


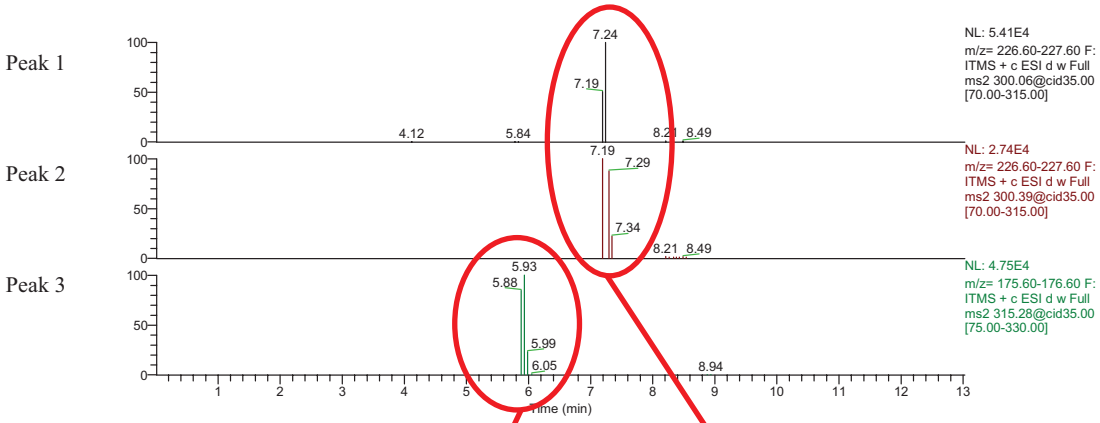
Peak Number	Compound Name	Code	SI	RSI	m/z	Expected RT	Actual RT	Intensity	Library Name
1	OH-LSD	m	756	922	356.20	6.20	6.90	15461	Tox_Library_LXQ

Figure 1. Example ToxID software short report showing ion chromatogram and a compound detected in urine positive for LSD.

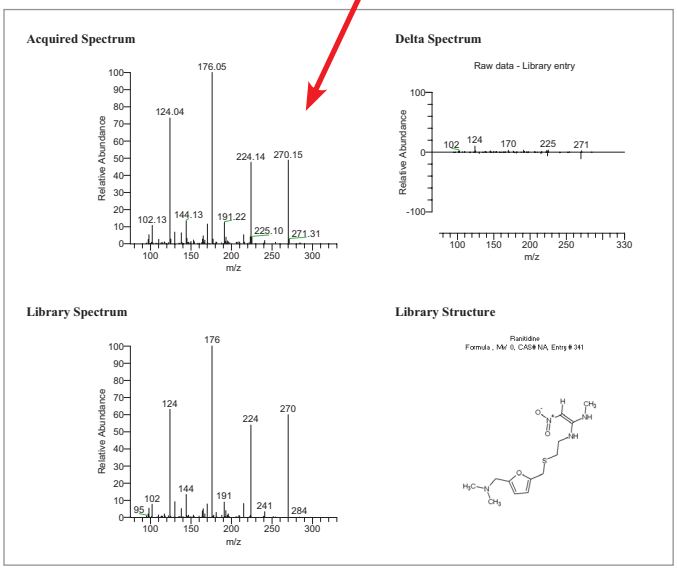
We confirmed the presence of ranitidine and metoclopramide through the mass spectra, which are very similar to that present in the library and the measured retention times are very similar to the expected (Figure 2). Moreover, some immunoassays are known to give cross-reactivity between ranitidine and amphetamines. As a consequence, we established that a false positive was found by the immunoassay and the ToxSpec Analyzer that identified the cross-reacting molecule as ranitidine.

Conclusion

The ToxSpec Analyzer was used to screen toxic compounds and their metabolites in urine based on LC-MS/MS. This method has been compared with other classical screening techniques such as HPLC-DAD and an immunoassay. LC-MS/MS demonstrated more reliable results than other techniques. In conclusion, the LC-MS/MS method provides rapid sample preparation, ease-of-use, sensitivity, specificity and a low cost per sample analysis, making the ToxSpec Analyzer an appropriate tool for non-targeted screening in a forensic toxicology laboratory.



Peak Number	Compound Name	Code	SI	RSI	*m/z*	Expected RT	Real RT	Intensity	Library Name
1	Metoclopramide	p	958	958	300.20	7.20	7.24	54117	Tox_Library_LXQ
2	Metoclopramide	p	984	984	300.20	7.20	7.19	27361	Tox_Library_LXQ
3	Ranitidine	p	792	802	315.20	5.45	5.93	47525	Tox_Library_LXQ



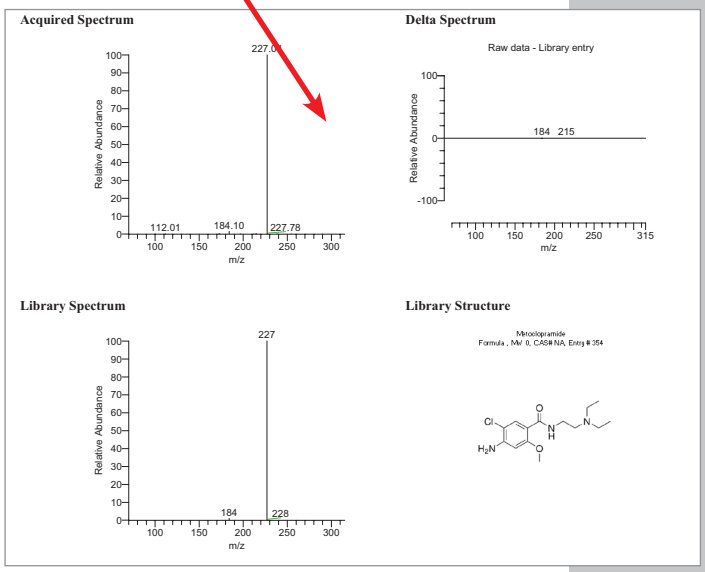


Figure 2. ToxID software long report showing ion chromatograms and MS/MS spectra of compounds detected in Urine 5. Mass spectra recorded for ranitidine and metoclopramide show a perfect match when compared with spectra from the database.

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Screening and Quantification of Multiple Drugs in Urine Using Automated Online Sample Preparation and Tandem Mass Spectrometry

Barbora Brazdova, Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

- Transcend System
- TSQ Quantum Access MAX
- ToxID Software
- Clinical Research

Introduction

Liquid chromatography-mass spectrometry (LC-MS) is a sensitive, accurate, and precise technique applied in clinical research for the analysis of a large number of compounds and metabolites from various drug classes, such as antidepressants, hypnotics, stimulants, cardiacs, and antihistamines. Thermo Scientific Transcend system powered by TurboFlow™ technology provides an alternative separation technique for complex biomatrices, simplifying sample preparation, increasing LC-MS/MS sensitivity, and reducing ion suppression.

Goal

To develop a fast and efficient LC-MS/MS method using Thermo Scientific TurboFlow technology for the analysis of 30 drugs and metabolites in urine.

Experimental

Sample Preparation

Eight internal standards were used in the study for the corresponding compounds: nicotine-d4, cotinine-d4, midazolam-d4, diphenhydramine-d3, promethazine-d3, norlfuoxetine-d6, chlorpromazine-d3, and fluoxetine-d6. For the other compounds, the internal standard with the closest retention time was assigned.

Human urine samples (100 µL) were diluted with 100 µL of methanol containing the internal standards in concentrations of 100 ng/mL. The samples were vortexed and centrifuged. Then, 10 µL of the supernatant was injected onto the TurboFlow column.

HPLC

HPLC analysis was performed using the Transcend™ system with a TurboFlow Cyclone MAX column (0.5 x 50 mm) and a Thermo Scientific Hypersil GOLD PFP analytical column (100 x 2.1 mm; 5 µm). Total analysis time was 9 minutes.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer equipped with a Thermo Scientific Ion Max source and an electrospray ionization (ESI) probe. Two selected reaction monitoring (SRM) transitions with scan times of 10 msec were collected for each analyte.

Results and Discussion

Quantitation of 30 drugs in urine was performed in 9 minutes with a calibration range of 1-1000 ng/mL for 14 compounds, 5-1000 ng/mL for 9 compounds, 10-1000 ng/mL for 5 compounds and 50-1000 ng/mL for 2 compounds. Figure 1 shows the chromatograms of the lowest calibration standard. Table 1 displays the calibration ranges and method precision for all analyzed drugs.

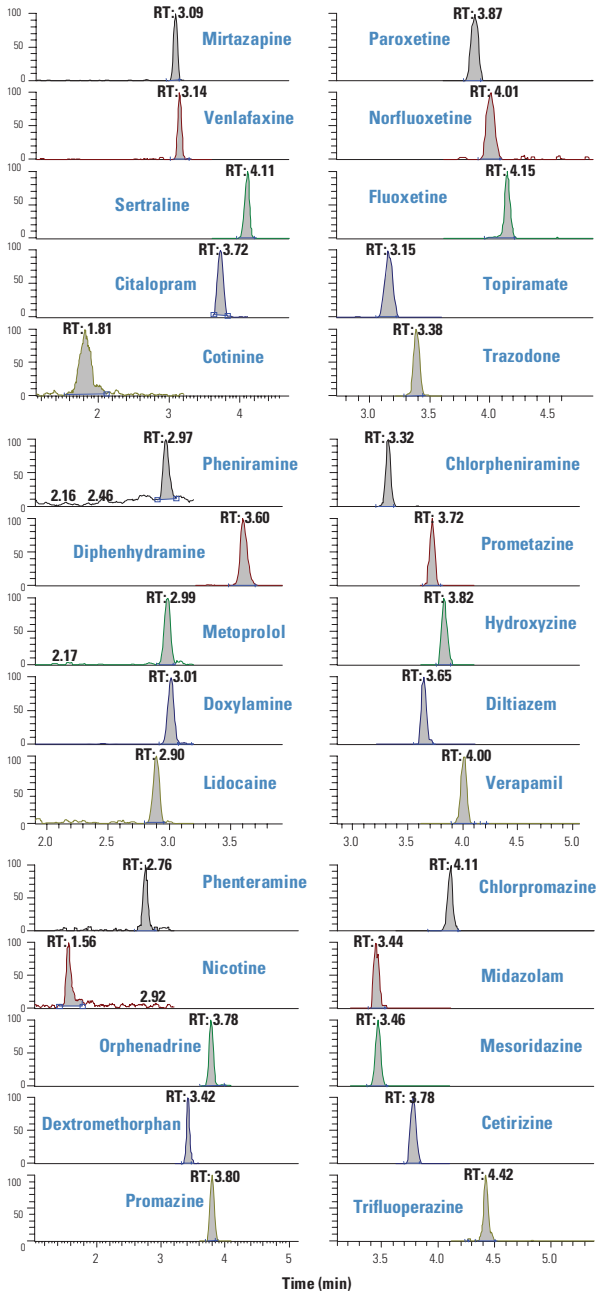

Figure 1: Chromatographs of the lowest calibration standard.

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Within-day and between-days precisions were determined with QC samples prepared by spiking blank urine to three concentrations: twice the lowest standard concentration (QC1), the middle of the calibration range concentration (QC2), and 80% of the highest standard concentration (QC3).

Table1: Calibration ranges, within-day precision, and between-days precision for the lowest QC sample.

Analyte	Calibration range (ng/mL)	Within-day (%RSD)	Between-days (%RSD)
Citalopram	1-1000	10.7	9.3
Fluoxetine	10-1000	10.4	9.1
Norfluoxetine	10-1000	16.4	11.0
Mirtazapine	1-1000	13.6	12.5
Paroxetine	10-1000	10.2	14.6
Sertraline	10-1000	8.0	15.7
Trazodone	1-1000	11.7	10.6
Venlaflaxine	1-1000	13.6	12.1
Diphenhydramine	1-1000	7.1	7.1
Chlorpheniramine	1-1000	8.2	7.2
Pheniramine	1-1000	7.6	5.6
Cetirizine	5-1000	15.4	15.1
Promethazine	50-1000	4.6	4.2
Nicotine	5-1000	10.7	7.1
Cotinine	5-1000	12.0	8.1
Dextromethorphan	1-1000	8.6	10.9
Topiramate	50-1000	13.2	10.4
Orphenadrine	1-1000	7.2	9.1
Lidocaine	1-1000	11.5	9.4
Phenteramine	10-1000	11.1	13.8
Mesoridazine	5-1000	3.4	4.4
Midazolam	1-1000	14.3	12.2
Chlorpromazine	5-1000	8.0	15.0
Promazine	5-1000	17.1	10.8
Trifluoperazine	5-1000	7.9	17.5
Diltiazem	1-1000	10.1	10.2
Metaprolol	5-1000	10.0	8.5
Verapamil	5-1000	8.7	9.2
Doxylamine	1-1000	14.4	11.7
Hydroxyzine	1-1000	17.9	14.0

Conclusion

An efficient (9 minute), sensitive (LOQ of 1-50 ng/mL), and precise LC-MS/MS method using TurboFlow technology was developed for the quantitation of 30 drugs in human urine. In clinical research, TurboFlow technology simplifies sample preparation and improves method robustness and sensitivity.

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Forensic Toxicology Screening with LC-MS/MS and Automated Online Sample Preparation

Guifeng Jiang, Marta Kozak, Subodh Nimkar, Thermo Fisher Scientific, San Jose, CA, USA

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Key Words

- ToxSpec Analyzer
- ToxID Software
- LXQ Linear Ion Trap
- TurboFlow Technology
- Forensic Toxicology

Introduction

The quality of liquid chromatography-mass spectrometry (LC-MS) data collected in forensic drug screening applications is largely affected by sample preparation methods. Offline solid phase extraction (SPE) and liquid-liquid extraction (LLE) are the most commonly used methods. Automated online sample preparation using Thermo Scientific TurboFlow technology provides a robust front end platform for forensic drug screening, which is convenient and labor-saving.

Goal

The goal is to evaluate the performance of three sample preparation techniques – TurboFlow™ technology, SPE, and LLE – to screen 300 basic, neutral, and acidic drug compounds for forensic toxicology use.

Experimental

SPE – Mixed-mode Thermo Scientific HyperSep Verify-CX SPE cartridges (200 mg; 6 mL) were used for offline SPE. Samples of 1 mL of urine were spiked to final concentrations of 10, 100 and 1000 ng/mL with analytes of interest, as well as 100 ng/mL of three deuterated internal standards, and loaded on the SPE column. Basic, acidic, and neutral fractions were collected, combined, evaporated to dryness, reconstituted in 100 µL, and injected onto the LC column.

LLE – Toxi-Tubes® A & B (Varian) were used for offline LLE. Samples of 1 mL of urine were spiked to final concentrations of 10, 100 and 1000 ng/mL with analytes of interest, as well as 100 ng/mL of three deuterated internal standards, and then applied to the Toxi-Tube. The organic layers were transferred, evaporated to dryness, reconstituted in 100 µL, and injected onto the LC-MS.

TurboFlow Method – Urine samples were diluted in ratio 1:1 v/v with 50% MeOH containing internal standards. Fifty (50) µL of diluted sample was injected onto the TurboFlow columns. Two different chemistry TurboFlow columns were used to extract chemically diverse compounds.

A 12-minute LC method was developed for TurboFlow and LLE samples. Samples were injected onto a Thermo Scientific Hypersil GOLD PFP 100 x 30 mm, 3 µm column. A gradient method was employed with flow rates of 600 µL/min. For offline SPE samples, a 13-minute LC gradient was used with a Thermo Scientific Hypersil GOLD PFP analytical column (50 x 2.1 mm, 5 µm) and a 200 µL/min flow rate.

Mass Spectrometry

All samples were analyzed on the Thermo Scientific ToxSpec Analyzer system equipped with a Thermo Scientific LXQ linear ion trap mass spectrometer and an electrospray ionization (ESI) source using a scan-dependent, polarity-switching method. Reports were automatically produced with Thermo Scientific ToxID automated forensic toxicology screening software, including lists of identified compounds and their matching MS/MS spectrum.

Results and Discussion

Table 1 shows limits of identification for representative compounds from the SPE, LLE, and TurboFlow methods. The lowest concentration validated was 10 ng/mL. All three methods showed comparable limits of identification. In addition, with the automated TurboFlow method, the sample quantity loaded on the column was one-quarter of that in the SPE method and one-eighth of that in the LLE method.

Table 1. Comparison of limits of identification for selected compounds

Compound	TurboFlow Method (ng/mL in urine)	SPE Method (ng/mL in urine)	LLE Method (ng/mL in urine)
Codeine	10	10	10
Hydrocodone	10	10	10
Cocaine	10	10	10
Amphetamine	10	10	1000
Stanozolol	100	100	10
Diazepam	10	10	10

Figure 1 shows the results of the identification limits of 300 drugs with the three sample preparation methods. Compared to traditional sample preparation methods, the automated TurboFlow method provides competent performance with automated online sample preparation.

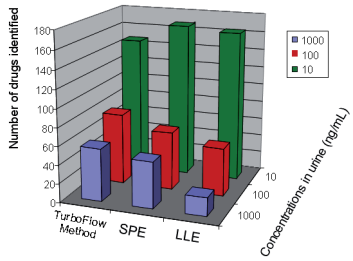

Figure 1: Limits of identification of 300 compounds

Conclusion

The TurboFlow method with the ToxSpec™ Analyzer allows for the identification of 300 drugs, with limits of detection (LODs) ranging from less than 10 ng to greater than 1000 ng per milliliter of urine. It provides an automated online sample preparation platform for forensic toxicology screening with competent performance and limits of identifications. The TurboFlow method is easier, faster, and cost efficient in comparison to traditionally used SPE and LLE methods.

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Application Note: 499

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Quantitative LC-MS Screening for Illicit Drugs Using Ultrahigh Resolution Mass Analysis and Accurate Mass Confirmation

Kevin J. McHale and Mark Sanders, Thermo Fisher Scientific, Somerset, NJ

Forensic toxicology use only.

Key Words

- Exactive LC-MS
- Accela UHPLC
- Drug Screening
- Drug Quantitation
- Forensic Toxicology

Introduction

There is increasing demand to rapidly identify and quantify illicit drugs in human samples for forensic purposes. By using ultrahigh resolution, accurate mass spectrometry detection coupled to liquid chromatography separation, a large number of both expected and unexpected compounds can be easily screened and quantified without prior knowledge.

Goal

To evaluate a simple LC-MS method using a benchtop ultrahigh resolution mass spectrometer to quantitatively screen for 46 illicit drugs of abuse in urine with little sample preparation.

Experimental

Sample Preparation

Blank human urine diluted with 25% acetonitrile was spiked with varying concentrations of 46 drugs of abuse and their corresponding isotopically-labeled standards at 50 ng/mL.

HPLC

HPLC analyses were performed using a Thermo Scientific Accela liquid chromatography system. Gradient elution with a Thermo Scientific Hypersil GOLD PFP column (100 x 2.1 mm; 3 µm) was used at a flow rate of 350 µL/min. The injection volume was 10 µL.

Mass Spectrometry

MS detection was carried out on a Thermo Scientific Exactive benchtop LC-MS system with a heated electrospray ionization (HESI) source in positive ion mode at a mass resolution of 50,000 FWHM via external mass calibration.

Results and Discussion

LC-MS quantification of 46 drugs of abuse was accomplished via the calculated area ratios of the compound to its heavy-labeled internal standard. Table 1 gives a listing of the targeted drugs of abuse, their limits of quantitation (LOQ), and their measured mass errors at the LOQ in urine.

Example extracted ion chromatograms for methamphetamine and benzoylecgonine in urine are shown in Figure 1. At a mass resolution of 50,000 FWHM, at least 10 data points were obtained across the LC peaks. Applying extracted ion chromatograms of ±5 ppm, along with the isotopically-labeled internal standards for confirmation, all drugs of abuse were easily identified.

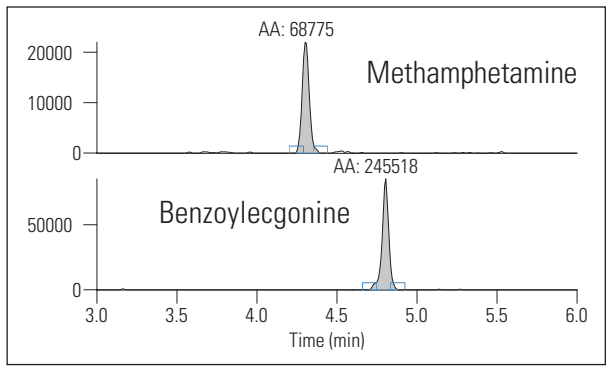

Figure 1: Example extracted ion chromatograms (± 5 ppm) at LOQs in urine

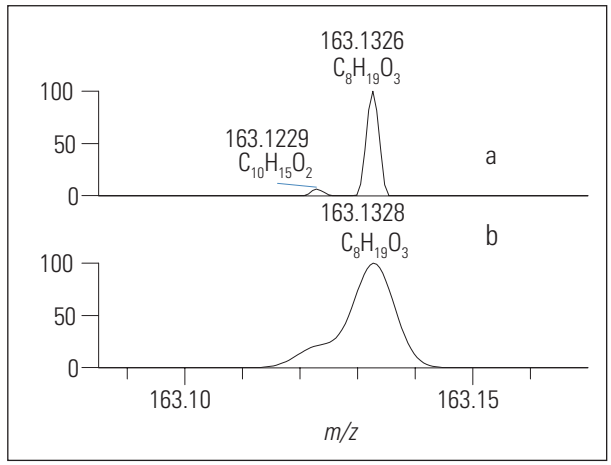

Figure 2: Nicotine mass spectrum in urine (a) and simulated spectrum (b) at 20,000 FWHM

Figure 2 shows the nicotine mass spectrum in urine (a) with an isobaric interference at *m/z* 163.1326 and a simulated spectrum (b) at 20,000 FWHM. Although nicotine and its isobaric interference have a large relative mass difference of ~60 ppm, the absolute mass difference is only 0.0097 u. As seen in the simulated spectrum (b) at 20,000 FWHM, the nicotine ion is not resolved from the interference. Only ultrahigh mass resolution of 50,000 FWHM provides the necessary selectivity to resolve these compounds and therefore allows for confident identification and quantification.

Conclusion

The Exactive benchtop LC-MS system provides easy confirmatory and quantitative analysis of 46 illicit drugs at LOQs of 0.5 – 5 ng/mL in urine for forensic toxicology. Owing to the sensitivity of the Exactive system, urine samples require only dilution with solvent to achieve this level of performance.

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Table 1: List of drugs of abuse monitored

Drug of Abuse	RT (min)	Exact m/z	Measured *m/z*	Error (ppm)	LOQ (ng/mL)
Nicotine	1.40	163.12298	163.12288	-0.6	0.5
Cotinine	1.55	177.10224	177.10204	-1.2	0.5
Morphine	2.35	286.14377	286.14362	-0.5	1.25
Hydromorphone	3.19	286.14377	286.14374	-0.1	1.25
Ephedrine	3.23	166.12264	166.12256	-0.5	1.25
Amphetamine	3.82	136.11208	136.11203	-0.4	2.5
Codeine	3.91	300.15942	300.15891	-1.7	1.25
Noroxycodone	4.17	302.13868	302.13828	-1.3	1.25
Methamphetamine	4.32	150.12773	150.12761	-0.8	1.25
MDA	4.36	180.10191	180.10181	-0.6	0.5
Oxycodone	4.36	316.15433	316.15384	-1.5	0.5
6-Acetylmorphine	4.44	328.15433	328.15384	-1.5	1.25
Hydrocodone	4.64	300.15942	300.15891	-1.7	1.25
MDMA	4.69	194.11756	194.11742	-0.7	0.5
Norketamine	4.77	224.08367	224.08351	-0.7	1.25
7-Amino-clonazepam	4.75	286.07417	286.07367	-1.7	0.5
Benzoylecgonine	4.81	290.13868	290.13806	-2.1	1.25
Ketamine	5.01	238.09932	238.09877	-2.3	0.5
Norfentanyl	5.03	233.16484	233.16470	-0.6	0.5
MDEA	5.31	208.13321	208.13304	-0.8	0.5
7-Amino-flunitrazepam	5.73	284.11937	284.11929	-0.3	0.5
Normeperidine	6.26	234.14886	234.14865	-0.9	0.5
Meperidine	6.45	248.16451	248.16428	-0.9	0.5
Cocaine	6.62	304.15433	304.15405	-0.9	0.5
Norbuprenorphine	6.74	414.26389	414.26257	-3.2	2.5
alpha-Hydroxymidazolam	7.29	342.08039	342.07971	-2.0	1.25
Oxazepam	7.30	287.05818	287.05768	-1.7	5
alpha-Hydroxytriazolam	7.41	359.04609	359.04562	-1.3	2.5
alpha-Hydroxyalprazolam	7.42	325.08507	325.08447	-1.8	2.5
Cocaethylene	7.43	318.16998	318.16962	-1.1	0.5
Lorazepam	7.45	321.01921	321.01797	-3.9	2.5
PCP	7.44	244.20598	244.20518	-3.3	0.5
Nitrazepam	7.54	282.08732	282.08658	-2.6	2.5
2-Hydroxyethylflurazepam	7.57	333.08006	333.07889	-3.5	1.25
Midazolam	7.64	326.08548	326.08493	-1.7	0.5
Nordiazepam	7.71	271.06327	271.06265	-2.3	0.5
Clonazepam	7.72	316.04835	316.04791	-1.4	2.5
Temazepam	7.83	301.07383	301.07349	-1.1	2.5
Fentanyl	7.90	337.22744	337.22635	-3.2	2.5
Alprazolam	8.00	309.09015	309.08957	-1.9	1.25
Triazolam	7.99	343.05118	343.05103	-0.4	0.5
Flunitrazepam	8.01	314.09355	314.09293	-2.0	1.25
Buprenorphine	8.12	468.31084	468.31006	-1.7	1.25
Diazepam	8.24	285.07892	285.07819	-2.6	0.5
EDDP	8.58	278.19033	278.18991	-1.5	0.5
Methadone	8.81	310.21654	310.21628	-0.8	0.5

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Screening in Equine Doping Control Analysis with Ultrahigh Resolution and Accurate Mass

Yves Moulard, Yves Bonnaire, Laboratoire des Courses Hippiques, Verrières-Le Buisson, France;
Bénédicte Duretz, Thermo Fisher Scientific, Les Ulis, France; Dennis Nagtalon, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Exactive
- LC/MS
- Orbitrap Technology
- Forensic Toxicology
- ToxID Software

Introduction

Triple quadrupole or tandem mass analyzers have been used most frequently in the accurate identification, confirmation, and quantitation of prohibited compounds in a single analysis. In addition, ion trap and quadrupole time-of-flight mass analyzers have been useful for screening and confirming results. However, these technologies cannot address the main requirements of equine doping control analysis such as:

- Data re-interrogation
- Analyze and monitor a vast number of compounds
- Fast and easy method development, instrument operation, and data interpretation
- Efficient separation of analytes from interferences present in the matrix
- Highly confident identification of compounds

Here we present a screening approach that uses ultrahigh resolution (R = 50,000) and accurate mass in positive and negative mode for the screening of illicit substances in urine matrix using the Thermo Scientific Exactive benchtop mass spectrometer. More than 120 analytes are screened using this method. Confirmation is made using the exact mass of the analytes in positive and negative mode (if available) and the retention time.

Goal

To demonstrate a new approach using ultrahigh resolution (> 50,000) and accurate mass for the screening of illicit substances in a urine matrix using the Exactive™ mass spectrometer, a new high performance benchtop LC/MS instrument equipped with Thermo Scientific Orbitrap technology, for doping control analysis.

Figure 1. Thermo Scientific Exactive high performance benchtop LC/MS system

Experimental

Sample preparation

Solid phase extraction (SPE) was used for sample pre-treatment and clean up. The details of the procedure are described below.

- To 5 mL of urine add 25 µL of hydrocortisone d3 at 10 µg/mL
- Add 1 mL of phosphate buffer
- Add 50 µL of β glucuronidase and 50 µL of protease
- Incubate for 1 hour at 55 °C
- Centrifuge at 4,000 rpm for 30 minutes
- Transfer the supernatant to a tube
- Add 5 mL of water
- Condition the C18-HF cartridge with 3 mL of methanol and 3 mL of water
- Load the sample and wash the cartridge with 3 mL of water and 3 mL of hexane
- Elute with 3 mL of a mixture containing dichloromethane and ethanol
- Evaporate to dryness
- Reconstitute with 100 µL of a mixture containing water and acetonitrile (80/20)

Instrumentation Method

HPLC conditions

Chromatographic analyses were performed using Shimadzu binary pumps LC-20ADxr (Champs sur Marne, France). The chromatographic conditions were as follows:

Column:	Reversed-phase, silica-based C18 (3.5 µm, 150 x 2.1 mm) column
Flow rate:	0.3 mL/min
Injection volume:	10 µL
Mobile phase:	A: Water containing 0.1% formic acid
	B: Acetonitrile containing 0.1% formic acid

Gradient:

T(min)	A(%)	B(%)
0.0	80	20
5.0	80	20
20.0	50	50
25.0	0	100
25.2	80	20
30.0	80	20

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Mass Spectrometry conditions

MS analysis was carried out on an Exactive benchtop mass spectrometer with an electrospray ionization (ESI) source (Figure 1). The MS conditions were as follows:

Ion Polarity:	Polarity switching scan dependent experiment
Spray Voltage:	4500 V in positive mode and –3900 V in negative mode
Sheath gas pressure (N_2):	45 (arbitrary units)
Auxiliary gas pressure (N_2):	3 (arbitrary units)
Capillary temperature:	300 °C
Resolution:	50,000 (FWHM)
AGC Target Value	500,000

Results and Discussion

The screening method was set up for the identification and confirmation of more than 100 compounds, including anabolic agents, steroids, anesthetics, anti-inflammatory agents, and diuretics, as listed in Table 1

Acquisition was performed using the full MS scan mode with polarity switching and external calibration. All data were reprocessed using 5 ppm mass accuracy. Figure 2 shows the sensitivity obtained for a urine sample spiked with 4 compounds: dexamethasone, flumethasone, triamcinolone acetonide, and triamcinolone. The injected concentrations were 50 pg/mL for dexamethasone and flumethasone and 1 ng/ml for triamcinolone and triamcinolone acetonide. In the positive mode, the analytes were identified as protonated species and in the negative mode, as formate adducts. As data acquired was in full scan MS mode, re-interrogation of the data file, particularly for non-targeted or unknown compounds or metabolites, is easily made possible.

Thousands of real urine samples have been analyzed using this approach. Figure 3 shows an example of a real sample that has been analyzed using this method.

All data have been processed using Thermo Scientific ToxID software. ToxID™ software for Exactive processes data using the mass accuracy and retention time of the analytes. An example of the automatically generated report can be seen in Figure 4.

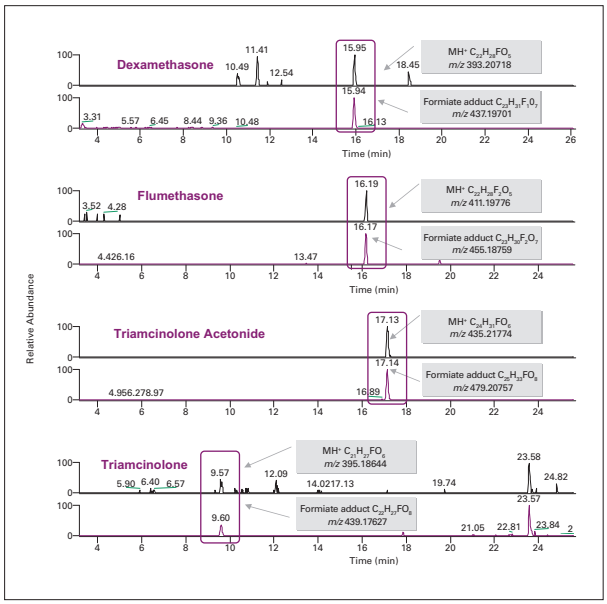


Figure 2: Extracted ion chromatograms for dexamethasone, flumethasone, triamcinolone acetonide, and triamcinolone in the positive and negative modes using 5 ppm mass accuracy

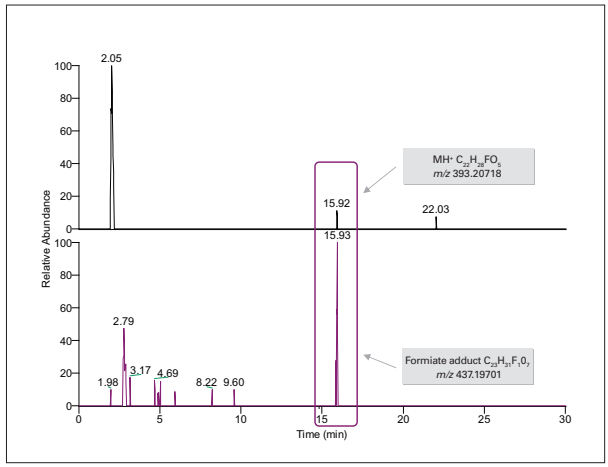
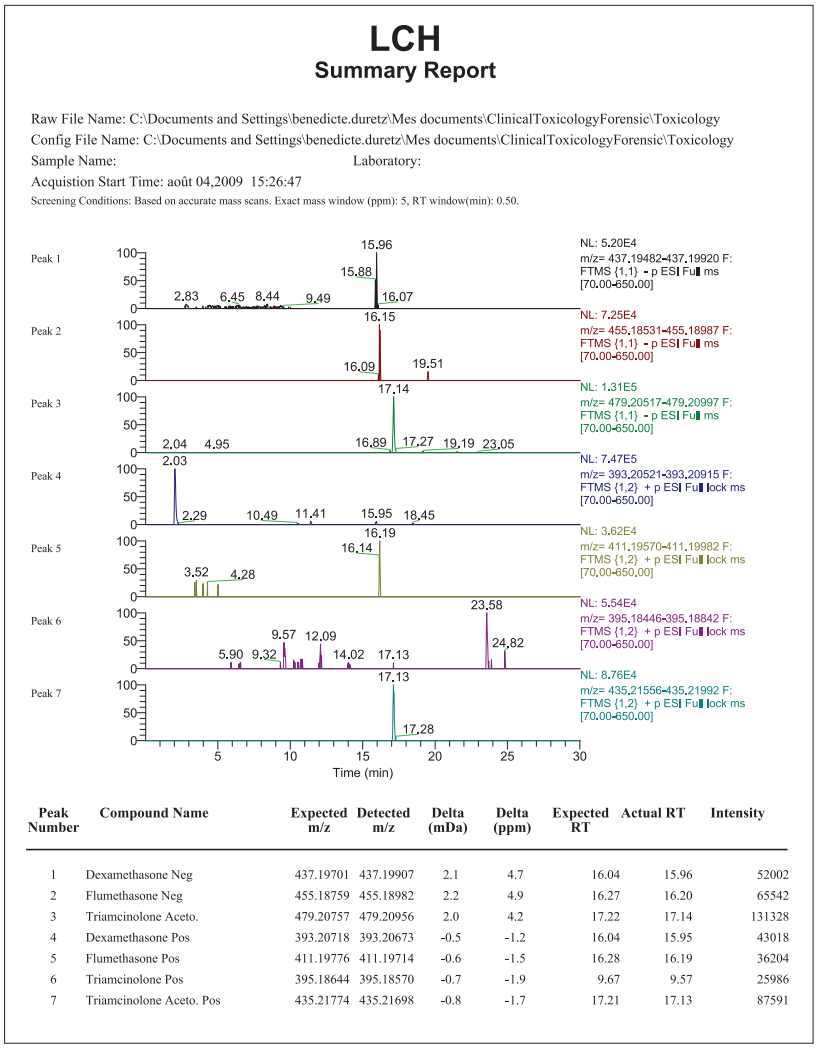


Figure 3: Dexamethasone identified in a real sample in positive and negative mode

Table 1: List of compounds monitored in the screening.

Index	Compounds	Index	Compounds	Index	Compounds
1	20 Beta dihydrocortisol	42	Diazoxide	83	Naftidrofuryl
2	4 Methylamino antypirine	43	Dichlorisone	84	Niketamide
3	5' Hydroxy Omeprazole	44	Diphenydramine	85	Nimesulide
4	Acepromazine	45	Diphylline	86	Nordazepam
5	Acide ethacrynic	46	Etamiphylline	87	Omeprazole
6	Althiazide	47	Etophylline (Etofylline)	88	Oxazepam
7	Ambroxol	48	Fenspiride	89	Oxyphenbutazone
8	Amcinonide	49	Fludrocortisone	90	Paramethasone
9	Amitryptylline	50	Flufenamic acid	91	Pentoxyphylline
10	Antipyrine (phenazone)	51	Flumethasone	92	Petidine (meperidine)
11	Beclomethasone	52	Flunisolid	93	Phenobarbital
12	Bendroflumethiazide	53	Flunixin	94	Phenylbutazone
13	Benzocaine	54	Fluocinolone acetonide	95	Phenytoin
14	Benzoylecgonine	55	Fluocinonide	96	Piroxicam
15	Benzydamine	56	Fluorometholone	97	Prednisolone
16	Betamethasone	57	Fluoroprednisolone	98	Prednisone
17	Budesonide	58	Flurandrenolide	99	Probenicid
18	Buflomedil	59	Fluticasone propionate	100	Procaine
19	Bumetanide	60	Furosemide	101	Prolintane
20	Bupivacaine	61	Guaifenesin	102	Promazine
21	Butorphanol	62	Halcinonide	103	Pyrilamine
22	Caffeine	63	Hydrochlorothiazide	104	Ranitidine
23	Capsaicine	64	Hydroflumethiazide	105	Sildenafil
24	Carbetapentane	65	Hydroxy Lidocaine	106	Sildenafil hydroxy
25	Chlorothiazide	66	Hydroxy Meloxicam	107	Sulindac
26	Chlorpheniramine	67	Hydroxy Piroxicam	108	Tenoxicam
27	Chlorpromazine	68	Hydroxy Tenoxicam	109	Tetracaine
28	Chlorthalidone	69	OH-Triamcinolone Aceto.	110	Tetrahydrogestrinone
29	Cimetidine	70	Imipramine	111	Tetramisole
30	Clenbuterol	71	Indapamide	112	Theobromine
31	Clobetasol	72	Isoflupredone	113	Theophylline
32	Cortisol	73	Ketamine	114	Timolol
33	Cortisol d3	74	Ketoprofen	115	Tixocortol pivalate
34	Cortivazol	75	Ketorolac	116	Tramadol
35	Cyclothiazide	76	Lidocaine	117	Triamcinolone
36	Dantrolene	77	Meloxicam	118	Triamcinolone acetonide
37	Dantrolene hydroxy	78	Mepivacaine	119	Triamcinolone hexacetonide
38	Desonide	79	Meprednisone	120	Trichlormethiazide
39	Desoximethasone	80	Methyl phenidate	121	Tripelennamine
40	Dexamethasone	81	Metocarbamol	122	Xipamide
41	Diazepam	82	Morphine	123	Xylazine



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Summary Report

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Sample Name: Laboratory:
Acquistion Start Time: août 04,2009 15:26:47
Screening Conditions: Based on accurate mass scans. Exact mass window (ppm): 5, RT window(min): 0.50.

Peak Number	Compound Name	Expected m/z	Detected m/z	Delta (mDa)	Delta (ppm)	Expected RT	Actual RT	Intensity
1	Dexamethasone Neg	437.19701	437.19907	2.1	4.7	16.04	15.96	52002
2	Flumethasone Neg	455.18759	455.18982	2.2	4.9	16.27	16.20	65542
3	Triamcinolone Aceto.	479.20757	479.20956	2.0	4.2	17.22	17.14	131328
4	Dexamethasone Pos	393.20718	393.20673	-0.5	-1.2	16.04	15.95	43018
5	Flumethasone Pos	411.19776	411.19714	-0.6	-1.5	16.28	16.19	36204
6	Triamcinolone Pos	395.18644	395.18570	-0.7	-1.9	9.67	9.57	25986
7	Triamcinolone Aceto. Pos	435.21774	435.21698	-0.8	-1.7	17.21	17.13	87591

Figure 4: ToxID report – short summary style

Conclusion

The Exactive high performance LC/MS demonstrates high resolving power (up to 100,000) and precise mass accuracy for easy, routine analysis and data re-interrogation of urine samples for illicit substances in equine doping control analysis.

Application Note: 489b

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Quantitation of 12 Benzodiazepines and Metabolites in Urine Using Ultrahigh Resolution LC-MS for Forensic Toxicology Use

Kent Johnson, Fortes Laboratories, Portland, OR; Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

- Exactive
- Accela HPLC
- Pain Management
- Forensic Toxicology

Goal

To demonstrate the quantitation of 12 benzodiazepines in urine using a liquid chromatography-mass spectrometry (LC-MS) method and ultrahigh resolution with the Thermo Scientific Exactive benchtop mass spectrometer for forensic analysis.

Experimental

Standards and Samples Preparation

Calibration standards were prepared by spiking blank urine with 12 benzodiazepines (lorazepam, nordiazepam, oxazepam, temazepam, hydroxytriazolam, 7-aminoclonazepam, 7-aminonitrazepam, hydroxyalprazolam, 7-aminoflunitrazepam, desalkylflurazepam, diazepam, and 2-hydroxyethylflurazepam) to final concentrations ranging from 10 ng/mL to 2,000 ng/mL.

Calibration standards and urine samples were spiked with internal standards (10 deuterated benzodiazepines), hydrolyzed and processed using a solid phase extraction (SPE) procedure.

Third party QC samples containing 6 benzodiazepines were processed and analyzed to obtain method accuracy and precision.

HPLC

HPLC analysis was performed using a Thermo Scientific Accela liquid chromatography system with a Thermo Scientific Hypersil GOLD PFP column (50 x 2.1 mm; 5 µm). A processed sample of 5 µL was analyzed with a 6-minute gradient method.

Mass Spectrometry

MS analysis was carried out on an Exactive™ benchtop LC-MS instrument with an electrospray ionization (ESI) source. Full scan data with resolution of 100,000 (FWHM) was acquired.

Results and Discussion

Figure 1 displays 6 of the 12 selected benzodiazepines at 10 ng/mL and internal standards. Chromatograms for compound detection and quantitation are reconstructed with a mass tolerance of 5 ppm.

Figure 2 shows the calibration curve for this set. Data results for the other six benzodiazepines are available upon request.

Conclusion

The Exactive benchtop LC-MS instrument provides excellent quantitative analysis of 12 benzodiazepines, from 10 ng/mL to 5000 ng/mL in urine, using ultrahigh resolution full scan data acquisition in a 6-minute method. The accuracy, precision, LOQ, and linearity range of the method meet the demands of today's forensic toxicology laboratories.

Method Performance Summary

Target Analytes	Benzodiazepines
Matrix	Urine
Limit of Quantitation (LOQ)	10 ng/mL
Recovery	> 85%
Assay Linearity	10 ng/mL – 5000 ng/mL
Precision (%CV)	< 4%
Carryover at Lower Limit of Quantitation (LLOQ)	< 1%
Sample Volume	2 mL
Analysis Time	6 minutes

Analyte	Mean Conc.(ng/mL)	% Recovery	%RSD
Oxazepam	248	99.3	1
Nordiazepam	234	93.5	1.4
Temazepam	218	87.1	4
Desalkylflurazepam	214	85.7	4
Lorazepam	227	90.8	0.4
Hydroxyalprazolam	255	102	0.4

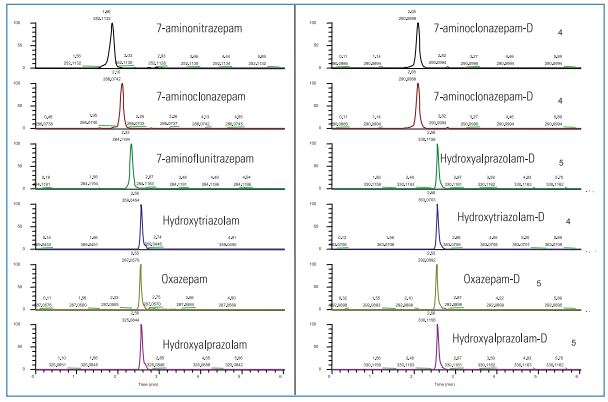

Figure 1: Chromatograms of 6 of the 12 selected benzodiazepines at 10 ng/mL and internal standards.

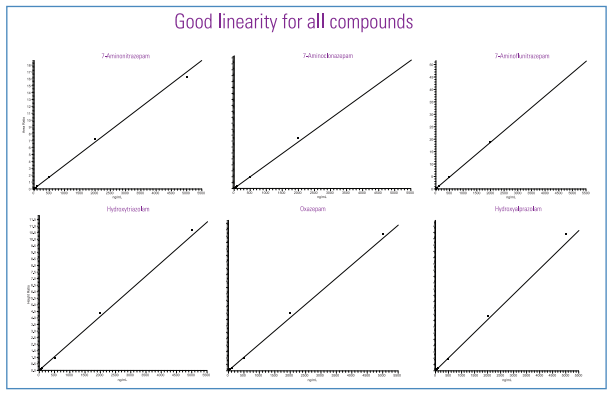

Figure 2: Calibration curves (10-5000 ng/mL) for all analytes

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Application Note: 488b

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Quantitation of Urinary Ethyl Glucuronide and Ethyl Sulfate Using Ultrahigh Resolution LC-MS

Forensic Toxicology Use Only

Kent Johnson, Fortes Laboratories, Portland, OR; Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

- Exactive
- Accela HPLC
- EtG / EtS
- Pain Management
- Forensic Toxicology

Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are sensitive and specific urinary biomarkers of recent alcohol intake that are of great interest in today's forensic toxicology laboratories.

Goal

To demonstrate the quantitation of EtG and EtS in urine using a liquid chromatography-mass spectrometry (LC-MS) method with ultrahigh resolution on the Thermo Scientific Exactive benchtop mass spectrometer.

Experimental

Calibration Standards and Sample Preparation

Calibration standards were prepared by spiking blank urine with EtG and EtS to final concentrations ranging from 25 ng/mL to 20,000 ng/mL.

Calibration standards and urine samples were spiked with internal standards (EtG-d5 and EtS-d5) and diluted 10 times with an LC mobile phase prior to injection onto the analytical column.

Commercial QC samples were used to obtain method accuracy and precision.

HPLC

HPLC analysis was performed using a Thermo Scientific Accela liquid chromatography system with a Thermo Scientific Hypersil GOLD C18 column (50 x 2.1 mm; 5 µm). A diluted sample of 20 µL was analyzed with a 6-minute gradient method.

Mass Spectrometry

MS analysis was carried out on the Exactive™ benchtop LC-MS instrument equipped with an electrospray ionization (ESI) source. Full scan data with resolution of 100,000 FWHM at *m/z* 200 was acquired.

Results and Discussion

Figure 1 shows the linear calibration curves for EtG (100-20,000 ng/mL) and EtS (100-20,000 ng/mL).

Figure 2 shows chromatograms of EtG and EtS at 25 ng/mL and the respective deuterated internal standards. Chromatograms for compound detection and quantitation are reconstructed with a mass tolerance of 5 ppm.

Conclusion

The Exactive benchtop LC-MS instrument provides excellent quantitative analysis of EtG and EtS in a 6-minute method. When applied to real samples, the method meets the demands of today's forensic toxicology laboratories with exceptional performance.

Method Performance Summary

Target Analytes	Ethyl glucuronide	Ethyl sulfate
Matrix	Urine	Urine
LOD	25 ng/mL	25 ng/mL or less
LOQ	100 ng/mL	100 ng/mL
Recovery	> 85%	> 85%
Precision	< 15%	< 15%
Assay Linearity	100 – 20,000 ng/mL	100 – 20,000 ng/mL
Carryover at LLOQ	< 1%	< 1%
Sample Volume	100 µL	100 µL
Analysis Time	6 minutes	6 minutes

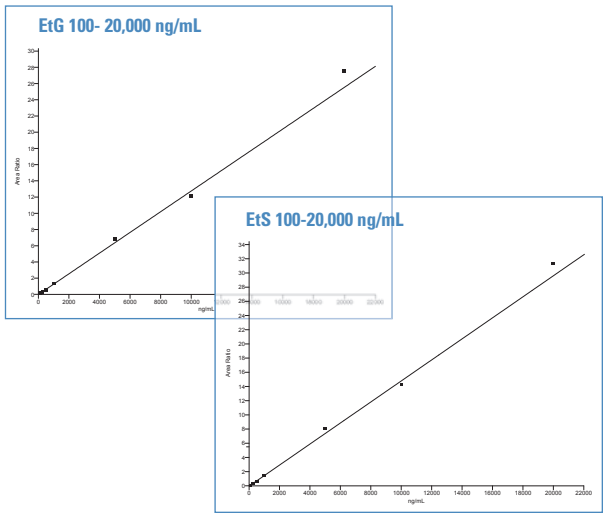

Figure 1: Linear calibration curves for EtG (100-20,000 ng/mL) and EtS (100-20,000 ng/mL).

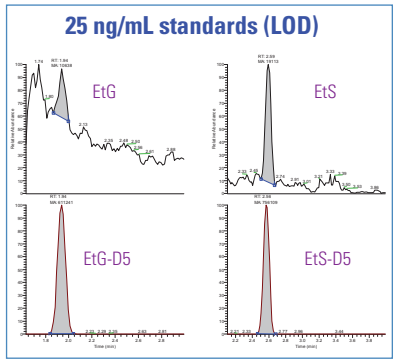

Figure 2: LOD chromatograms of EtG and EtS at 25 ng/mL with deuterated internal standards.

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Application Note: 486b

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Simultaneous Analysis of Opiates and Benzodiazepines in Urine in Under 3 Minutes per Sample Using LC-MS/MS

Forensic Toxicology Use Only

Christopher L. Esposito, Matthew Berube, Francois Espourteille, Thermo Fisher Scientific, Franklin, MA

Key Words

- Transcend TLX-2 system
- TSQ Quantum Access Max
- Forensic Toxicology
- H-ESI
- SRM

Introduction

A two-channel liquid chromatography separation method has been developed for the simultaneous analysis of opiates and benzodiazepines in urine for forensic use. A Thermo Scientific Transcend TLX-2 system powered by multiplexing and automated online sample preparation technology was used to run two LC-MS/MS methods, one for each class of compounds. The multiplexing technology and data windowing of the system increase throughput with minimal operator intervention.

Experimental Conditions

Sample Preparation

Urine samples were spiked with a deuterated internal standard mix. Opiate samples were acidified to hydrolyze the metabolites, and then all samples were centrifuged.

HPLC

HPLC analysis was performed using the Transcend™ TLX-2 system. Samples were separated from the matrix using Thermo Scientific TurboFlow Cyclone-P polymer columns. Chromatographic separation was performed using a Thermo Scientific Hypersil GOLD C18 column (50 x 3 mm; 5 ! m) for benzodiazepines and a Hypersil GOLD™ PFP column (100 x 3 mm; 3 ! m) for opiates.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer with a heated electrospray ionization source (H-ESI). The selective reaction monitoring (SRM) mode was used for mass spectrometry detection.

Results and Discussion

The analysis of directly-injected urine is accomplished for both drug classes. Seven benzodiazepines and internal standards and seven opiates and internal standards were analyzed. Figures 1 and 2 display data-windowed runs for selected benzodiazepines and opiates, respectively. Table 1 provides calibration curve statistics for several benzodiazepines and opiates.

Conclusion

The Transcend TLX-2 system with its unique multiplexing technology successfully runs two totally independent channels for forensic use. Limits of detection were 1 ng/mL (25 ng/mL for morphine). Quantitative analysis ranges were 5-5000 ng/mL for benzodiazepines and 50-25,000 ng/mL for opiates. Multiplexing both channels for analysis of benzodiazepines and opiates produces very significant time savings. The total MS data collection run times are efficiently reduced to less than 3 minutes per sample, inclusive of online sample preparation, thus resulting in more than 50% time savings versus running the analyses separately.

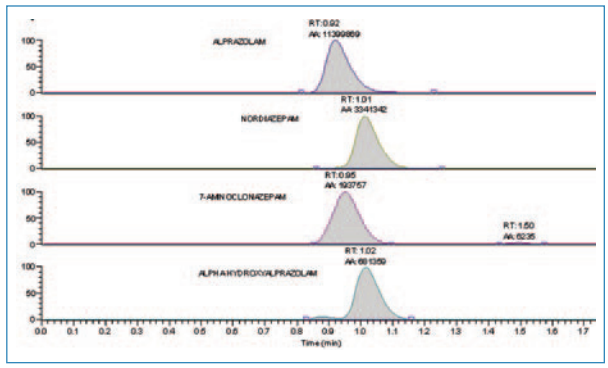

Figure 1: Data-windowed run for selected benzodiazepines

Assay performance summary

Target Analytes	Benzodiazepines	Opiates
Matrix	Urine	Urine
LOD	1 ng/mL	1 ng/mL (25 ng/mL morphine)
LOQ	5 ng/mL	50 ng/mL
Assay Linearity	1 ng/mL – 5 µg/mL	1 ng/mL – 25 µg/mL
Precision (%CV)	±15% (20% at LLOQ)	±15% (20% at LLOQ)
Sample Volume	10 µL	20 µL
Analysis Time	5.5 minutes, with a 2.5 minute data collection window	7 minutes, with a 3 minute data collection window

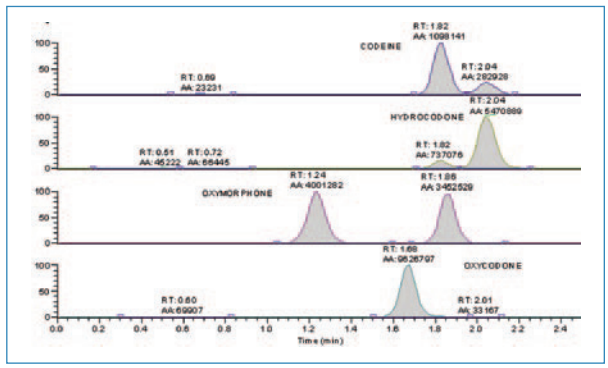

Figure 2: Data-windowed run for selected opiates

Table 1: Calibration curve statistics of 4 analytes

Analyte	R^2 (1/x weighing)	Range (ng/mL)	LOD (ng/mL)
Nordiazepam	0.9900	5-5000	1
Clonazepam	0.9960	5-5000	1
Oxymorphone	0.9903	50-25000	1
Hydromorphone	0.9950	50-25000	1

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Screening Drugs and Toxic Compounds with LC-MS/MS: An Alternative to LC-UV for Research Toxicology Labs

Jordan Velardo[1], Monique Manchon[1], Bénédicte Duretz[2], Dennis Nagtalon[3], Marta Kozak[3];
[1]Laboratory of Toxicology, Lyon Sud Hospital, Pierre-Bénite, France; [2]Thermo Fisher Scientific, Les Ulis, France; [3]Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- ToxSpec Analyzer
- ToxID software
- LXQ Linear Ion Trap
- Accela UHPLC System

Introduction

Screening for drugs of abuse and other toxic compounds in biological samples has quickly become a routine assay conducted in many research toxicology laboratories. The main challenge is to get rapid and accurate results amidst the generally large number of potential analytes to be identified within complex biological matrices. One of the techniques widely used in this area is high pressure liquid chromatography (HPLC) combined with photo diode array detection (DAD) or ultra-violet (UV) detection. The most popular LC-UV platform has been the Bio-Rad® REMEDi™ HS drug profiling system. When this platform was recently discontinued, a significant technological gap became apparent. Now this gap is rapidly being filled by newer, more effective high pressure liquid chromatography - mass spectrometry (HPLC-MS) technologies.

Here we present the workflow and results obtained by using the Thermo Scientific ToxSpec Analyzer, a new UHPLC-MS system based on ultra-high pressure liquid chromatography and linear ion trap mass spectrometry technology.

Goal

Evaluate an LC-MS/MS method for screening and semi-quantitation of drugs and toxic compounds in serum and urine matrices to determine if this approach can provide an alternative to REMEDi technology for research toxicology.

Experimental

The ToxSpec™ Analyzer combines hardware, software, and screening methodologies designed to significantly simplify and improve the screening assay workflow. LC-MS² data is acquired by using a pre-configured instrument method, and the data is automatically processed, post-acquisition, by Thermo Scientific ToxID automated drug screening software.

The LC-MS screening was performed on Thermo Scientific instrumentation including an LXQ™ linear ion trap mass spectrometer coupled to an Accela™ UHPLC system using a polarity-switching and scan-dependent MS/MS experiment (Figure 1). The MS² spectra generated were processed through ToxID™ software. Using a novel screening algorithm, the software program identifies target analytes through a MS² library search against a large spectral library of known analytes as well as expected retention times. Semi-quantitative data results can also be generated concurrently from the MS² spectral intensity ratios between the target analyte and the corresponding internal standard.

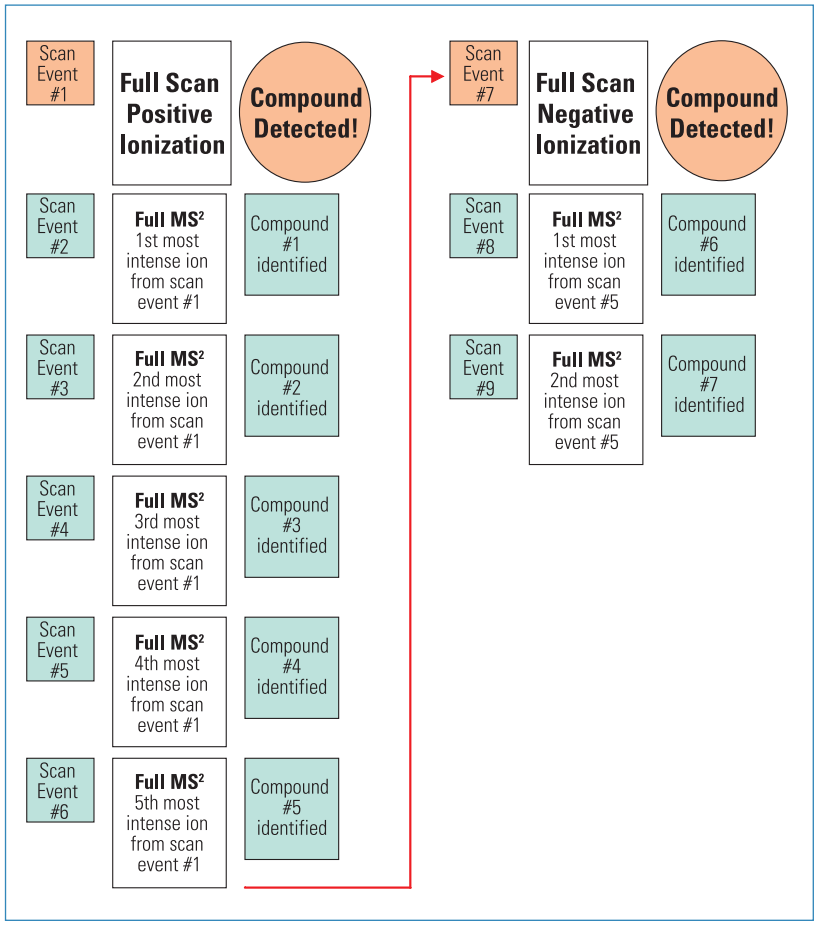

Figure 1: Polarity-switching and scan-dependent MS/MS experiment

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The ToxSpec Analyzer includes a diverse and easily-expandable MS/MS library of 300 compounds that it screens using a single pre-configured method. In our laboratory, we have expanded the library by more than 50 entries to date.

Sample preparation

The extraction procedure was performed by using liquid/liquid extraction (LLE) with Toxi-Tube A® (Varian, les Ulis, France). Details of the procedure are described below.

- Vortex the Toxi-Tube A for 10 seconds.
- Add 1 mL of serum or urine into the Toxi-Tube A.
- Add 200 µL of a solution of internal standard [haloperidol-d4, chlorpromazine-d3, and prazepam-d5 at the following concentrations: 100 ng/mL, 1 µg/mL and 100 ng/mL, respectively, in 70/30 of A/B (A: water containing 10 mM ammonium acetate and 0.1% formic acid; B: acetonitrile containing 0.1% formic acid)].
- Add 5 mL of water.
- Vortex for 10 seconds.
- Mix for 5 minutes.
- Centrifuge for 5 minutes at 2700 rpm.
- Transfer the upper layer to a tube and evaporate to dryness at 40 °C.
- Reconstitute the sample in 200 µL of 70/30 of A/B.

HPLC Conditions

Chromatographic analyses were performed using the Thermo Scientific Accela UHPLC system. The chromatographic conditions were as follows:

Column:	Thermo Scientific Hypersil GOLD PFP 5 µm, 150 x 2.1 mm		
Flow rate:	0.2 mL/min		
Mobile phase:	A: water containing 10 mM ammonium acetate and 0.1% formic acid; B: acetonitrile containing 0.1% formic acid		
Injection volume:	10 µL		
Gradient:	T (min)	A (%)	B (%)
	0.0	95	5
	5.0	55	45
	18.0	30	70
	20.0	5	95
	27.0	5	95
	27.1	95	5
	32.0	95	5

Mass Spectrometry Conditions

MS analysis was carried out on a our LXQ linear ion trap mass spectrometer with an electrospray ionization (ESI) source. The MS conditions were as follows:

Ion polarity:	Polarity-switching scan-dependent experiment
Spray voltage:	5000 V
Sheath gas (N_2) pressure:	30 (arbitrary units)
Auxiliary gas (N_2) pressure:	8 (arbitrary units)
Capillary temperature:	275 °C
Microscan:	1
Wideband Activation™:	Activated
Stepped normalized collision energy:	35% ± 10%

Results and Discussion

More than 150 real laboratory samples (serum and urine) have been analyzed. Table 1 reports some of the data obtained from both the REMEDi HS LC/UV system and the ToxSpec Analyzer UHPLC/MS system. Among the 12 samples reported here, 22 compounds have been identified using both the REMEDi HS and the ToxSpec Analyzer. Notably however, the ToxSpec Analyzer system identified 24 additional compounds that were not detected with the REMEDi HS due in most cases to a lack of sensitivity, specificity, and coelution capability.

The ToxSpec Analyzer also provided a better response for some classes of compounds, like benzodiazepines. With the REMEDi HS system, the retention time for this class of compounds was close to the dead volume of the column. For that reason, the signals that interfered with matrix components were rather difficult to identify. It was also observed that haloperidol (sample #5) and paroxetine (sample #10) gave a much better signal with the ToxSpec Analyzer.

Sample #	Compounds identified using ToxSpec Analyzer	Compounds identified using REMEDi HS
1	Acetaminophen	**Not detected**
	Nortriptyline	**Not detected**
	Amitriptyline	Amitriptyline
	Oxazepam	**Not detected**
2	Nordiazepam	Nordiazepam
	Alprazolam	**Not detected**
	Cyamemazine	Cyamemazine
3	Acetaminophen	**Not detected**
	Nordiazepam	Nordiazepam
	Venlafaxine	Venlafaxine
	Oxazepam	Oxazepam
	Alprazolam	**Not detected**
4	Nordiazepam	**Not detected**
	Diazepam	Diazepam
	Oxazepam	**Not detected**
	Temazepam	**Not detected**
	Levomepromazine	Levomepromazine
	Zopiclone	Zopiclone
5	Oxazepam	**Not detected**
	Clomipramine	Clomipramine
	Quinidine	Quinine
	Haloperidol	**Not detected**
	Clonazepam	**Not detected**
6	Acetaminophen	**Not detected**
	Bisoprolol	Bisoprolol
7	Venlafaxine	Venlafaxine
	Risperidone	**Not detected**
8	Quinine	Quinine
	Hydromorphone	Hydromorphone
	Morphine	Morphine
9	Lidocaine	**Not detected**
	Nortriptyline	**Not detected**
	Mirtazapine	**Not detected**
	Amitriptyline	Amitriptyline
	Cyamemazine	Cyamemazine
	Levomepromazine	Levomepromazine
	Zopiclone	**Not detected**
10	Bromazepam	Bromazepam
	Paroxetine	**Not detected**
11	Sertraline	**Not detected**
	Hydrocortisone	**Not detected**
12	Acetaminophen	**Not detected**
	Alprazolam	Alprazolam
	Prednisolone	**Not detected**
	Hydroxyzine	Hydroxyzine
	Fexofenadine	**Not detected**
TOTAL	46 Molecules	22 molecules

Table 1: List of psychoactive molecules identified in real laboratory samples using the ToxSpec Analyzer compared to the REMEDi HS system

Our aim was to quickly and confidently identify toxic compounds in the samples by spectral library searching while performing a semi-quantification calculation for identified compounds. To perform the semi-quantification, a response factor that correlated the intensity of the MS^2 spectra to a concentration was calculated for each molecule present in the library using internal standards. The semi-quantification result was automatically calculated using ToxID software. An example of the automatically-generated report can be seen in Figure 2. The report includes a list of compounds identified in a real laboratory sample and their respective calculated concentrations.

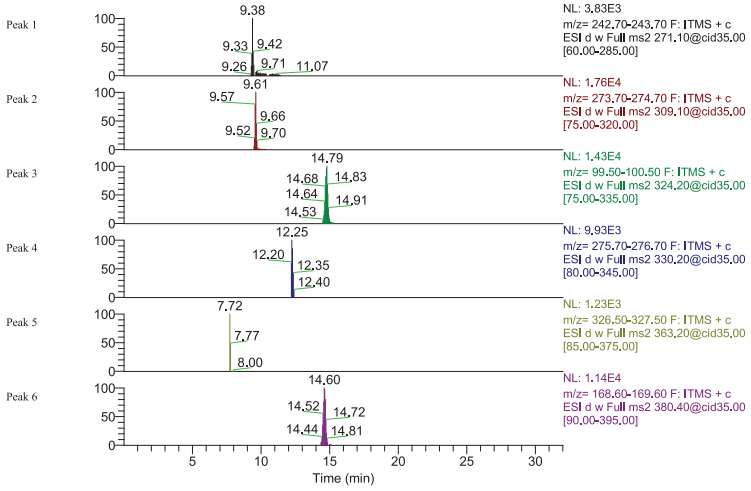
One important aspect of this method is the ability to reprocess data retrospectively from the MS spectra. The ToxID report is based on MS^2 spectra library searching. This means that if the entry corresponding to the compound is not currently available in the library, ToxID will not be able to identify the analyte. However, as data are acquired in MS mode, it is possible to reprocess the MS trace and check that all major ions have been identified by ToxID. If not, it is then possible to re-inject the sample and perform MS^2 acquisition on specific ions.

Conclusion

The ToxSpec Analyzer is a good replacement for the REMEDi HS system in research toxicology laboratories because it offers increased sensitivity, greater specificity, and lower cost-per sample analysis.

Centre Hospitalier Lyon Sud
Summary Report

Raw File Name: C:\Xcalibur\Validation REMEDI\Data\Real samples\090107\375489.RAW
Config File Name: C:\Xcalibur\Validation REMEDI\CSV fi...\LXQ_GUS_config_30min_TOXID Semi-quanti.csv
Sample Name: Laboratory: Laboratoire de Toxicologie
Acquistion Start Time: 1/7/2009 1:23:18 PM
Screening Conditions: Based on Full MS2 scans. m/z window (amu): 0.50, RT window (min): 2.00, MS2 Search libraries: Tox_Library_LXQ,Use full MS scan to confirm.



Peak Number	Compound Name	Code	SI	RSI	m/z	Expected RT	Actual RT	Concentration mg/l	Library Name
1	Nordiazepam	p	700	708	271.10	8.60	9.38	1.05	Tox_Library_LXQ
2	Alprazolam	p	811	814	309.10	9.10	9.61	0.84	Tox_Library_LXQ
3	Cyamemazine	p	818	819	324.20	15.00	14.79	0.25	Tox_Library_LXQ
4	Prazepam-D5	i	890	894	330.20	11.70	12.25	0.87	Tox_Library_LXQ
5	Hydrocortisone	p	863	865	363.20	7.50	7.72	0.11	Tox_Library_LXQ
6	Haloperidol-D4	i	845	861	380.40	14.90	14.60	1.00	Tox_Library_LXQ

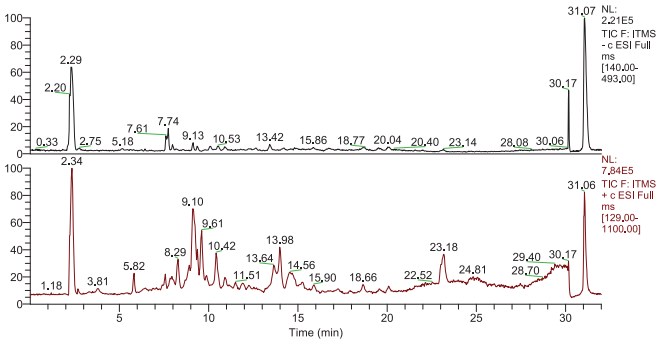


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Figure 2: ToxID report – short summary style

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Forensic Analysis of Opiates in Whole Blood by LC-MS/MS Using Automated, Online Sample Preparation

Peter Ashton, Alex Allan, Bob Ardrey, Triple A Forensics Ltd., Oldham, UK
Shane McDonnell, Sarah Robinson, Thermo Fisher Scientific, Hemel Hempstead, UK

Key Words

- Transcend TLX-1
- TurboFlow Technology
- TSQ Quantum Ultra
- Forensic Toxicology

Introduction

Forensic use of free- and protein-bound opiate analytes in whole blood by LC-MS/MS traditionally requires rigorous sample cleanup via solid phase extraction (SPE) or liquid-liquid extraction (LLE). The method described here can be used in place of these laborious offline sample preparation methods.

Goal

The goal is to quantitate opiate compounds in whole blood by using a simple, fast, low-volume protein precipitation step followed by a Thermo Scientific TurboFlow method coupling automated, online sample preparation and chromatography with selective reaction monitoring (SRM) tandem mass spectrometry.

Experimental

Sample Preparation

Horse blood was spiked with a mixture of opiates [codeine, morphine, 6-monoacetyl morphine (6-MAM), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), and d6-codeine (internal standard)] at concentrations ranging from 1 ng/mL to 500 ng/mL. A 150 µL sample of spiked whole blood was mixed with 200 µL acetonitrile, vortexed, and centrifuged for 10 minutes at 300 rpm. For analysis, 10 µL of supernatant was used.

HPLC

HPLC analysis was performed using the Thermo Scientific Transcend TLX-1 system. Whole blood supernatant samples were extracted using a TurboFlow™ Cyclone MAX column (0.5 x 50 mm). Chromatographic separation was performed using a Thermo Scientific Hypersil GOLD aQ column (50 x 2.1 mm, 5 µm).

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer with a heated electrospray ionization (H-ESI) source. The SRM mode was used for mass spectrometry detection.

Results and Discussion

The extracted ion chromatograms of the lowest concentration sample are presented in Figure 1. The calibration curves for morphine (Figure 2), codeine and M3G/M6G covered 10–500 ng/mL and the curve for the 6-MAM metabolite covered 1–50 ng/mL. All calibration curves were linear over the concentration range, and carryover was calculated at less than 1% for all analytes.

Conclusion

The use of a simple, rapid work-up followed by a TurboFlow method on the Transcend™ TLX-1 system followed by MS/MS analysis allowed the specific and sensitive analysis of various common opiates and their metabolites from a small volume of whole blood. The 4 minute method allows 15 samples per hour to be completed, and the throughput can be doubled or quadrupled with the use of multiplexing. Significant time is saved with the absence of SPE or LLE sample preparation.

The forensic toxicologist can use this method to assist with the determination of time of heroin injection (presence of 6-MAM) and the detection of M3G and M6G to determine prior use or accumulation following heavy use of opiates.

Assay performance summary

Target Analytes	codeine, morphine, 6-MAM, M3G, M6G
Matrix	whole blood
Assay Linearity	1 - 50 ng/mL (6-MAM) 10 - 500 ng/mL (all other analytes))
Carryover at LLOQ	< 1% for all analytes
Sample Volume	10 µL
Analysis Time	~ 4 minutes

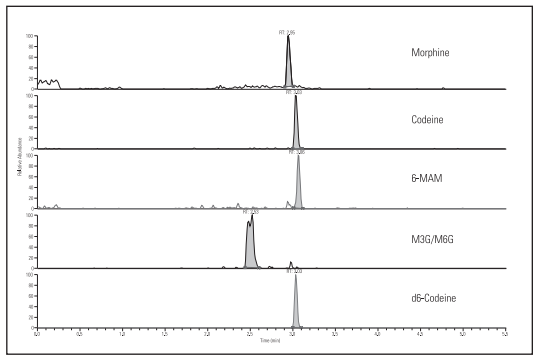

Figure 1: Extracted ion chromatogram for the lowest standard of each analyte

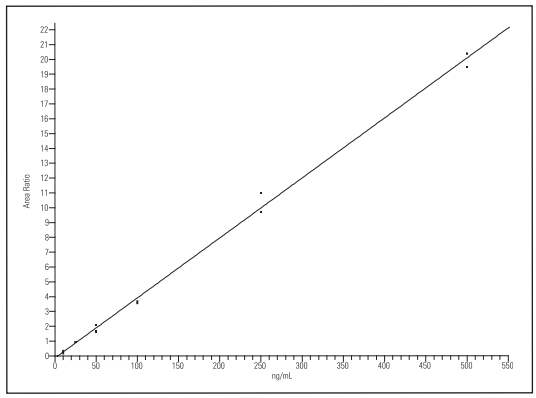

Figure 2: Calibration curve for the analyte morphine from 10–500 ng/mL

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Rapid Analysis of Opiates from Low Volume Whole Blood Samples by LC-MS/MS Utilizing TurboFlow Methods

Peter Ashton, Alex Allan, Bob Ardrey, Triple A Forensics Ltd., Oldham, UK
Shane McDonnell, Sarah Robinson, Thermo Fisher Scientific, Hemel Hempstead, UK

Key Words

- Transcend TLX-1
- TurboFlow Technology
- TSQ Quantum Ultra
- Whole Blood
- Opiates

Introduction

The opiate morphine, and its derivatives, are medicines often used for pain-relief, cough-relief and as anti-diarrhoeals. For example, codeine and dihydrocodeine (morphine derivatives) are available in over-the-counter preparations in combination with paracetamol (acetaminophen) and are slowly metabolized to morphine and dihydromorphine respectively. However, the semi-synthetic opiate diacetylmorphine (heroin) is subject to wide abuse and has become such a major social problem that it is responsible for almost half of the drug-related deaths in the UK.[1]

Heroin is deacetylated very rapidly (half-life ca. 3 mins in plasma) to its major active metabolite 6-monoacetylmorphine (6-MAM), which readily penetrates the blood-brain barrier to produce the desired euphoric effects.[2] 6-MAM also has a short plasma half-life of about 38 minutes (producing morphine), and thus, its detection in blood is very important to the forensic toxicologist in establishing the recent use of heroin.[3] As a product of heroin metabolism, via 6-MAM, or from its own administration, morphine also undergoes further metabolism. The conjugation step produces inactive morphine-3-glucuronide (M3G) and the potently active morphine-6-glucuronide (M6G) along with other minor ones, including diglucuronides.

The forensic toxicologist is often asked to interpret results and possibly account for time of death in opiate (especially heroin) abuse cases. This task can be made easier if it is possible to identify and quantify the components such as 6-MAM, morphine, codeine, dihydrocodeine and the glucuronides in whole blood rather than urine. The volume of a human whole blood sample, however, may often only be available in the low microlitre range, thus presenting sample preparation and analysis sensitivity issues.

The analysis of free- and protein-bound opiate analytes in human whole blood by LC-MS/MS is routinely done after rigorous sample cleanup via solid phase extraction or liquid-liquid extraction in order to minimize ion suppression in the ionization source of the mass spectrometer. These clean up steps can be lengthy, laborious and expensive. Here we present a method to quantitatively analyze opiate compounds present in whole blood utilizing a simple, fast, low-volume extraction procedure followed by a Thermo Scientific TurboFlow method, an online extraction and chromatography coupled with selected reaction monitoring tandem mass spectrometry.

Goal

To replace laborious off line sample preparation with TurboFlow™ methodology and tandem mass spectrometry for the analysis of opiates in acetonitrile extracts from low volume whole blood samples.

Experimental

Sample Preparation

Horse blood was spiked with a mixture of opiates (codeine, morphine, 6-MAM, M3G, M6G and d6-codeine) at concentrations ranging from 1 ng/mL to 500 ng/mL. 150 µL spiked whole blood was mixed with 200 µL acetonitrile and vortexed. The resulting sample was then centrifuged for 10 min at 300 rpm. The supernatant was placed into a 96-well microtitre plate and 10 µL of the supernatant was used for the analysis.

TurboFlow Methodology

Thermo Scientific Transcend TLX-1 system	
Column:	Thermo Scientific TurboFlow Cyclone MAX 0.5 x 50 mm
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.1% formic acid in acetonitrile
Mobile phase C:	10 mM ammonium bicarbonate pH 9
Mobile phase D:	10 mM ammonium acetate pH 6

Analytical LC

Column:	Thermo Scientific Hypersil GOLD aQ 50 x 2.1 mm, 1.9 µm
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.1% formic acid in acetonitrile

The eluent gradients for both pumps are shown in Table 1.

			TurboFlow Method								Analytical			
Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	00:00	30	1.50	Step	-	-	100	-	====	out	0.30	Step	100	0
2	00:30	60	0.20	Step	100	-	-	-	T	in	0.10	Step	100	0
3	01:30	60	1.50	Step	-	-	-	100	====	in	0.30	Ramp	5	95
4	02:30	120	1.50	Step	99	1	-	-	====	in	0.30	Step	5	95
5	04:30	60	1.50	Step	-	-	100	-	====	out	0.30	Step	100	0

Table 1: Thermo Scientific Aria operating software gradient programs for the Transcend™ TLX-1 system with TurboFlow method and analytical LC method. Flow rate is reported as mL/min.

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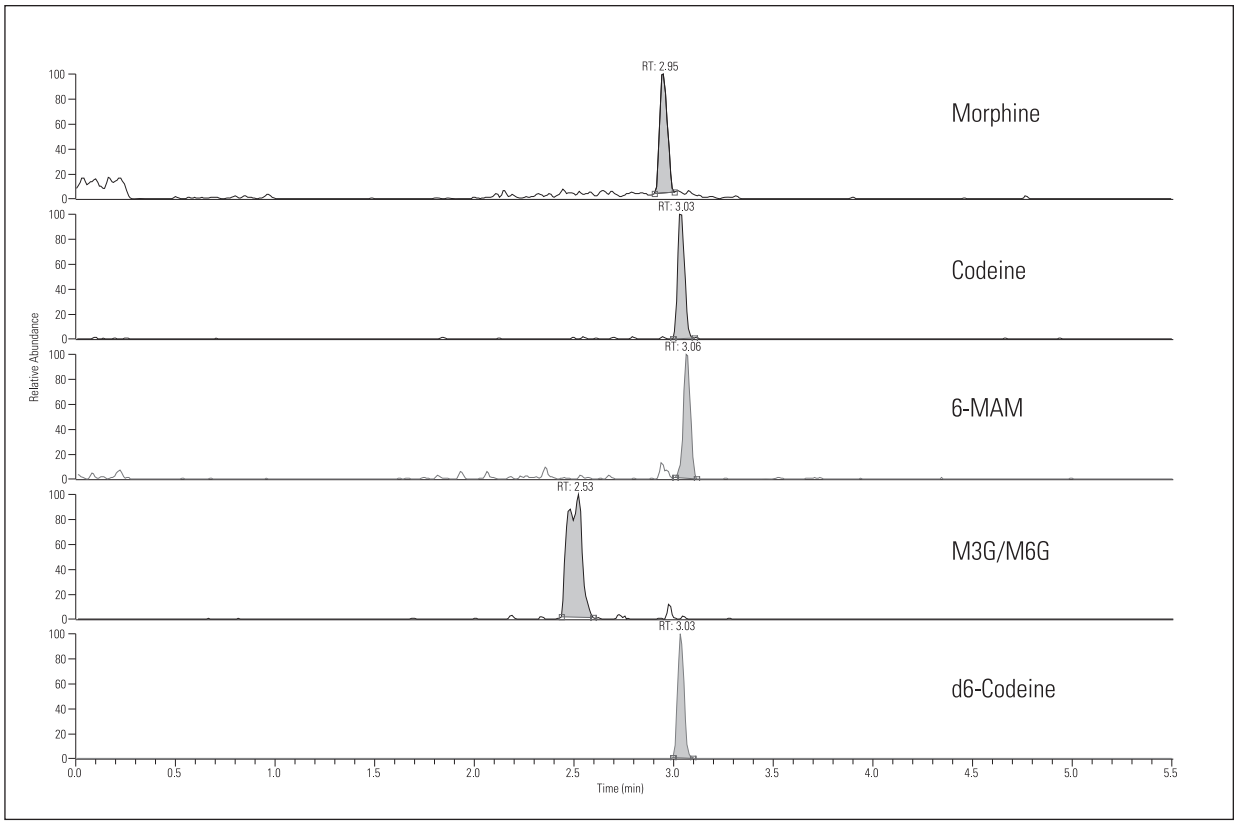

Figure 1: Extracted ion chromatogram for the lowest standard of each analyte

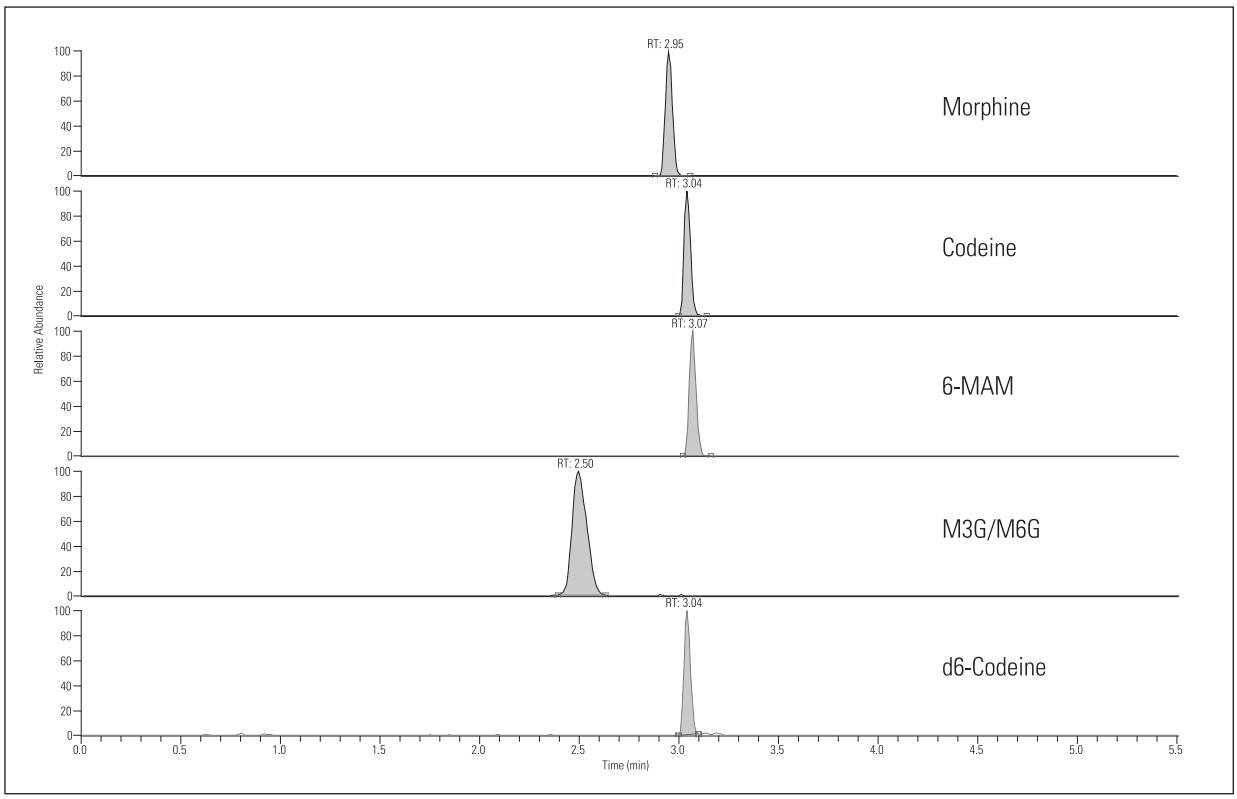

Figure 2: Extracted ion chromatogram for the highest standard of each analyte

Mass Spectrometry	
Thermo Scientific TSQ Quantum Ultra	
Ion Source & polarity:	HESI, positive ion mode
Spray Voltage:	4750 V
Vaporizer Temperature:	450 °C
Sheath Gas:	50 units
Ion Sweep Gas:	5 units
Auxillary Gas:	60 units
Capillary Temperature:	200 °C
Collision Gas Pressure:	1.5 mTorr

The SRM transitions used for this experiment are presented in Table 2.

Analyte	Parent	Product	Scan Time	Collision Energy	Tube Lens
Morphine	286.13	165 201	5 ms 5 ms	39 25	133 133
Codeine	300.14	165 215	5 ms 5 ms	38 26	148 148
6-MAM	328.13	165 211	5 ms 5 ms	38 25	145 145
M3G/M6G	462.16	286	5 ms	31	155

Table 2: SRM transitions monitored in the experiment

Results and Discussion

Prior to the analysis of spiked whole blood samples, opiate analytes were spiked into 100% acetonitrile and analyzed by the TurboFlow and LC-MS/MS method in order to demonstrate that the high organic content of the sample did not affect peak shape (peak splitting, etc.). The extracted, spiked whole blood samples were analyzed using the same TurboFlow method. Samples were run from low to high concentration with a solvent blank sample submitted after the highest concentration sample to calculate carryover. In all analyses, 10 µL of the extracted sample was injected and replicated to generate a calibration curve.

The extracted ion chromatograms of the lowest concentration sample and highest concentration sample are presented in Figures 1 and 2 respectively. The calibration curves for morphine, codeine and M3G/M6G covered 10–500 ng/mL (Figure 3, 4 and 6) and for the 6-MAM metabolite the curve covered 1–50 ng/mL (Figure 5). The isotopically labeled internal standard (d6-codeine) was spiked into each sample at 50 ng/mL. The concentration data for each analyte are provided as blood equivalents, i.e. the concentration in the blood before extraction. For example, 1 ng/mL blood equivalent was actual 0.43 ng/mL in the sample vial (150 µL diluted with 350 µL acetonitrile). Therefore, the equivalent on column amount of the lowest 6-MAM standard was 4.3 pg.

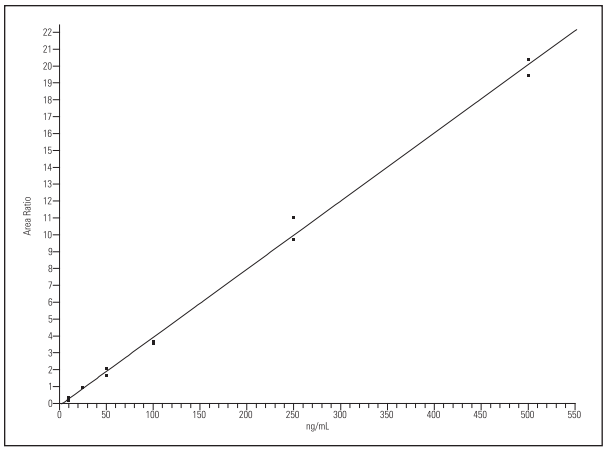

Figure 3: Calibration curve for the analyte morphine from 10–500 ng/mL

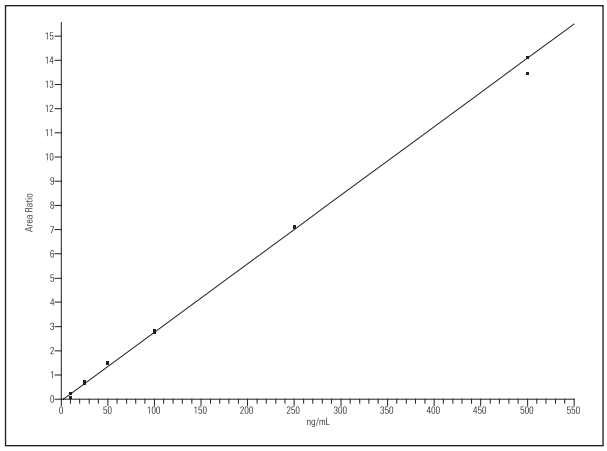

Figure 4: Calibration curve for the analyte codeine from 10–500 ng/mL

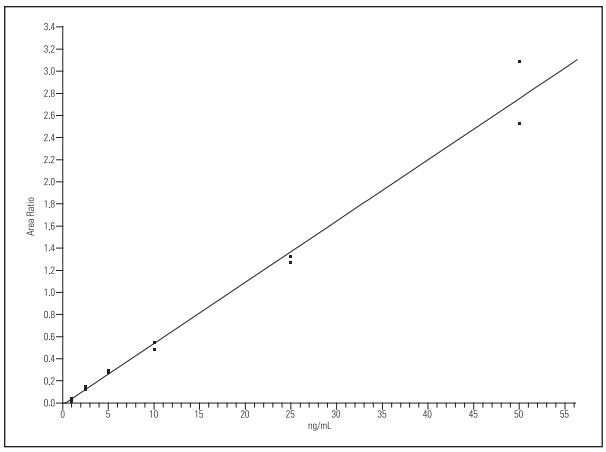

Figure 5: Calibration curve for the analyte 6-MAM from 1–50 ng/mL

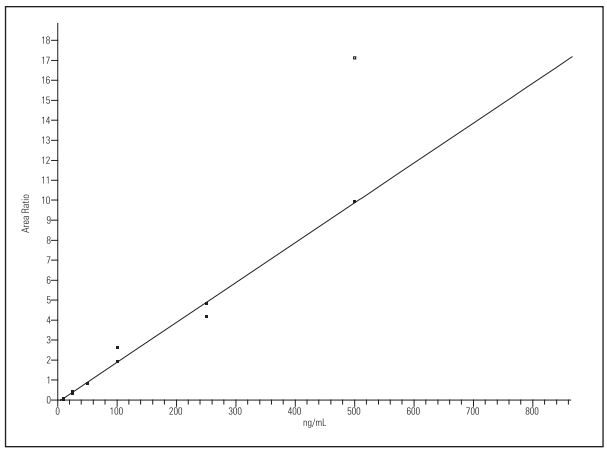

Figure 6: Calibration curve for the analyte M3G/M6G from 10–500 ng/mL

Conclusion

The use of a simple rapid acetonitrile work-up followed by a TurboFlow method (online extraction and chromatography) on the Thermo Scientific Transcend TLX-1 system with tandem MS/MS allowed the specific and sensitive analysis of various common opiates and their metabolites from a small volume of whole blood. Moreover, a limited portion of the acetonitrile extract volume was utilized in the analysis, thus, the method presents potential to scale down to a volume of blood achievable from a finger prick (5–10 µL). The calibration curves for all analytes analyzed were linear over the concentration range and carryover was calculated at less than 1% for all analytes. Since the method is ~ 4 minutes, 15 samples per hour may be completed, or indeed, doubled/quadrupled with the use of multiplexing. Significant time is saved in the absence of SPE sample preparation.

The method enables the forensic toxicologist to produce a full picture of the opiates and metabolites in blood to assist with the determination of time of injection (presence of 6-MAM) and the detection of M3G and M6G to determine prior use or accumulation following heavy use.

References and Acknowledgements

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2. Therapeutic Drugs, Second Edition, Ed C Dollery, Churchill & Livingstone, London, 1998
3. Disposition of Toxic Drugs and Chemicals in Man. 8th Edition, Ed Randall C Baselt. Biomedical Publications, Foster City, Ca, 2008

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Quantitation of Fentanyl and Norfentanyl from Urine Using On-line High Throughput System

Francois A. Espourteille, Ph. D., Thermo Fisher Scientific, Franklin, MA

Key Words

- TurboFlow Technology
- TSQ Quantum Access
- Clinical Research
- Aria TLX-4

Introduction

The use of the Thermo Scientific Aria TLX-4 system with TurboFlow™ methods for automated on-line sample cleanup of a biological sample is well documented in the literature[1]. The Aria™ TLX-4 system enhances the sensitivity, specificity, and precision for mass spectrometric detection of fentanyl and norfentanyl. Increasing demand in clinical research laboratories for higher sample throughput has put the emphasis on automated methods and platforms that have the ability to quickly ramp up throughput to meet demand.

The Aria TLX-4 system extracts both fentanyl and its metabolite, norfentanyl, from the many interferences found in urine and chromatographically separates them from each other, before sending them to the mass spectrometer. TurboFlow extraction methods exclude both high molecular weight species and salts while the stationary phase coating retains the analyte(s) through reverse phase column chemistry. This results in fast, efficient, on-line separation of fentanyl and its metabolite prior to introduction into the mass spectrometer.

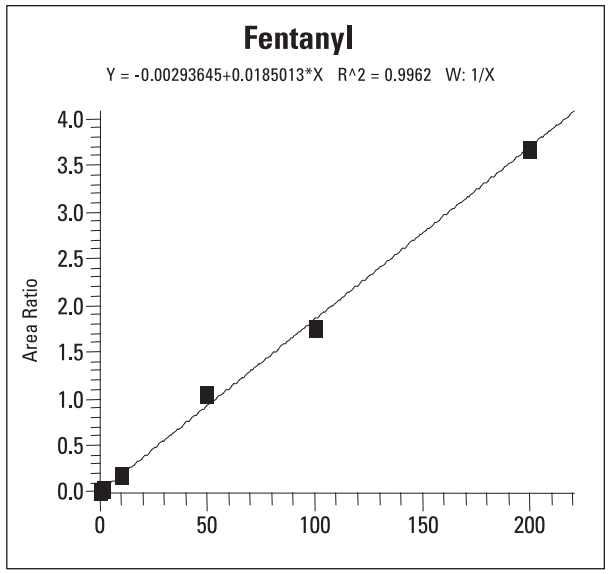

Figure 1: Calibration curves of Fentanyl from 4 channels of Aria TLX-4 System. Data courtesy of Dennis Crouch, Ameritox, LTD.

Goal

- Eliminate the need for SPE extraction of urine samples for fentanyl/norfentanyl assay
- Significantly increase sample throughput by running multiple samples simultaneously in front of one mass spectrometer
- Confirm the stability of the on-line assay

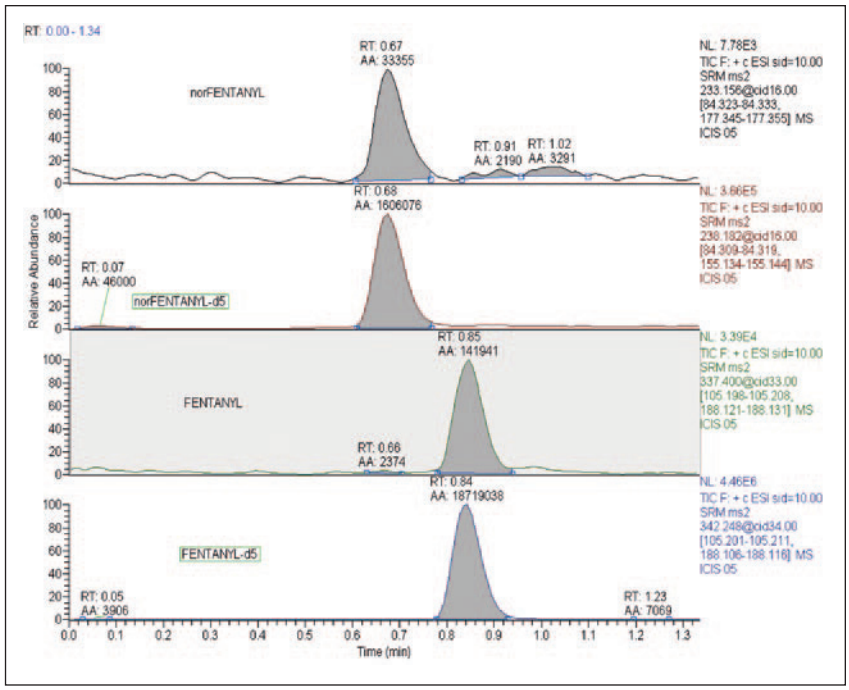

Figure 2: Excellent Signal/Noise at LOQ for (A) Norfentanyl and (B) Fentanyl at 0.5 ng/mL calibration. Data courtesy of Dennis Crouch, Ameritox, LTD.

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Methods

This method describes the analysis for the determination of fentanyl and its metabolite, norfentanyl, from a urine sample. Human urine was used as the test matrix. An LOQ of 0.5 ng/mL was seen in human urine, with an LOD below 0.1 ng/mL. Instrumentation used is identified in Table 1.

Table 1. Instrumentation used in this method

LS-MS/MS:	Aria TLX-4 with Thermo Scientific TSQ Quantum Access triple quadrupole mass spectrometer
Extraction column:	Thermo Scientific TurboFlow XL C18 P 0.5x50 mm
Analytical column:	Thermo Scientific Hypersyl GOLD aQ 3x50, 5 µm

Experimental Conditions:

A working solution containing fentanyl and norfentanyl at 1000 ng/mL was made. Subsequent dilutions yielded a curve from 200 ng/mL to 0.5 ng/mL. An internal standard solution containing both fentanyl-D5 and norfentanyl-D5 was added to all standards. Samples were vortexed and then centrifuged at 10,000 RCF for 5 minutes and analyzed immediately.

Results:

The data in Figure 1 shows linear regression for 0.5 ng/mL to 200 ng/mL, with 1/x weighing. Figure 2 demonstrates the limit of quantitation with excellent signal to noise ratio.

Conclusion:

The Aria TLX-4 system powered by TurboFlow technology provides a fast, efficient, and automated on-line separation technology for the extraction and analysis of fentanyl and its metabolite, norfentanyl. The ability to run 5.5 minute methods on four channels further decreases analysis time and increases the efficiency of the TSQ Quantum Access™ mass spectrometer. The Aria TLX-4 coupled with the TSQ Quantum Access can run one sample every 86 seconds with a 92.9% sample completion rate with 7.1% re-injection[2]. The method run time was 5.5 minutes. This system provides a reliable high throughput method of fentanyl and norfentanyl for clinical research labs.

References

1. Sauvage et al. **2006**. Therapeutic Drug Monitoring 28(1), pp. 123-130.
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A Complete Toxicology Screening Procedure for Drugs and Toxic Compounds in Urine and Plasma Using LC-MS/MS

Marta Kozak, Taha Rezai, Thermo Fisher Scientific, San Jose, CA

Key Words

- ToxSpec Analyzer
- ToxID Software
- LXQ Linear Ion Trap
- Clinical Toxicology
- General Unknown Screening

Introduction

Toxicology laboratories commonly use automated immunoassays, gas chromatography-mass spectrometry (GC-MS) and high pressure liquid chromatography-diode array detector (HPLC-DAD) techniques to perform toxicology screening analyses. None of these techniques are able to identify all the drugs and toxic compounds that are potentially present in a sample. Implementation of liquid chromatography-mass spectrometry (LC-MS) for toxicology screening provides specific and sensitive analysis of drugs and toxic substances. The benefits of the LC-MS/MS screening methodology include a simple sample preparation procedure, ease of adding new compounds to the screening method and fewer limitations based on compound volatility and thermal stability. In addition, Thermo Scientific ToxID automated toxicology screening software is able to automatically generate both Summary and Long Reports, avoiding the need for manual analysis of each sample chromatogram. This application note describes the use of the Thermo Scientific LXQ ion trap mass spectrometer equipped with an ESI source and HPLC for identification of unknown compounds in human urine and human plasma.

Goal

To develop a complete LC-MS/MS screening methodology which includes a sample preparation method, LC-MS method, spectra library, and data processing and reporting software.

Experimental Conditions

An MS/MS spectral library of 275 drugs and toxic compounds was created. Sample preparation of spiked human urine or human plasma was carried out using a solid-phase extraction (SPE) cartridge for basic, neutral and acidic compounds. A 13-minute LC method implementing a Perfluorophenyl (PFP) column was developed. Samples were analyzed using electrospray ionization (ESI) on an ion trap mass spectrometer in polarity switching scan dependent MS/MS experiments (see Figure 1), with retention time windows specified for each listed parent mass. The method allows acquisition of MS^2 spectra for co-eluting compounds and analysis of positively and negatively ionized compounds with a single run. Figure 2 shows the overall application workflow.

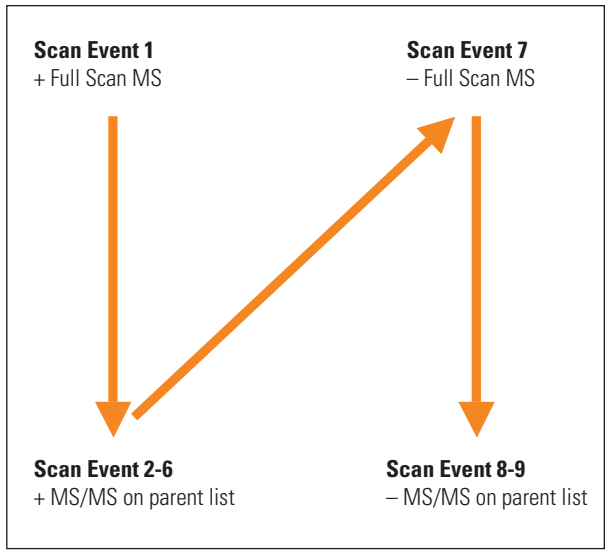

Figure 1: MS scan events

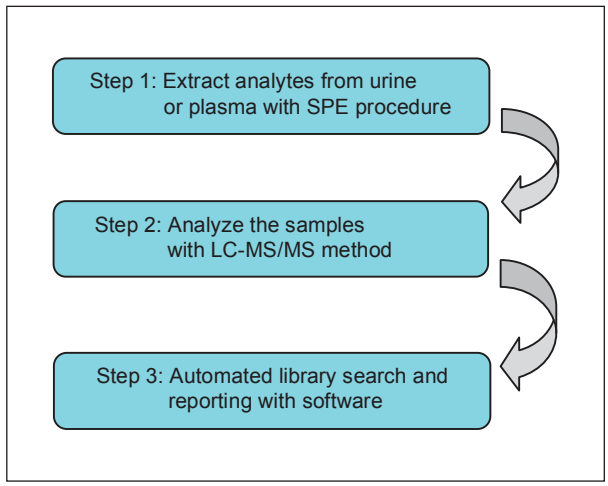

Figure 2: Step-by-step application workflow

Sample Preparation

Samples (1 mL of urine or 0.5 mL of plasma) were spiked with 0.1 mL of an internal standard solution at a concentration of 1 µg/mL (Chlorpromazine-D3, Haloperidol-D4 and Prazepam-D5) and diluted with 2 mL of 0.1 M phosphate buffer pH 6.0. The resulting mix was extracted with an SPE (Thermo Scientific Hypersep Verify-CX 200 mg mixed mode cartridges) procedure prior to injection onto LC-MS.

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Chromatography

HPLC separation was performed with a Thermo Scientific Accela pump using a Thermo Scientific Hypersil GOLD PFP column (50 x 2.1 mm; 5 µm particles). Flow rate was set to 200 µL/min. The gradient is summarized in Table 1 (solvent A = water/0.1% formic acid/10 mM ammonium formate, solvent B = acteonitrile/0.1% formic acid). Injection volume was 10 µL.

Table 1. Thirteen-minute LC method

Time (minutes)	%A	%B
0	95	5
0.5	95	5
5.5	5	95
8.5	5	95
8.6	5	95
13	95	5

MS Conditions

Instrument:	LXQ ion trap mass spectrometer
Ionization:	ESI, Thermo Scientific Ion Max source
Capillary temperature:	275 °C
Spray voltage:	5.0 kV
Sheath gas:	30
Aux gas:	8
Data acquisition mode:	Polarity switching scan dependent experiment
Microscans:	1
WideBand Activation™:	On
Stepped Normalized Collision Energy:	35% ± 10%

Method Validation and Results:

The method was prequalified by processing and analyzing urine samples spiked with 10 randomly selected compounds in concentrations of 10 ng/mL, 100 ng/mL and 1000 ng/mL. Table 2 lists the concentration at which each analyte in the toxicology screen for urine samples is identified. The presence of an analyte at 10, 100 or 1000 ng/mL implies that the limit of detection is likely below that value. Of the 275 compounds analyzed, 70% were detected at 10 ng/mL, 20% at 100 ng/mL, 8% at 1000 ng/mL and 2% were detected at a concentration above 1000 ng/mL.

Table 2. Results for spiked urine samples in toxicology screen by LC-MS/MS

LXQ – 13 min method Compound	**Concentration Tested (ng/mL)** 10	100	1000
All barbiturates require an APCI source for detection.	*P=Drug present.*	*N=Drug not present.*	
11-Hydroxy-delta-9-THC	N	N	>1000
11-nor-9-carboxy-Delta-9-THC	N	N	P
2-Bromo-Alpha-Ergocryptine	P	P	P
2-Hydroxyethylflunitrazepam	N	P	P
3-Hydroxystanozolol	N	N	>1000
4-Hydroxynordiazepam	N	P	P
6-Acetylcodeine	P	P	P
6-Acetylmorphine (6-MAM)	P	P	P
7-Amino-Clonazepam	P	P	P
7-Amino-Flunitrozepam	P	P	P
Acebutolol	P	P	P
a-Hydroxy-Alprazolam	P	P	P
a-Hydroxy-Triazolam	P	P	P
Albuterol	P	P	P
alpha-Hydroxymidazolam	N	P	P
Alprazolam	P	P	P
Alprenolol	P	P	P
Aminorex	N	P	P
Amiodarone	P	P	P
Amitriptyline	P	P	P
Amlodipine	N	N	P
Amobarbital	P	P	P
Amoxapine	P	P	P
Amphetamine	P	P	P
Anhydroecgonine MethylEster	N	P	P
Antipyrine	N	N	>1000
Apomorphine	N	N	>1000

LXQ – 13 min method	Concentration Tested (ng/mL)		
Compound	10	100	1000
Astemizole	N	P	P
Atenolol	P	P	P
Atropine	N	P	P
BDB	N	P	P
Benzocaine	N	N	P
Benzoylecgonine	N	P	P
Betaxolol	P	P	P
Bisacodyl	P	P	P
Bisoprolol	P	P	P
Bromazepam	P	P	P
Brompheniramine	P	P	P
Bupivocaine	P	P	P
Buprenorphine	P	P	P
Bupropion	P	P	P
Buspirone	P	P	P
Butalbital	N	P	P
Butorphanol	P	P	P
Cannabidiol	N	N	>1000
Cannabinol	N	N	>1000
Captopril	N	N	P
Carbamazepine	P	P	P
Carbinoxamine	N	P	P
Carisoprodol	N	N	P
Cathinone	N	N	P
Chlordiazepoxide	P	P	P
Chlorothiazide	N	P	P
Chlorpheniramine	P	P	P
Chlorpromazine	P	P	P
Chlorpromazine-D3	N	P	P
Chlorprothixene	N	N	>1000
Cinnarizine	P	P	P
cis-4-Methylaminorex	N	P	P
Cisapride	N	P	P
Citalopram	P	P	P
Clenbuterol	P	P	P
Clenbuterol	N	P	P
Clobazam	N	P	P
Clomipramine	P	P	P
Clonazepam	P	P	P
Clonidine	P	P	P
Clopidogrel	P	P	P
Clozapine	P	P	P
Cocaethylene	P	P	P
Cocaine	P	P	P
Codeine	P	P	P
Cyclobenzaprine	P	P	P
Delta9-THC	N	P	P
Desalkylflurazepam	N	P	P
Desipramine	N	P	P
Desmethyldoxepin	P	P	P
Dextromethorphan	P	P	P
Diazepam	P	P	P
Diflunisal	P	P	P
Digoxin	N	N	P
Dihydrocodeine	P	P	P
Dihydroergotamine	P	P	P
Diltiazem	P	P	P
Diphenhydramine	P	P	P
Dipyridamole	N	N	P
Disopyramide	P	P	P
Dothiepin	N	P	P
Doxepin	P	P	P
Doxylamine	P	P	P
Ecgonine-Methyl-Ester	N	N	P
EDDP	P	P	P
EMDP	P	P	P
Enalapril	P	P	P
Ephedrine	N	P	P

LXQ – 13 min method	Concentration Tested (ng/mL)		
Compound	10	100	1000
Ergotamine	P	P	P
Estazolam	N	P	P
Felcainide	P	P	P
Fendiline	P	P	P
Fenfluramine	P	P	P
Fentanyl	P	P	P
Fexofenadine	P	P	P
Flumethasone	N	N	P
Flunitrazepam	P	P	P
Flunixin	N	P	P
Fluoxetine	P	P	P
Fluoxymesterone	N	P	P
Fluphenazine	P	P	P
Flurazepam	P	P	P
Fluvoxamine	P	P	P
Furosemide	N	P	P
Gabapentin	N	N	P
Gliclazide	N	N	P
Glimepiride	N	P	P
Glipizide	P	P	P
Glyburide	P	P	P
Haloperidol	P	P	P
Haloperidol-D4	N	P	P
Heroin	P	P	P
HMMA	N	N	>1000
Hydrochlorothiazide	N	N	P
Hydrocodone	P	P	P
Hydromorphone	P	P	P
Hydroxyzine	N	P	P
Imipramine	P	P	P
Indomethacin	N	N	>1000
Isradipine	P	P	P
Ketamine	P	P	P
Ketoconazole	P	P	P
Ketoprofen	N	N	>1000
Ketorolac	N	N	>1000
Labetolol	N	P	P
Lamotrigine	P	P	P
LAMPA	P	P	P
Lidocaine	P	P	P
Lometazepam	N	P	P
Loratadine	P	P	P
Lorazepam	P	P	P
LSD	P	P	P
Maprotiline	P	P	P
MBDB	N	P	P
MDA	P	P	P
MDEA	N	P	P
MDMA	P	P	P
Melatonin	N	N	>1000
Meperidine	P	P	P
Mepivocaine	N	P	P
Meprobamate	N	P	P
Mescaline	P	P	P
Mesoridazine	P	P	P
Metaprolol	P	P	P
Methadienone	P	P	P
Methadone	P	P	P
Methamphetamine	P	P	P
Methaqualone	N	N	>1000
Methcathinone	N	N	P
Methenolone	P	P	P
Methohexital	P	P	P
Methoxyverapmil	P	P	P
Methylphenidate	P	P	P
Metoclopramide	P	P	P
Metronidazole	N	P	P
Mexiletine	N	N	>1000

LXQ – 13 min method
Compound
Mianserin
Miconazole
Midazolam
Mirtazapine
Molsidomine
Morphine
Morphine-3-b-glucuronide
Nalbuphine
Nalorphine
Naloxone
Naltrexone
NAPA
N-DemethylTrimipramine
N-Desmethyl-cis-tramadol
N-Desmethylflunitrazepam
N-Desmethylselegiline
N-DesmthylClomipramine
N-Ethylamphetamine
Nicardipine
Nicotine
Nitrazepam
Nitrendipine
Nizatidine
Norbenzoylecgonine
Norbuprenorphine
Norclomipramine
Norcocaethylene
Norcocaine
Norcodeine
Nordiazepam
Nordoxepin
Norethandrolone
Norfentanyl
Norfluoxetine
Norketamine
NOR-LSD
Normeperidine
Normorphine
Noroxycodone
Noroxymorphone
Norproproxyphene
Nortriptyline
Noscapine
OH-LSD
Ondansetron
Opipramol
Oxazepam
Oxcarbazepine
Oxycodone
Oxymorphone
Papaverine
Paraxanthine
Paroxetine
PCP
Pentazocine
Pentobarbital
Perphenazine
Pheniramine
Phenobarbital
Phenolphthalein
Phentermine
Phenylbutazone
Phenyltoloxamine
Physostigmine
Pindolol
Piroxicam
PMA
PMMA

Concentration Tested (ng/mL)		
10	100	1000
P	P	P
P	P	P
P	P	P
P	P	P
N	N	>1000
N	P	P
N	N	>1000
P	P	P
P	P	P
P	P	P
P	P	P
P	P	P
P	P	P
N	N	P
N	P	P
N	P	P
N	P	P
N	P	P
P	P	P
P	P	P
N	N	>1000
P	P	P
N	N	P
N	N	>1000
N	N	>1000
P	P	P
P	P	P
P	P	P
N	P	P
P	P	P
P	P	P
N	P	P
N	P	P
P	P	P
N	P	P
P	P	P
P	P	P
N	N	P
N	P	P
N	N	>1000
P	P	P
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N	N	P
P	P	P
N	P	P
P	P	P
N	N	>1000
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P	P	P
P	P	P
P	P	P
P	P	P
N	P	P
P	P	P
P	P	P
N	N	P
N	N	P
N	N	P
P	P	P
N	N	P
P	P	P
N	N	P
N	P	P

LXQ – 13 min method	Concentration Tested (ng/mL)		
Compound	10	100	1000
Prazepam-D5	N	P	P
Prazosin	P	P	P
Prilocaine	N	N	P
Procainamide	N	P	P
Promazine	P	P	P
Promethazine	N	P	P
Prometryn	N	P	P
Propafenone	P	P	P
Propoxyphene	P	P	P
Propranolol	P	P	P
Protriptyline	P	P	P
Psilocin	N	P	P
Pyrilamine	P	P	P
Quetiapine	P	P	P
Quinidine	P	P	P
Quinine	N	P	P
Ranitidine	N	N	P
Risperidone	P	P	P
Scopolamine	P	P	P
Secobarbital	P	P	P
Selegiline	N	P	P
Sertraline	P	P	P
Sotalol	N	P	P
Spironolactone	N	P	P
Stanozolol	N	P	P
Telmisartan	P	P	P
Temazepam	P	P	P
Terfenadine	P	P	P
Tetracine	P	P	P
Thiamylal	N	P	P
Thiopental	P	P	P
Thioridazine	P	P	P
Thiothixene	P	P	P
Timolol	P	P	P
Topiramate	P	P	P
Trazodone	P	P	P
Triazolam	P	P	P
Trimethoprim	P	P	P
Trimipramine	P	P	P
Venlafaxine	P	P	P
Verapamil	P	P	P
Vincristine	P	P	P
Warfarin	P	P	P
Zimelidine	P	P	P
Zolpidem	P	P	P
Zopiclone	N	N	P

All barbiturates require an APCI source for detection. *P=Drug present.* *N=Drug not present.*

Table 3. Results for spiked plasma samples in toxicology screen by LC-MS/MS

LXQ – 13 min method Compound	Concentration Tested (ng/mL) 10	100	1000
BDB	N	P	P
Benzocaine	N	P	P
Benzoylecgonine	P	P	P
Betaxolol	P	P	P
Bisacodyl	P	P	P
Bisoprolol	P	P	P
Bromazepam	N	P	P
Brompheniramine	N	P	P
Bufotenine	N	P	P
Bupivocaine	P	P	P
Buprenorphine	P	P	P
Bupropion	N	P	P
Buspirone	P	P	P
Butorphanol	P	P	P
Cannabidol	N	P	P
Cannabinol	N	P	P
Captopril	N	N	>1000
Estazolam	N	P	P
Carbamazepine	P	P	P
Carbinoxamine	P	P	P
Carisoprodol	N	P	P
Cathinone	N	N	>1000
Chlordiazepoxide	N	P	P
Chloroquine	N	P	P
Chlorpheniramine	P	P	P
Chlorpromazine	N	P	P
Chlorprotixene	P	P	P
Clozapine N-Oxide	N	P	P

All barbiturates require an APCI source for detection. P=Drug present. N=Drug not present.

For selected sets of compounds the method was also prequalified by processing and analyzing spiked plasma samples. Table 3 lists the concentration at which each analyte in the toxicology screen for plasma samples is identified. In general, detection limits for urine and plasma are comparable.

In addition, the assay performance was verified by analyzing patient urine samples obtained from the Johns Hopkins University Hospital Clinical Laboratory and data were compared to the results from established LC-UV and immunoassay analytical techniques. The result is shown in Table 4. The LC-MS/MS method has consistently identified more analytes present in the sample than either LC-UV or immunoassays.

Table 4. Urine sample analyzed with LC-MS/MS, LC-UV and Immunoassay methods

LC-MS	LC-UV	Immunoassay
Nortriptyline	Nortriptyline	Barbiturates
Amitriptyline	Amitriptyline	Benzodiazepines
Benzoylecgonine	Benzoylecgonine	Cocaine
Cocaine	Cocaine	Opiates
Norcocaethylene	Cocaethylene	THC
Norbenzoylecgonine	-	-
Morphine	-	-
Norcocaine	-	-
Quinidine/Quinine	-	-
Hydroxyzine	-	-
Noskapine	-	-
Diltiazem	-	-
Morphine-3-beta-Glucuronide	-	-

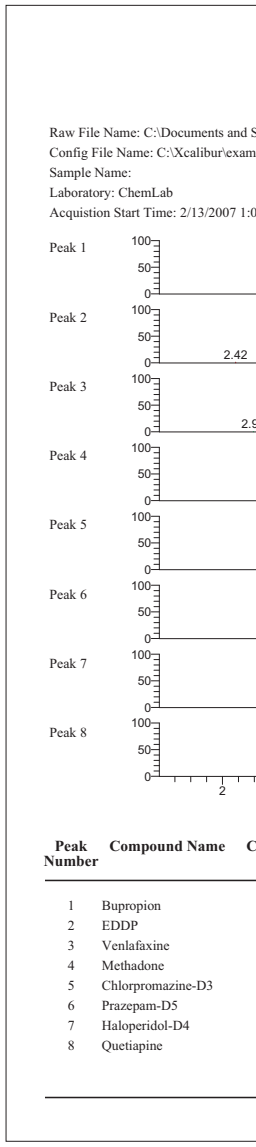

Figure 3: The ToxID Summary Re

Company Name
ToxID Summary Report

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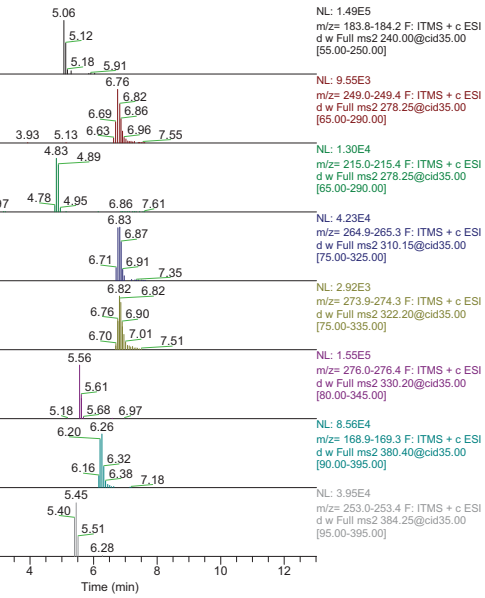


ode	SI	RSI	m/z	Expected RT	Real RT	Intensity	Library Name
p	909	909	240.0	5.20	5.06	148721	Tox_Library
p	857	873	278.2	6.60	6.76	9549	Tox_Library
p	816	837	278.2	4.90	4.83	12964	Tox_Library
p	932	932	310.2	6.70	6.83	42262	Tox_Library
i	859	859	322.2	6.80	6.82	2924	Tox_Library
i	969	974	330.2	5.60	5.56	154827	Tox_Library
i	830	837	380.4	6.20	6.26	85589	Tox_Library
p	870	871	384.2	5.40	5.45	39512	Tox_Library

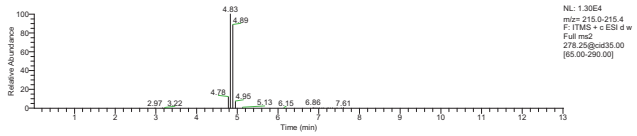
port is designed for a quick synopsis of the data.

Company Name ToxID Long Report

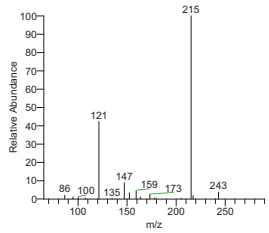
Raw File Name: C:\Documents and Settings\marta.kozak\Desktop\Desktop\Application_Notes\ToxID\2J.RAW
Config File Name: C:\Xcalibur\examples\ToxID\ToxID_config_13min.csv
Sample Name:
Laboratory: ChemLab
Acquistion Start Time: 2/13/2007 1:04:54 AM

Peak Number	Compound Name	Code	SI	RSI	m/z	Expected RT	Real RT	Intensity	Library Name
3	Venlafaxine	p	816	837	278.2	4.90	4.83	12964	Tox_Library

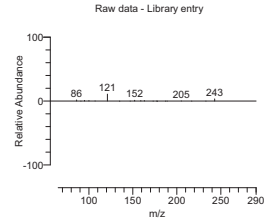
Peak 3



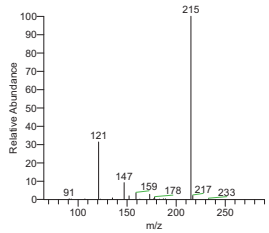
Acquired Spectrum



Delta Spectrum



Library Spectrum



Library Structure

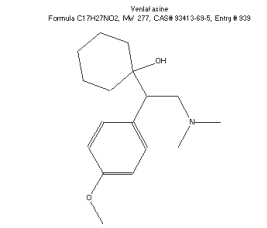


Figure 4: The ToxID Long Report is designed for a more thorough examination of the data.

Table 5. Simple workflow for adding new analytes

STEP 1: Directly infuse analyte to obtain MS^2 spectra, then add spectra to the library	10 Minutes
STEP 2: Run analyte on column to obtain retention times	13 Minutes
STEP 3: Update Parent Mass Table in instrument method with parent masses and retention times	2 Minutes
STEP 4: Update ToxID with name, parent masses, the most intense product ion and retention times	2 Minutes

ToxID™ Software Automates Reporting, Reduces Manual Analysis

ToxID software identifies compounds present in the sample based on MS/MS spectra and retention times. Positive hits are automatically reported via ToxID software. Reports are automatically generated, reducing the time necessary for manual analysis of each sample chromatogram. An example of a Summary Report is shown in Figure 3. A Long Report with one page per detected compound is shown in Figure 4.

Adding New Compounds to the Application

This LC-MS/MS workflow allows the user to quickly and easily add new analytes to the screening method. This feature is very important for toxicology screening because new target compounds are continually being added to the target list. As shown in Table 5, new compounds can typically be added in less than 1 hour.

Conclusion

The comprehensive, turn-key toxicology screening methodology described in this application note utilizes an LXQ ion trap, and includes an SPE procedure and LC method that enables the identification of 275 compounds in human urine and human plasma. Accompanying ToxID software performs automatic data analysis and reporting. This eliminates the need for manual data interpretation and increases confidence in compound identification. It is worth noting that when compared to other screening methods, the LC-MS/MS screening methodology identifies more analytes.

Mass Spectrometers are general purpose laboratory instruments. They have not been cleared or approved by the United States Food and Drug Administration, the European IVD Directive or any other agency for diagnostic, clinical or other medical use.

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Application Note: 439

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UHPLC/MS: An Efficient Tool for Determination of Illicit Drugs

Guifeng Jiang, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Accela™ UHPLC System
- MSQ Plus MS Detector
- Drugs of Abuse
- Hypersil GOLD™ PFP Columns
- Sensitivity

Goal

Optimize a UHPLC/MS method with respect to stationary phase, mobile phase, and detector settings to achieve picogram level quantitation of fourteen drugs and metabolites employing a 12 minutes separation.

Introduction

Gas chromatography-mass spectrometry (GC-MS) is commonly employed for the separation and identification of drugs and metabolites in forensic toxicology, using electron impact (EI) or chemical ionization (CI).[1] This methodology has become a "gold standard" in terms of admissibility and defensibility in court because of its good sensitivity, excellent selectivity and a high degree of standardization.[2] However, laborious and time consuming derivatization procedures and sample clean ups are mandatory in most cases.

LC/MS methods eliminate the need to derivatize and often simplify sample preparation. However, long run times and low separation efficiency limit the utility of conventional HPLC. Ultra high performance liquid chromatography (UHPLC) performs separations 5 to 10 times faster than conventional HPLC by employing sub-2 µm diameter particles. The 1-2 second peak widths and relatively high separation efficiency of UHPLC are more competitive with capillary GC, making UHPLC-MS an attractive alternative method for illicit drug analysis.

This application note illustrates the separation and detection of a mixture of 14 illicit drugs/metabolites by ultra high performance liquid chromatography-mass spectrometry (UHPLC-MS). The drugs/metabolites are separated on a Hypersil GOLD PFP, 1.9 µm, 100 x 2.1 mm column and detected by a fast scanning single quadrupole mass spectrometer.

Experimental Conditions

1. Drug Standard Preparation

Pseudoephedrine, ephedrine, amphetamine, methamphetamine, 3,4-methylenedioxy-N-methamphetamine (3,4-MDMA), oxycodone, hydrocodone, clonazepam, noscapine, cocaine, caffeine, tetrahydrocannabinol (THC), cannabinol and cannabidiol standards (1 mg/mL in methanol) were purchased from Alltech-Applied Science (State College, PA, USA). The above fourteen compounds were mixed with the optimized molar ratio in the range of 1 to 100 and diluted to 0.1 ppm with methanol to make the drug mixture standards.

2. Chromatographic Conditions

Chromatographic analyses were performed using the Accela UHPLC system (Thermo Scientific, San Jose, CA). The chromatographic conditions were as follows:

LC Column:	Hypersil GOLD, 1.9 µm, 20 x 2.1 mm Hypersil GOLD, 1.9 µm, 50 x 2.1 mm Hypersil GOLD, 1.9 µm, 100 x 2.1 mm Hypersil GOLD, aQ (polar endcapped C18), 1.9 µm, 100 x 2.1 mm Hypersil GOLD PFP (perfluorinated phenyl), 1.9 µm, 100 x 2.1 mm Hypersil GOLD PFP (perfluorinated phenyl), 1.9 µm, 50 x 2.1 mm
Column Temperature:	45 °C
Injection:	1 µL Partial Loop Injection, 25 µL Loop Size Syringe Speed: 8 µL/sec Flush Speed: 100 µL/sec Flush Volume: 400 µL Wash Volume: 100 µL Flush/Wash Source: Bottle with methanol
Gradients:	**Method I** Column: Hypersil GOLD PFP 1.9 µm, 100 x 2.1 mm A: Water (0.06% acetic acid) B: Acetonitrile (0.06% acetic acid) C: Methanol (0.06% acetic acid) Flow Rate: 1000 µL/min

Time (min)	Eluent A%	Eluent B%	Eluent C%
0.00	95.0	1.0	4.0
0.10	88.0	2.4	9.6
5.00	85.0	3.0	12.0
13.00	5.0	19.0	76.0
13.90	5.0	19.0	76.0
14.00	95.0	1.0	4.0
15.00	95.0	1.0	4.0

For the other gradient methods used, see Appendix A for details.

Thermo SCIENTIFIC

3. Mass Spectrometer Conditions

MS analysis was carried out on a MSQ Plus single quadrupole LC-MS detector (Thermo Scientific, San Jose, CA). The MS conditions were as follows:

Ionization:	Electrospray (ESI)
Polarity:	Positive
Probe Temperature:	450 °C
Cone Voltage:	60 V
Scan Mode:	Full scans (100-500 *m/z*) and/or Selected ion monitoring (SIM)
ESI Voltage:	4.5 kV

Results and Discussion

1. MS Detection

Both positive and negative electrospray analysis were performed using the polarity switch function of the Xcalibur software. All of the analytes exhibited higher ionization efficiency in the positive ion mode compared with the negative mode. The MS spectra of the drug standards show both molecular ion signals of $[M+H]^+$ and acetonitrile adducts of the form $[M+ACN+H]^+$. For 13 of the analytes, the signal from the molecular ion was more intense than the signal from the acetonitrile adduct. For amphetamine, the most intense signal was from the acetonitrile adduct $[M+ACN+H]^+$ at *m/z* of 177.2 (data not shown).

2. Separations with Standard Stationary Phases

Three columns were evaluated to separate the illicit drug mixtures: Hypersil GOLD, Hypersil GOLD aQ and Hypersil GOLD PFP (Figure 1). The UHPLC method with each column type was optimized individually. Hypersil GOLD aQ, a polar endcapped C18 phase which offers more retention of polar compounds, did not resolve the early eluting compounds including methamphetamine, oxycodone, caffeine, MDMA and hydrocodone.[3] The separation on Hypersil GOLD aQ may have been impaired by interactions between the polar endcapped stationary phase and the polar analytes. Hypersil GOLD, with LI or C18 selectivity, showed improved selectivity for all analytes except caffeine (peak 1) and oxycodone (peak 7). Hypersil GOLD uses highly pure silica and endcapping procedure to minimize unwanted interactions between analytes and the acidic silanols of the silica support. Hypersil GOLD PFP enabled the optimal separation of all 14 analytes by improving the resolution of the earlier eluting compounds. Hypersil GOLD PFP introduces a fluorine group into the stationary phase to improve selectivity towards halogenated compounds, as well as polar compounds containing hydroxyl, carboxyl, nitro or other polar groups.[3]

3. Separations using Acetic Acid and Trifluoroacetic Acid (TFA) as Eluent Modifier

Trifluoroacetic acid, formic acid and acetic acid can be added into the mobile phase to generate differences in selectivity. Separation of 14 illicit drugs on a Hypersil GOLD PFP column was evaluated by using either trifluoroacetic acid, formic acid or acetic acid as eluent modifier. The separation method with 0.02% TFA (Figure 2A) provided fast separation performance with good resolution and sharp peaks. However, the use of TFA is generally not recommended with MS detection due to its effect on signal suppression.

All of the analytes are well resolved with 0.1% formic acid as modifier (Figure 1C), but only when 100% water is used at the beginning of the gradient method (Method C). Prolonged use of 100% water may degrade the stationary phase and shorten the column lifetime, so gradient method C is not suited for routine use.

Most of the analytes are well separated with adequate resolution using 0.06% acetic acid as eluent modifier (Figure 2B). However, under such conditions, a few pairs of compounds, such as oxycodone and methamphetamine (peaks 7 & 6), hydrocodone and 3, 4-MDMA (peaks 5 & 8), cocaine and noscapine (peaks 10 & 11), are not baseline resolved.

4. Separations with Hybrid Column Phases

Three hybrid stationary phases were evaluated after connecting different stationary phase columns in series:

Figure 3A: 50 x 2.1 mm Hypersil GOLD + 50 x 2.1 mm Hypersil GOLD PFP
Figure 3B: 50 x 2.1 mm Hypersil GOLD PFP + 50 x 2.1 mm Hypersil GOLD
Figure 3C: 100 x 2.1 mm Hypersil GOLD PFP + 20 x 2.1 mm Hypersil GOLD

Separations of 14 illicit drugs with these three hybrid stationary phases demonstrated great variation in selectivity. In general, the hybrid column phases improved selectivity between THC and cannabinol, cocaine and noscapine, but reduced selectivity between earlier eluting compounds, such as oxycodone, MA, hydrocodone and MDMA, compared with the Hypersil GOLD PFP phase.

5. Separation with Ternary Gradient

The separation of the drug mixtures was dramatically improved by using three solvents: water, acetonitrile and methanol (Figure 4). Baseline resolution of all 14 drugs was achieved. Methanol, a weaker eluent compared with acetonitrile, provided better resolution for most of the analytes. However, the flow rate had to be reduced to accommodate high column backpressure caused by the high viscosity of methanol. Adding acetonitrile reduced the column backpressure so as to maintain the same separation speed.

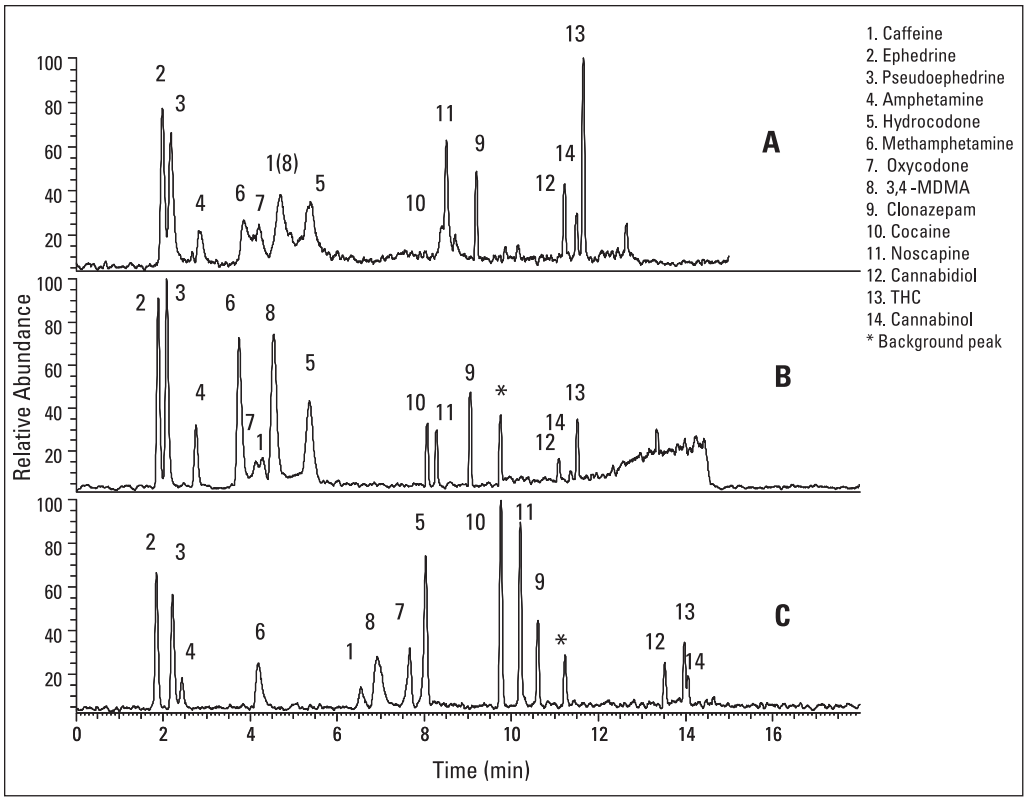

Figure 1: Comparison of 1.9 µm Hypersil GOLD stationary phases for the UHPLC separation of 14 illicit drugs. A) Hypersil GOLD aQ, Method A was applied; B) Hypersil GOLD, Method B was applied; C) Hypersil GOLD PFP, Method C was applied. *See Appendix A for methods details.*

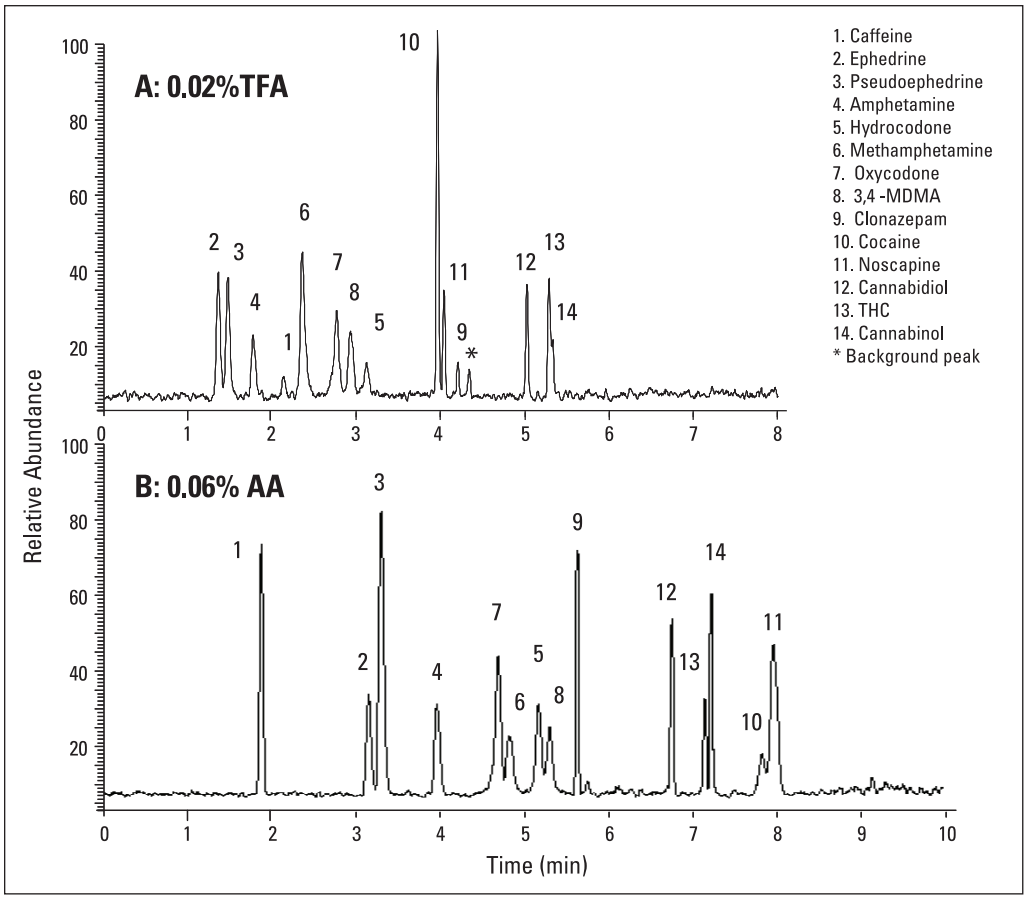

Figure 2: UHPLC/MS chromatograms of the 14 illicit drugs with acidic solvent modifiers. A) 0.02%TFA (Method D); B) 0.06% acetic acid (Method E). *See Appendix A for methods details.*

6. Calibration Curve and Sensitivity

Calibration curves for the drug standards were constructed over the concentration range listed in Table 1 with 10 calibration levels (Figure 5). Each calibration level was injected three times and the mean area responses were plotted against the concentrations. Correlation coefficients with $R^2 = 0.995$ or better were achieved for all illicit drug compounds.

The limit of quantitation (LOQ) and the limit of detection (LOD) of the drug compounds were determined based on the calibration curve of signal-to-noise ratio versus concentration and the definitions of LOQ and LOD using s/n = 10 and 3, respectively. LOQs for all drugs were in the range of 0.96-300 ng/mL, while LODs were from 0.29 to 90.0 ng/mL (Table 1). The outstanding sensitivity by this method was highlighted by the achievement of picogram level quantitation for 10 illicit drugs with 1 µL sample injection.

Analyte	LOQ (ng/mL)	LOD (ng/mL)	Linear Range (ng/mL)
ephedrine	1.21	0.36	1.3-2000
pseudoephedrine	1.25	0.38	1.3-1670
amphetamine	1.78	0.53	1.3-1670
methamphetamine	0.96	0.29	1.3-1670
3,4-MDMA	1.09	0.33	1.3-1670
hydrocodone	6.80	2.04	4.1-10000
oxycodone	3.48	1.04	3.3-10000
clonazepam	7.39	2.22	3.3-3000
cocaine	1.17	0.35	0.3-1000
noscapine	3.79	1.14	0.7-10000
cannabidiol	300	90.0	274-44400
cannabinol	251	75.4	123-20000
THC	191	57.4	68.5-11100

Table 1: LOQ and LOD of the thirteen drug compounds with 1 µL sample injection.

Conclusions

Fourteen illicit drugs and metabolites are baseline separated in twelve minutes by employing UHPLC/MS with a ternary solvent gradient. Various selectivities are achieved by different column surface chemistry, acidic solvent modifier and eluent system. These results are useful for method developments of drug identification and quantitation. Detection by single quadrupole MS at the ppb (ng/mL) level is more than sufficient to identify and quantify illicit drugs in real samples.

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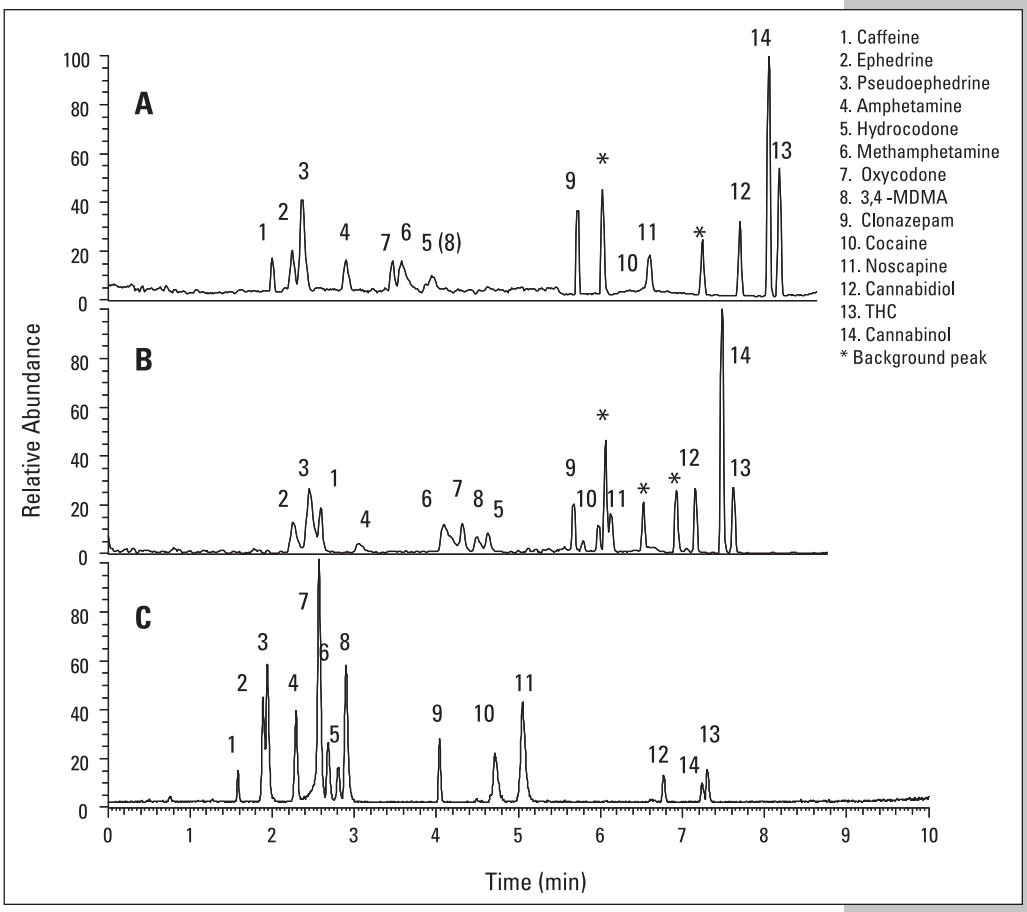

Figure 3: Comparison of hybrid stationary phase chemistry for the separation of 14 illicit drugs. A) 50 x 2.1 mm Hypersil GOLD + 50 x 2.1 mm Hypersil GOLD PFP, Method F; B) 50 x 2.1 mm Hypersil GOLD PFP + 50 x 2.1 mm Hypersil GOLD, Method G; C) 100 x 2.1 mm Hypersil GOLD PFP + 20 x 2.1 mm Hypersil GOLD, Method H. *See Appendix A for method details.*

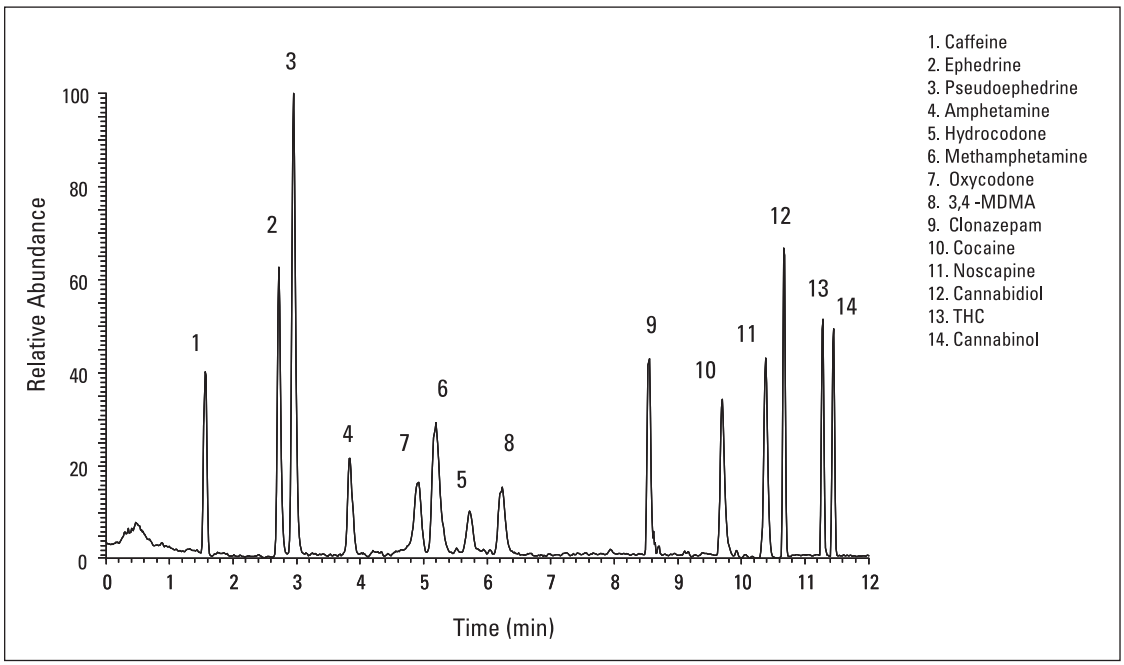

Figure 4: Optimized UHPLC/MS separation of 14 illicit drugs with ternary gradient, listed in Method I.

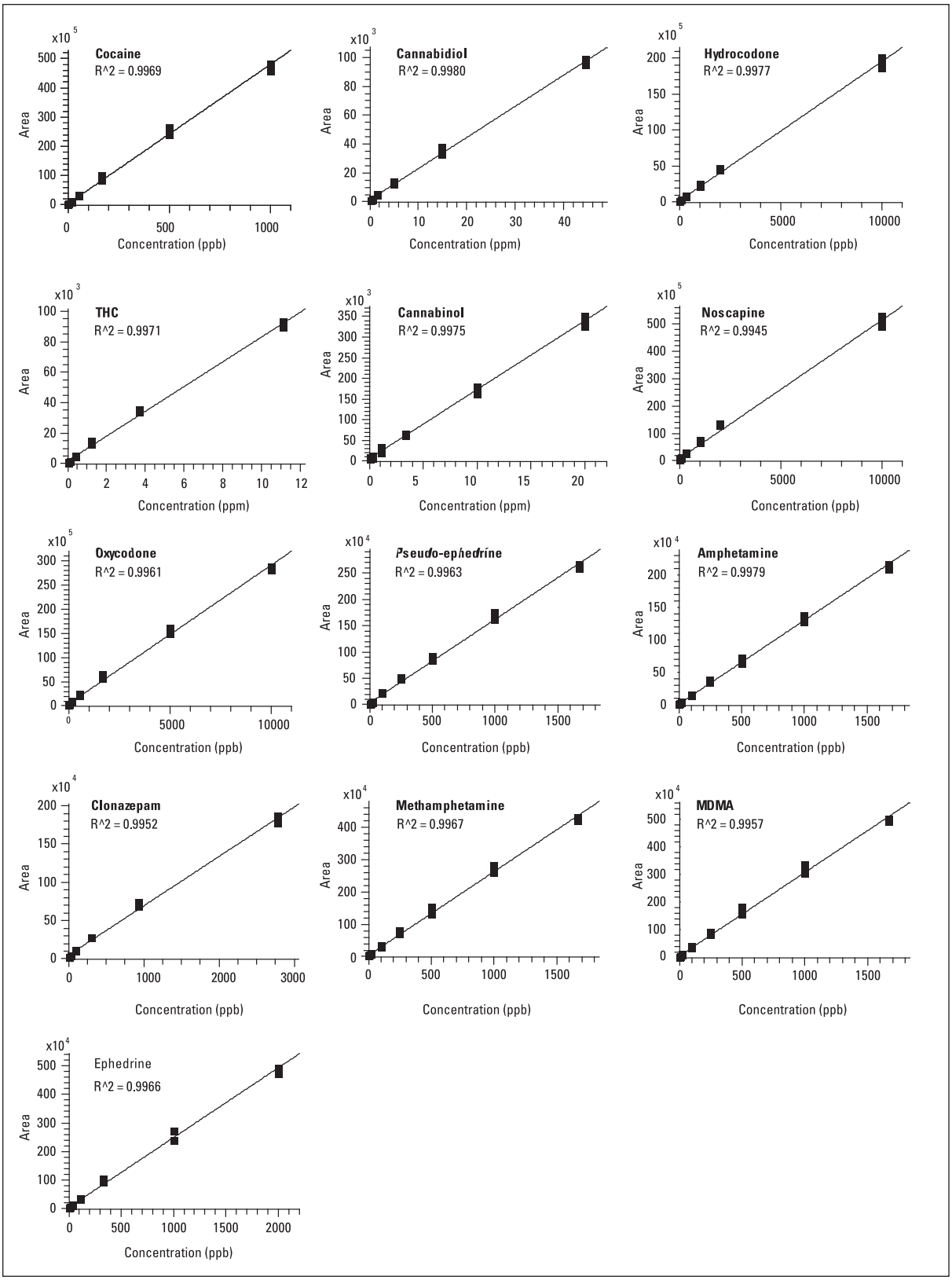

Figure 5: Calibration curves for illicit drugs.

Appendix A

Method A

Column: Hypersil GOLD aQ, 1.9 µm, 100 x 2.1 mm
A: Water, 0.1% FA
B: Acetonitrile, 0.1% FA
Flow Rate: 750 µL/min

Time (min)	Eluent B%
0.00	2.0
6.00	2.0
12.00	95.0
14.00	95.0
14.10	2.0
16.00	2.0

Method B

Column: Hypersil GOLD, 1.9 µm, 100 x 2.1 mm
A: Water, 0.1% FA
B: Acetonitrile, 0.1% FA
Flow Rate: 1000 µL/min

Time (min)	Eluent B%
0.00	1.0
6.00	1.0
12.00	95.0
14.00	95.0
14.10	1.0
16.00	1.0

Method C

Column: Hypersil GOLD PFP, 1.9 µm, 100 x 2.1 mm
A: Water, 0.1% FA
B: Acetonitrile, 0.1% FA
Flow Rate: 1000 µL/min

Time (min)	Eluent B%
0.00	0.0
6.00	0.0
10.00	30.0
14.00	60.0
14.10	0.0
16.00	0.0

Method D

Column: Hypersil GOLD PFP, 1.9 µm, 100 x 2.1mm
A: Water, 0.02% TFA
B: Acetonitrile, 0.02% TFA
Flow Rate: 1000 µL/min

Time (min)	Eluent B%
0.00	2.0
2.80	10.0
4.00	55.0
4.50	60.0
5.00	60.0
5.10	95.0
5.70	95.0
5.80	2.0
8.00	2.0

Method E

Column: Hypersil GOLD PFP, 1.9 µm, 100 x 2.1 mm
A: Water (0.06% AA)
B: Acetonitrile (0.06% AA)
Flow Rate: 1000 µL/min

Time (min)	Eluent B%
0.00	2.0
5.00	20.0
5.10	50.0
7.00	60.0
8.60	95.0
9.90	95.0
10.00	2.0
12.00	2.0

Method F

Hypersil GOLD, 1.9 µm, 50 x 2.1 mm
Hypersil GOLD PFP, 1.9 µm, 50 x 2.1 mm
A: Water (0.06% AA)
B: Acetonitrile (0.06% AA)
Flow Rate: 1000 µL/min

Time (min)	Eluent B%
0.00	2.0
5.00	20.0
5.10	50.0
7.00	60.0
8.60	95.0
10.0	95.0
10.10	2.0
12.00	2.0

Method G

Hypersil GOLD PFP, 1.9 µm, 50 x 2.1mm
Hypersil GOLD, 1.9 µm, 50 x 2.1 mm
A: Water (0.06% AA)
B: Acetonitrile (0.06% AA)
Flow Rate: 1000 µL/min

Time (min)	Eluent B%
0.00	1.5
3.00	5.0
4.50	25.0
6.00	65.0
9.50	95.0
10.0	95.0
10.10	1.5
12.00	1.5

Method H

Hypersil GOLD PFP, 1.9 µm, 100 x 2.1 mm
Hypersil GOLD, 1.9 µm, 20 x 2.1 mm
A: Water (0.06% AA)
B: Acetonitrile (0.06% AA)
Flow Rate: 1000 µL/min

Time (min)	Eluent B%
0.00	2.0
10.00	95.0
11.00	95.0
11.10	2.0
12.00	2.0

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Improved Signal-to-Noise Ratio in the Antidoping Analysis of Clenbuterol in Urine Using LC–FAIMS–H-SRM

James Kapron[1] and Rohan Thakur[2]
[1]Thermo Fisher Scientific, Ottawa, Canada; [2]Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- TSQ Quantum Ultra™
- Surveyor™ HPLC
- Drug Screening
- FAIMS Technology
- Improved Selectivity

Introduction

Clenbuterol is a beta-2 agonist drug with anabolic properties that is commonly used as a bronchodilator in veterinary medicine (Figure 1). Its use in humans has been banned in many countries, including the United States, because of serious cardiovascular and pulmonary side effects. The short-term effects of clenbuterol are similar to stimulant drugs like amphetamine or ephedrine and include increased heart rate, temperature, and blood pressure. Clenbuterol also increases lean muscle mass while reducing fat deposition. Some athletes and body-builders use the drug for these thermogenic and anti-catabolic effects, and as a result clenbuterol has been banned by the World Anti-Doping Agency (WADA).

Doping control laboratories must therefore routinely monitor clenbuterol in biological samples. Although the analysis of clenbuterol by LC-MS/MS is selective, endogenous matrix interferences often produce a high chemical background. Reducing the chemical background leads to improved detection of clenbuterol for monitoring purposes. The selectivity of the LC-MS/MS method is increased with the addition of both the gas-phase selectivity of FAIMS (high-Field Asymmetric waveform Ion Mobility Spectrometry) and H-SRM (Highly-Selective Reaction Monitoring).

FAIMS and H-SRM work together to increase assay selectivity. In the interface between the ion source and the mass spectrometer, FAIMS selects which ions are allowed into the vacuum region. By applying alternating low and high electric fields, interferences are filtered out. The result is LC-MS/MS chromatograms with reduced chemical background and endogenous interferences. H-SRM, in turn, provides higher analyte selectivity through improved mass resolution of the precursor ion with Q1 while maintaining high transmission efficiency. The net result is cleaner chromatograms and more reliable results.

Goal

To improve the selectivity of an LC-MS/MS method for the analysis of clenbuterol in urine using FAIMS in combination with H-SRM.

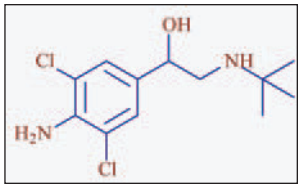

Figure 1: Structure of Clenbuterol

Experimental Conditions

Sample Preparation

Standard calibration samples of human urine fortified with clenbuterol were prepared at the following nine concentrations: 0.5, 1, 1.5, 2.5, 5, 10, 25, 50, and 100 ng/mL. Quality control samples of human urine fortified with clenbuterol were prepared at the following four concentrations: 0.5, 1.5, 50, and 100 ng/mL. Blank samples of human urine without the reference standard were also prepared.

To prepare each sample for analysis, 75 µL of sample was added to 225 µL of water. After mixing, 10 µL was injected. No internal standard was used.

HPLC

HPLC analysis was performed using the Surveyor HPLC System (Thermo Scientific, San Jose, CA). The 10 µL samples were injected directly onto a 4.6 ! 50 mm polar RP column. The mobile phase was acidified acetonitrile/water delivered at a flow rate of 400 µL/min. The gradient is described in Table 1.

Time (min.)	%B
0	10
2	67
2.2	67
2.5	10
3	10

Table 1: Gradient profile

Mass Spectrometry

MS analysis was carried out on a TSQ Quantum Ultra triple quadrupole mass spectrometer with a heated electrospray ionization (H-ESI) probe (Thermo Scientific, San Jose, CA). The MS and FAIMS conditions were as follows:

Mass Spectrometry Conditions

Ion source polarity: Positive ion mode heated ESI
Spray voltage: 3500 V
Vaporizer temperature: 400°C
Sheath gas pressure (N_2): 100 units
Auxiliary gas pressure (N_2): 60 units
Ion transfer tube temperature: 300°C
Scan Type: SRM and H-SRM

The clenbuterol transitions monitored were *m/z* 277.042→*m/z* 202.954 at a Q1 peak width of 0.7 u FWHM for SRM scans and *m/z* 277.078→*m/z* 202.958 at a Q1 peak width of 0.1 u FWHM for H-SRM scans. For both, the collision energy was 18 V and the scan time was 100 ms.

FAIMS Conditions

Dispersion voltage: –4500 V
Outer bias voltage: 35 V
Compensation voltage: –14 V
Inner electrode temperature: 50 °C
Outer electrode temperature: 70 °C
FAIMS gas: 50% He in N_2 at 3 L/min

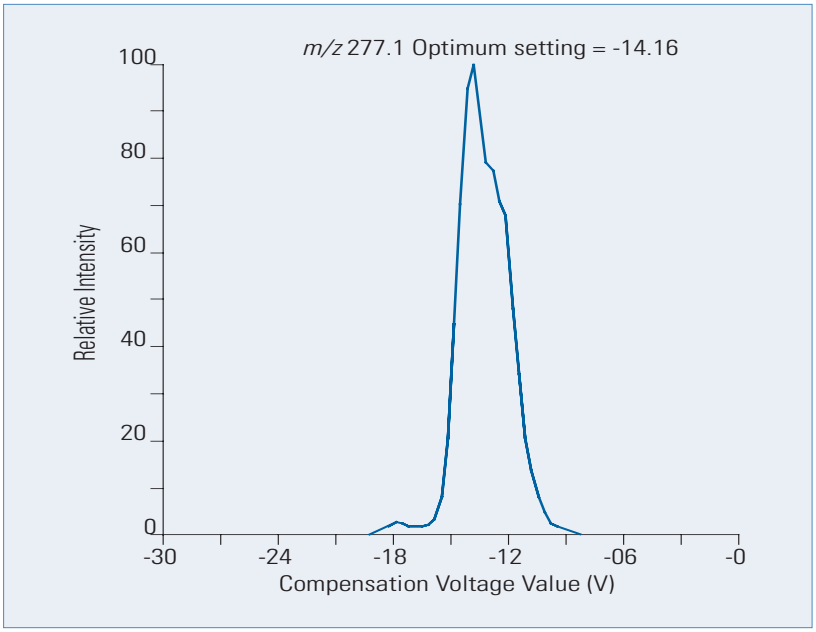

Figure 2: Compensation voltage scan from the infusion of a clenbuterol reference standard. The maximum response for clenbuterol occurred at a compensation voltage of –14V.

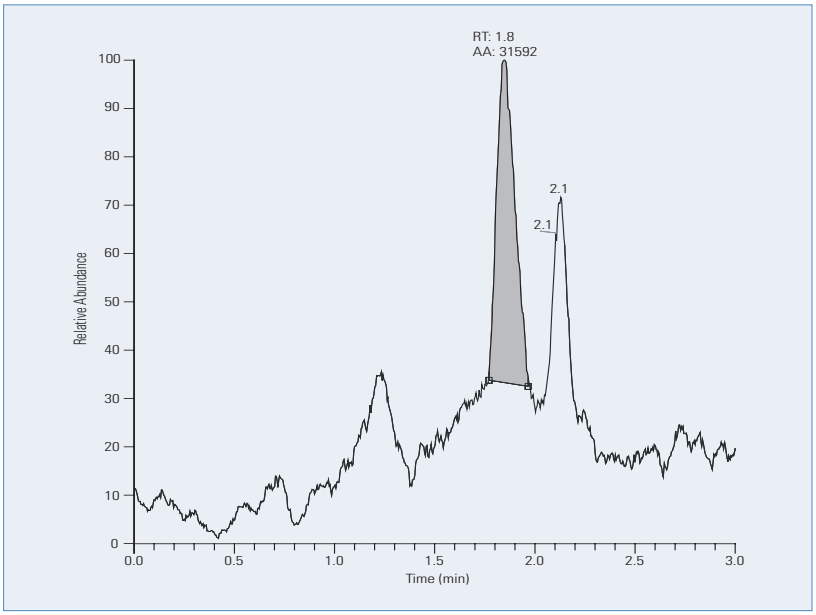

Figure 3: Representative LC–SRM chromatogram for clenbuterol in human urine, obtained with unit mass resolution and without FAIMS selectivity.

Implementing FAIMS requires the establishment of conditions for the transmission of the desired analyte(s) through the interface. Stable conditions for ion transmission can be expressed by the compensation voltage (CV). In these experiments, the CV was ramped from –30 to 0 V in 1.5 min. The maximum response for clenbuterol occurred at –14 V and indicated the appropriate CV for LC–FAIMS–H-SRM analysis as shown in Figure 2.

Results and Discussion

A representative LC-SRM chromatogram for the analysis of clenbuterol in human urine collected using unit mass resolution (0.7 u in both Q1 and Q3) is shown in Figure 3. Although LC-MS/MS is a selective technique, many isobaric interferences appear in the chromatogram. These isobaric interferences increase the chemical background and can make reproducible integration of the analyte peak difficult.

An increase in selectivity is achieved by using the FAIMS device to improve ion separation. A representative LC–FAIMS–SRM chromatogram for the analysis of clenbuterol in human urine is shown in Figure 4. The selectivity offered by FAIMS provides a cleaner chromatogram than the corresponding trace in Figure 3, but some contribution from interferences remain.

By utilizing both FAIMS and H-SRM in the LC-MS/MS analysis, an even further increase in selectivity is achieved. A representative LC–FAIMS–H-SRM chromatogram for the analysis of clenbuterol in human urine is shown in Figure 5. The selectivity offered by the combination of LC, FAIMS, and H-SRM results in the further removal of interferences.

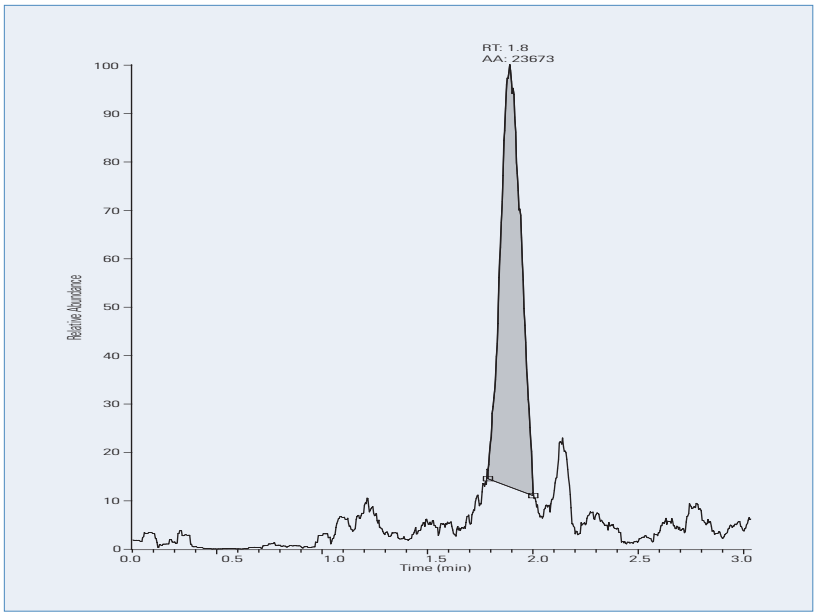

Figure 4: Representative LC–FAIMS–SRM chromatogram for clenbuterol in human urine, obtained with unit resolution and with FAIMS selectivity

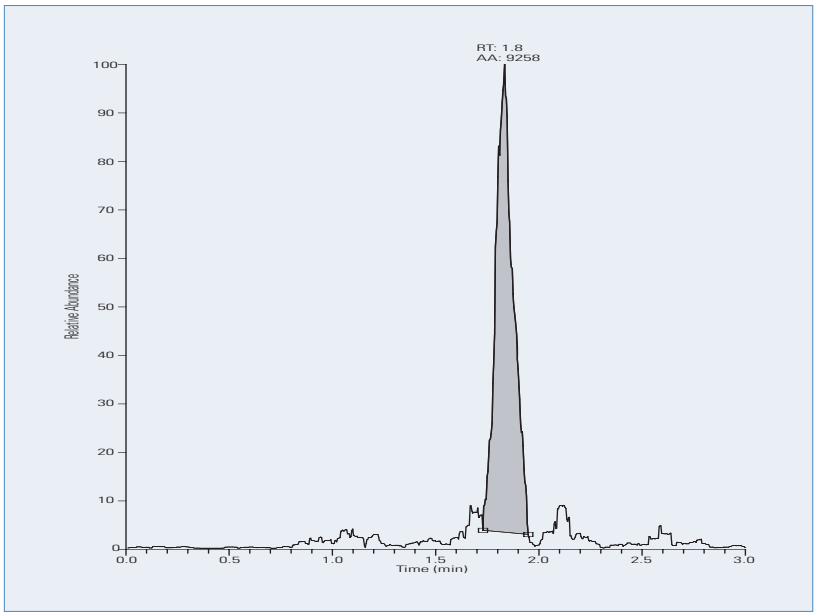

Figure 5: Representative LC–FAIMS–H-SRM chromatogram for clenbuterol in human urine, obtained with high (0.1 FWHM) resolution combined with FAIMS selectivity.

After analysis of the human urine samples, the chromatographic peaks were integrated and the peak areas were used to perform regression analysis. A representative calibration line is shown in Figure 6. The inset shows the calibration line between the LLOQ (0.5 ng/mL) and 5 ng/mL.

Quality control samples were analyzed as shown in Table 2. Combining FAIMS with H-SRM provides excellent selectivity, which results in improved accuracy and precision at the LLOQ.

The interferences that result in 31% RSD and –20% accuracy are removed using FAIMS and H SRM. The final precision is 12% RSD and the accuracy is 8% of theoretical.

Conclusion

The combination of FAIMS and H-SRM provides excellent selectivity for the analysis of clenbuterol in human urine. Compared to the LC–SRM method, the use of LC–FAIMS–H-SRM reduced the chemical background and resulted in cleaner chromatograms and more reproducibly integrated chromatographic peaks. At the LLOQ, the clenbuterol assay in human urine shows the best accuracy and precision via LC–FAIMS–H-SRM.

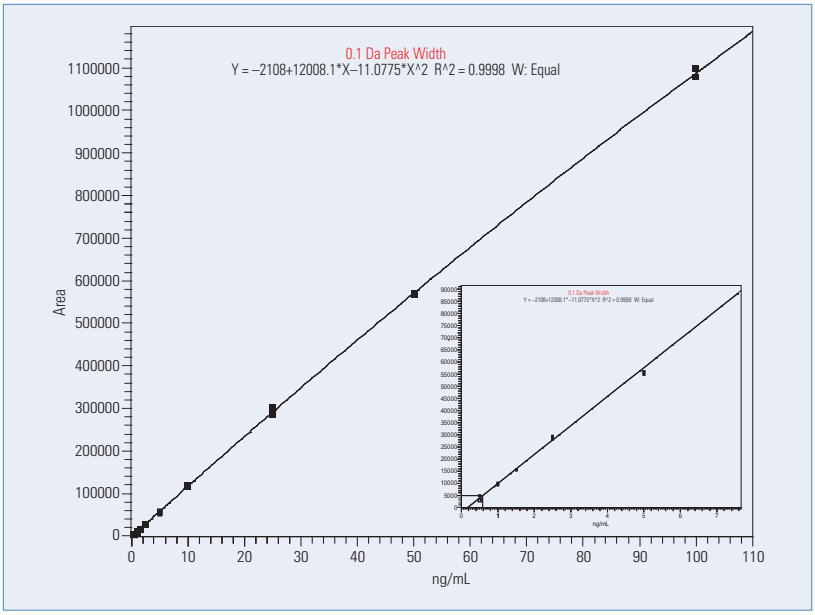

Figure 6: Representative regression analysis from the LC–FAIMS–H-SRM analysis of clenbuterol in human urine, using high resolution (0.1 FWHM) combined with FAIMS selectivity

	LC - SRM				LC - *f* - H-SRM			
Nominal	QC_Low 0.5	QC_3x 1.5	QC_Mid 50	QC_High 100	QC_Low 0.5	QC_3x 1.5	QC_Mid 50	QC_High 100
Replicate 1	0.340	1.53	51.1	99.1	0.624	1.40	47.4	94.4
Replicate 2	0.474	1.50	51.4	98.5	0.566	1.44	49.6	93.1
Replicate 3	0.504	1.64	50.9	97.6	0.433	1.52	50.1	92.6
Replicate 4	0.379	1.52	50.8	97.7	0.544	1.38	49.7	93.0
Replicate 5	0.517	1.47	50.0	98.1	0.535	1.29	47.9	99.1
Replicate 6	0.195	1.57	51.7	90.0	0.517	1.35	47.1	93.1
Mean	0.402	1.54	51.0	98.3	0.537	1.39	48.6	94.2
RSD	30.7	3.7	1.1	0.6	11.7	5.7	2.7	2.6
%Difference	-19.7	2.4	2.0	-1.7	7.3	-7.0	-2.8	-5.8

Table 2: Comparison of QC samples from LC–SRM and LC–FAIMS–H-SRM analysis. Concentrations exceeding bioanalytical criteria are framed. The precision and accuracy were improved to within the guidance criteria by using FAIMS and H-SRM.

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A Quantitative Test for Multiple Classes of Illicit Drugs and Their Primary Metabolites in Human Biological Fluids by LC-MS/MS for Forensic Use

Kevin J. McHale,[1] Joyce Ho,[2] and Angela Springfield[2]
[1]Thermo Fisher Scientific, Somerset, NJ, USA; [2]Tarrant County Medical Examiner, Fort Worth, TX, USA

Key Words

- TSQ Quantum Discovery MAX
- Surveyor HPLC
- Forensic drugs of abuse testing
- SRM

Introduction

Currently, GC/MS is the method of choice for quantifying drugs of abuse. In recent years, however, many forensic labs have been switching to LC-MS/MS methods, which do not require time-consuming derivatization or extensive sample cleanup necessary in GC/MS analyses. Yet, many of the LC-MS/MS methods described in the literature either assay a limited number of illicit drug classes or do not include their primary metabolites of these illicit drugs (see table 1).[1-5] Herein is described a method to assay multiple drugs of abuse including opiates, stimulants, depressants, and the primary metabolites of these illicit drugs.

Goal

To apply a single LC-MS/MS method to screen for 32 illicit drugs of abuse and their metabolites in biological fluids.

Experimental Conditions

Sample Preparation

Whole blood or urine samples (0.1–0.4 mL) were spiked with 20 ng of isotopically labeled internal standards and purified by solid phase extraction (SPE). Extracted samples were reconstituted to yield solutions with the internal standards at 25 ng/mL.

HPLC

HPLC analysis was performed using the Thermo Scientific Surveyor HPLC System. Each 10 µL sample was injected directly onto a Thermo Scientific Hypersil GOLD PFP 50×2.1 mm, 3 µm analytical column. A gradient LC method used mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.3 mL/min.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Discovery MAX triple stage quadrupole mass spectrometer with an electrospray ionization (ESI) probe. The MS conditions were as follows:

Ion source polarity: Positive ion mode
Ion transfer tube temperature: 370 °C
Scan Type: SRM
SRM scan time: 10 ms per transition
Q1, Q3 resolution: unit (0.7 Da FWHM)

Two SRM transitions were monitored for each component to provide ion ratio confirmations (IRC). Table 1 summarizes these SRM transitions.

	Drug of Abuse	Parent *m/z*	Quantifier Product *m/z*	Qualifier Product *m/z*	Ion Ratio
A	*Morphine*	286	201	165	87
B	7-amino-nitrazepam	252	121	94	14.5
C	*Ephedrine*	166	115	133	95
D	Hydromorphone	286	185	157	56
E	Amphetamine	136	119	91	86
F	*Codeine*	300	165	215	97
G	7-amino-clonazepam	286	222	250	85
H	Noroxycodone	302	187	227	97
I	*Methamphetamine*	150	91	119	67
J	Oxycodone	316	241	256	65
K	MDA	180	135	105	92
L	6-MAM	328	165	211	68
M	Norketamine	224	125	179	43
N	Hydrocodone	300	199	171	28
O	*Benzoylecgonine*	290	168	105	24
P	7-amino-flunitrazepam	284	135	227	52
Q	MDMA	194	163	135	30
R	*Ketamine*	238	125	179	40
S	*MDEA*	208	163	135	32
T	Meperidine	248	220	174	55
U	Oxazepam	287	241	269	54
V	*Nordiazepam*	271	140	208	82
W	Cocaine	304	182	82	11.1
X	Lorazepam	321	275	229	25
Y	Nitrazepam	282	236	180	38
Z	Alprazolam	309	281	205	85
AA	Temazepam	301	255	177	11.8
BB	*Clonazepam*	316	270	214	28
CC	Diazepam	285	193	154	70
DD	*Cocaethylene*	318	196	82	15
EE	Flunitrazepam	314	268	239	34
FF	*Methadone*	310	265	105	18

Table 1: Summary of SRM transitions for 32 illicit drugs.

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Results and Discussion

Figures 1 and 2 demonstrate the separation of 32 illicit drugs in less than 10 minutes. Using an SRM dwell time of 10 ms per transition yielded a minimum of 15 data points across an LC peak. The limits of quantitation (LOQs) were determined as either 0.5 ng/mL (lowest calibrator concentration used) or as the concentration where the percent relative errors and %CVs were less than 20% for five replicate injections.

As shown in Figure 3, most calibration curves were fit using linear regression. Some standards (for example, cocaine) yielded better statistical calibration curves using quadratic regression. In these select cases, the target compound used a structurally different isotopically labeled internal standard (for example, cocaine used D5-nordiazepam as internal standard).

The assay of biological sample extracts identified multiple drugs of abuse and related metabolites. Figures 4A and B demonstrate examples of urine and whole blood extracts assayed for the presence of illicit drugs with the developed LC-MS/MS method. Note that cocaine and benzoylecgonine were detected and qualified below the assay LOQs in a whole blood extract (Figure 4B), indicating that lower LOQs are possible for these compounds.

Conclusion

An LC-MS/MS method for assaying illicit drugs and their metabolites at an LOQ of 0.5–2.5 ng/mL in biological fluids for forensic use has been demonstrated. Confirmation of the drugs of abuse was achieved by monitoring two SRM transitions per compound and measuring their area ratios to within ±20%. Utilizing a low SRM dwell time of 10 ms per transition to achieve sufficient data points across a chromatographic peak had no adverse effects, such as SRM cross-talk, on the quantitation and confirmation of these illicit drugs. To authenticate this assay, extracts from biological fluids were analyzed, showing the presence of several drugs of abuse and their metabolites.

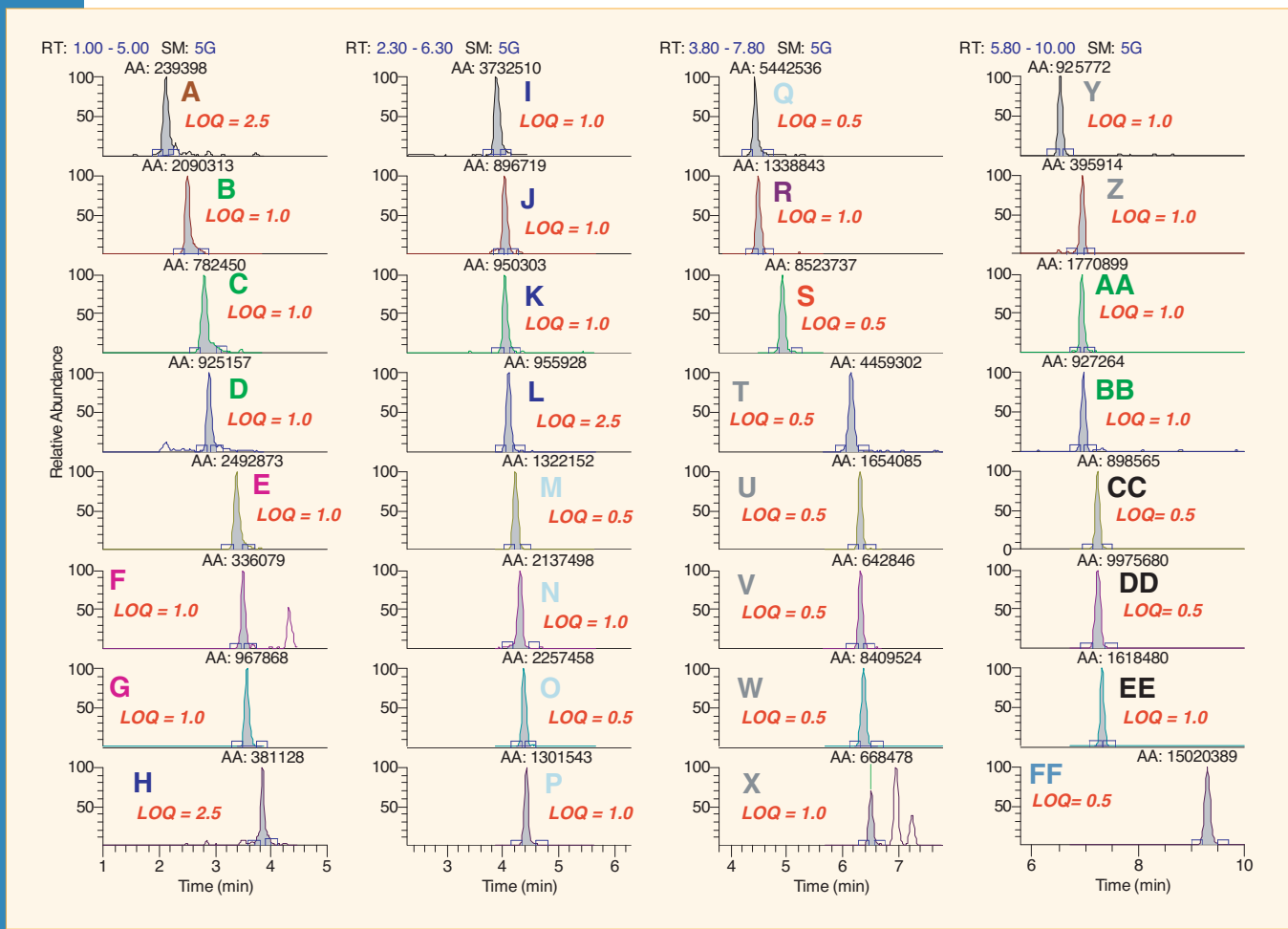

Figure 1: Quantifier SRM transitions for the 2.5 ng/mL standard. For the compound designators, refer to the legend in Table 1.

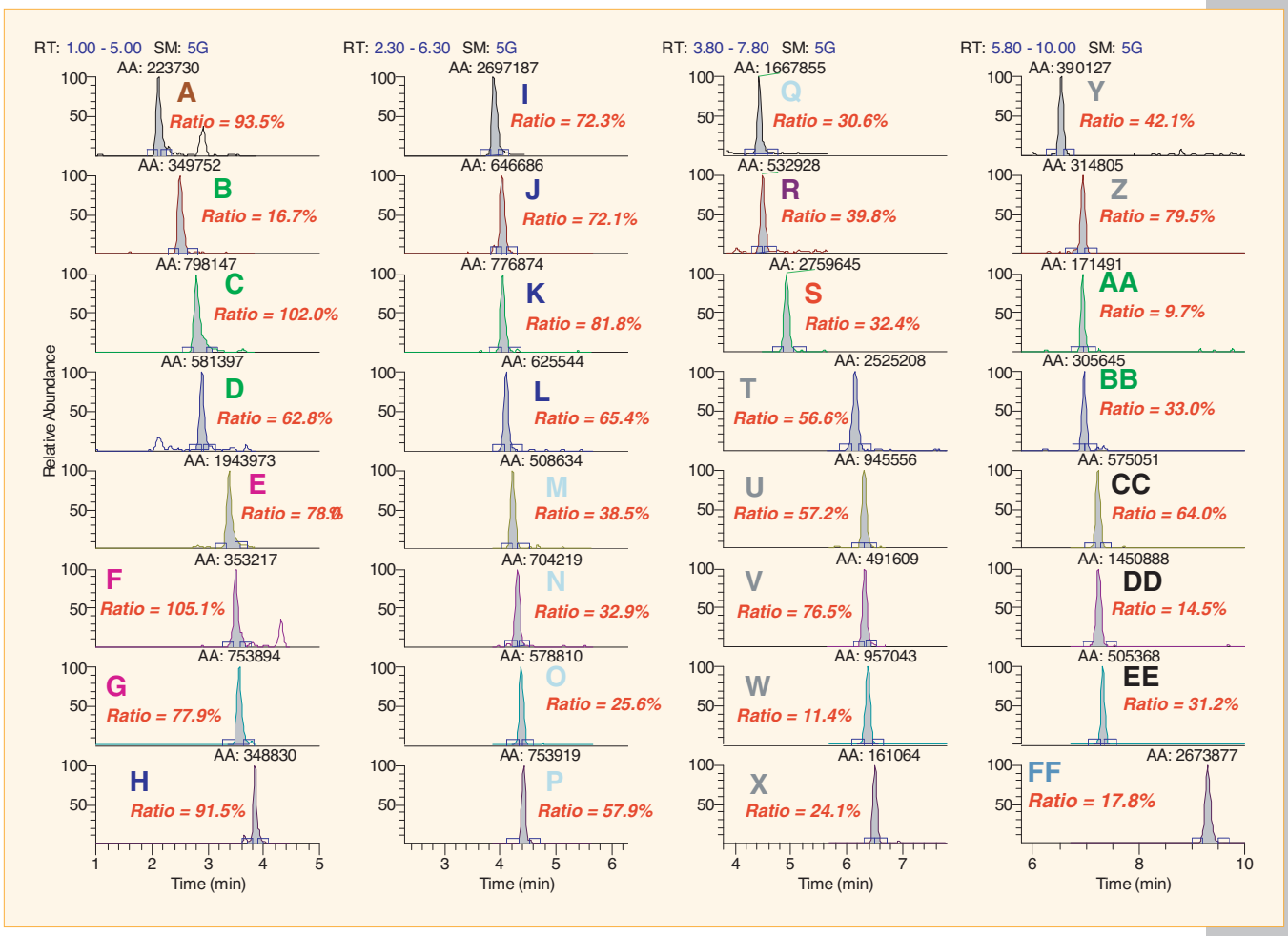

Figure 2: Qualifier SRM transitions for the 2.5 ng/mL standard. For the compound designators and the target ion ratio %, see Table 1.

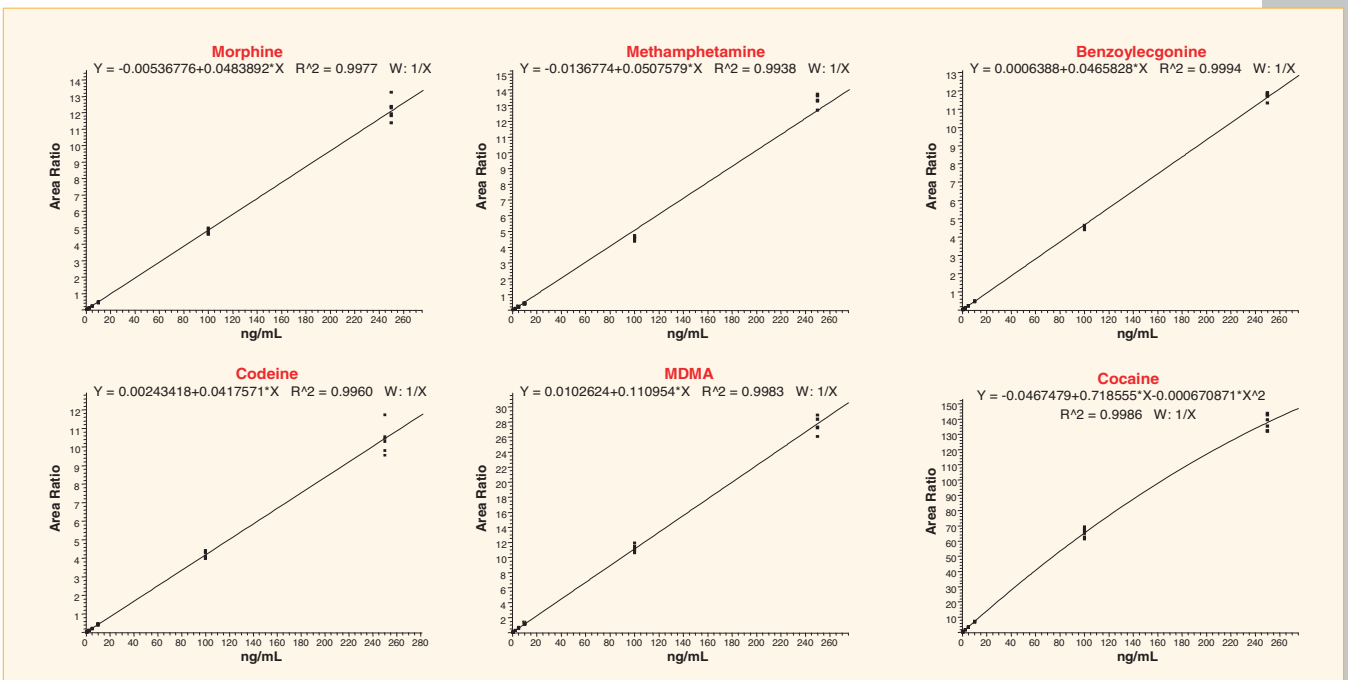

Figure 3: Calibration curves for select drugs of abuse. Regression curve fitting used 1/x weighting from five replicate injections, where $R^2 > 0.993$ for all standards.

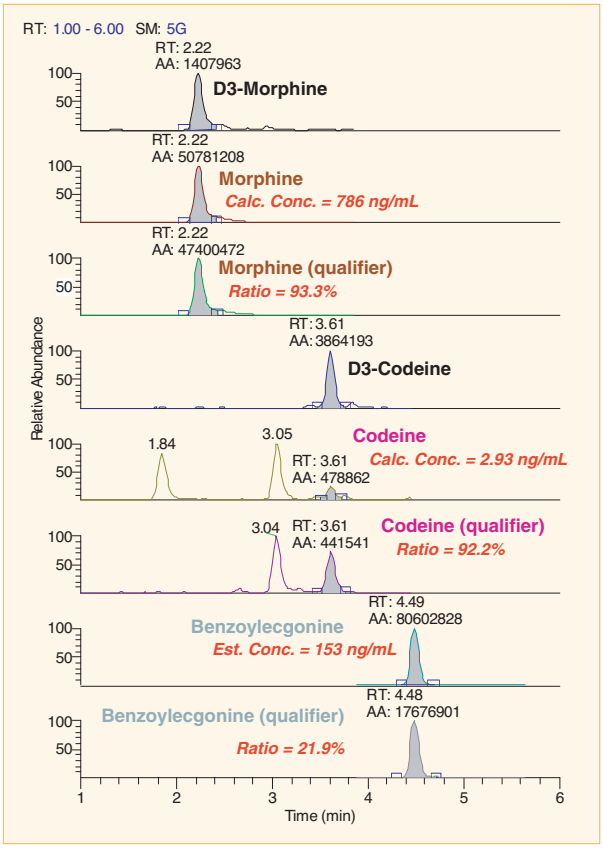

Figure 4A: Assay of urine extract (#423) targeting morphine and its metabolites. The concentration of benzoylecgonine is estimated because a labeled internal standard was not added to the sample extract.

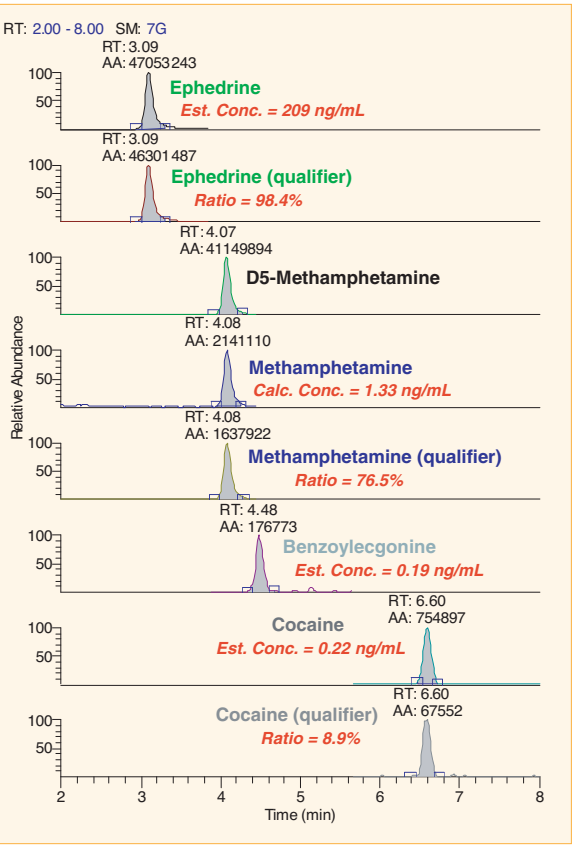

Figure 4B: Assay of whole blood extract (#473) targeting amphetamine and its metabolites. The concentrations of ephedrine, benzoylecgonine and cocaine are estimated because labeled internal standards were not added to sample extract.

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Determination of LSD and Its Metabolites in Human Biological Samples by Liquid Chromatography–Tandem Mass Spectrometry

François-Ludovic Sauvage[1], Pierre Marquet[1,2]

[1]*Department of Pharmacology-Toxicology, University Hospital, Limoges, France.*
[2]*Laboratory of Pharmacology, Faculty of Medicine, University of Limoges, France.*

Key Words

- TSQ Quantum
- Drugs of Abuse
- Forensic analysis
- LC-MS/MS
- LSD (Lysergic acid diethylamide
- LSD metabolites
- Toxicology

Introduction

Lysergic acid diethylamide (LSD) is a very potent hallucinogenic drug involving, particularly, behavioral disorders and is also extensively metabolized in man. Moreover, LSD and its major metabolites are present at low concentration in biological fluids, such as whole blood or urine. Identification and quantitation of such compounds for forensic use necessitate a sensitive and specific method. This study aims to describe a method using liquid chromatography/tandem mass spectrometry and permitting to quantify LSD and its metabolites at low concentrations.

Goal

The goal of this study was to identify and quantify LSD, iso-LSD, nor-LSD, nor-iso-LSD and 2-oxo-3-hydroxy-LSD in biological matrices. This report demonstrates the use of the TSQ Quantum for this application.

Experimental Conditions/Methods

Chemicals and Reagents

Lysergic acid diethylamide (LSD), d_3-LSD (internal standard), 2-oxo-3-hydroxy-LSD, iso-LSD, nor-LSD were purchased from Cerilliant (Austin, TX, USA). Ammonium formate and formic acid (>99 % pure) were purchased from Sigma. All reagents and solvents used in the extraction procedures were of analytical grade.

Sample Preparation

To 2 mL of serum, urine or whole blood content were added 100 µL of a 0.025 µg/mL aqueous solution of d_3-LSD (Internal Standard), 1 mL of a solution of pH 9.50 carbonate buffer and 8 mL of dichloromethane-isopropanol (95:5 by volume). The tubes were vortex-mixed and shaken on an oscillatory mixer. After centrifugation at 3,400 g for 5 min, the organic phase was poured in a conical glass tube and evaporated under a stream of nitrogen at 37°C. The dried extracts were reconstituted in 25 µL of acetonitrile : pH 3.0, 2 mmol/L ammonium formate (30:70 by volume) and 10 µL were injected into the chromatographic system.

Instrumentation Methods

HPLC Conditions

The chromatographic system consisted of a Shimadzu 10ADvp micro-flow rate, high-pressure gradient pumping system with a Rheodyne® Model 7725 injection valve equipped with a 5 µL internal loop. A C18, 5 µm (50×2.1 mm) column, maintained at 25°C, was used with a linear gradient of mobile phase A (pH 3.0, 2 mmol/L ammonium formate) and mobile phase B (acetonitrile:pH 3.0, 2 mmol/L ammonium formate [90:10; v/v]), flow rate of 200 µL/min, programmed as follows: 0-1.5 min, 5% B; 1.5-9 min, 5 to 50% B; 9-10 min, 50 to 90% B; 10-10.5 min, decrease from 90 to 5% B; 10.5-13 min, equilibration with 5% B.

MS Conditions

Mass Spectrometer: Thermo Scientific TSQ Quantum
Source: ESI mode
Ion Polarity: Positive
Spray Voltage: 4000 V
Sheath/Auxiliary gas: Nitrogen
Sheath gas pressure: 25 (arbitrary units)
Auxiliary gas pressure: 15 (arbitrary units)
Ion transfer tube temperature: 250°C
Scan type: SRM
Collision gas: Argon
Collision gas pressure: 1.5 mTorr

SRM Conditions

Settings were optimized by infusing at 5 µL/min a 1 µg/L solution containing the studied compound in acetonitrile: pH 3.0, 2 mmol/L ammonium formate (30:70, by volume). The structure of these compounds is shown in Figure 1.

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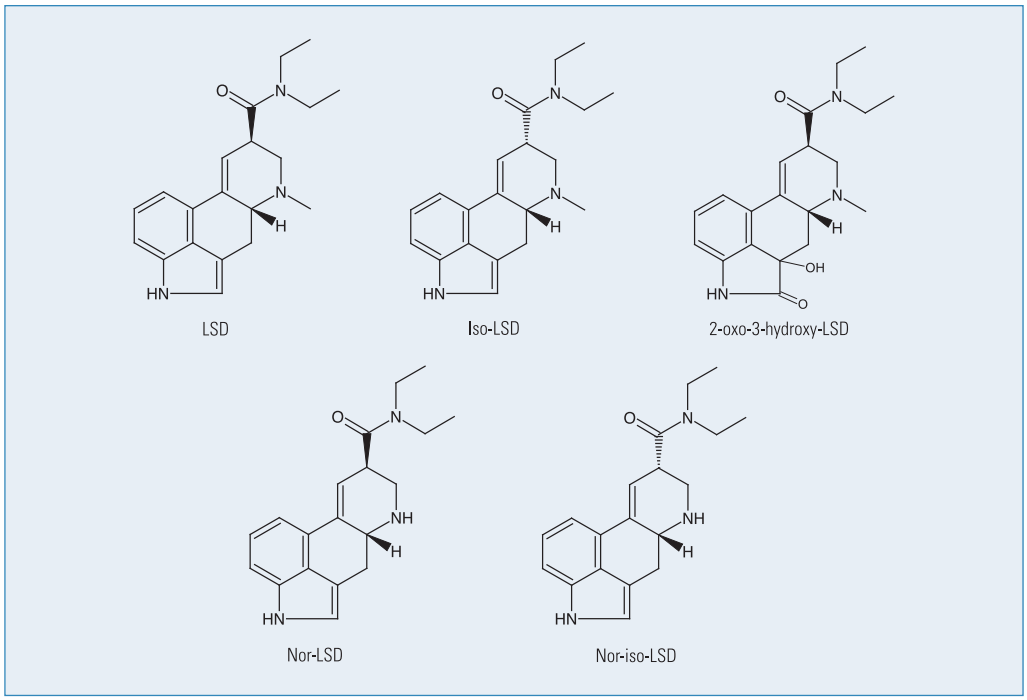

Figure 1: Structures of investigated compounds

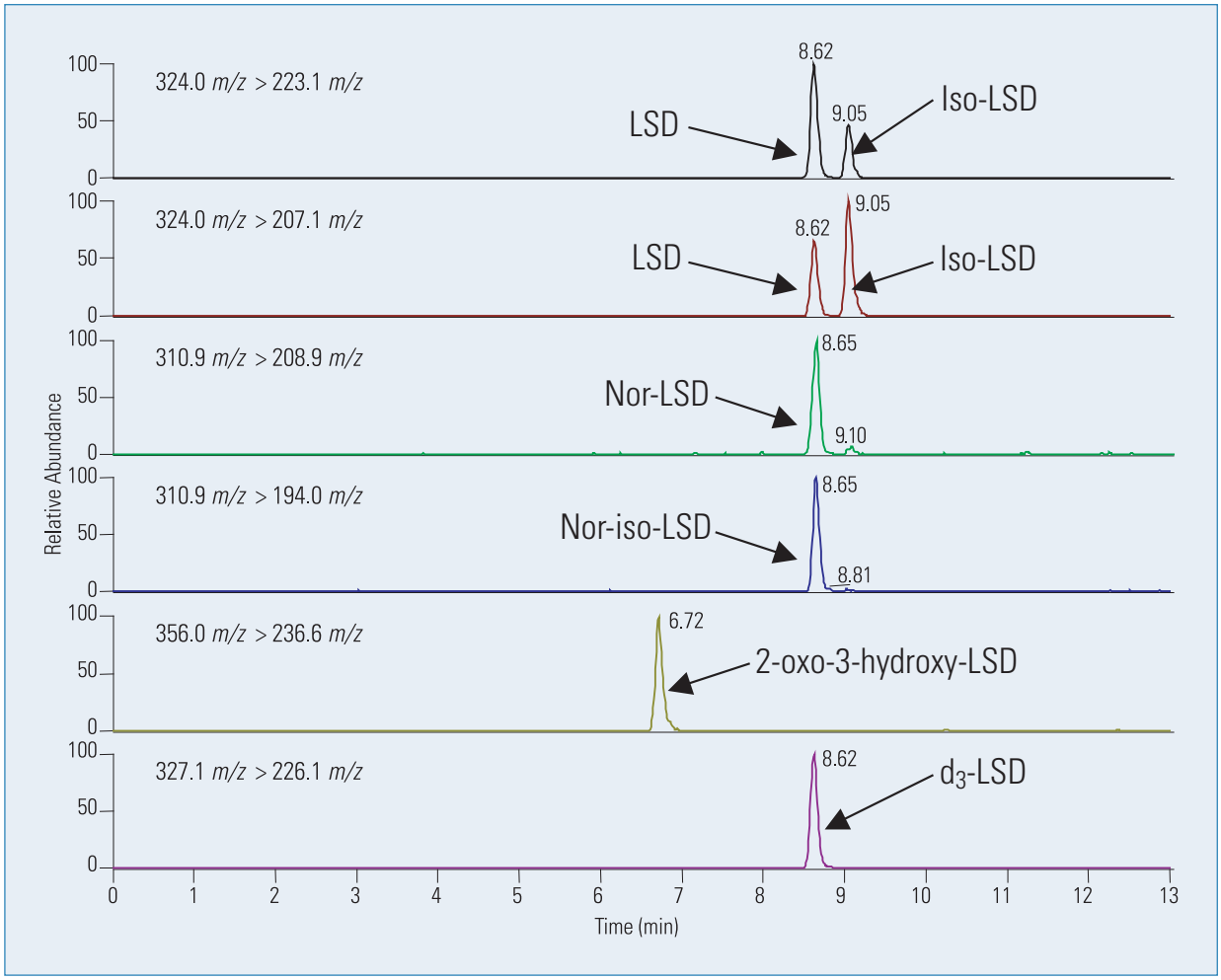

Figure 2: Chromatogram of a urine spiked at 0.5 ng/mL

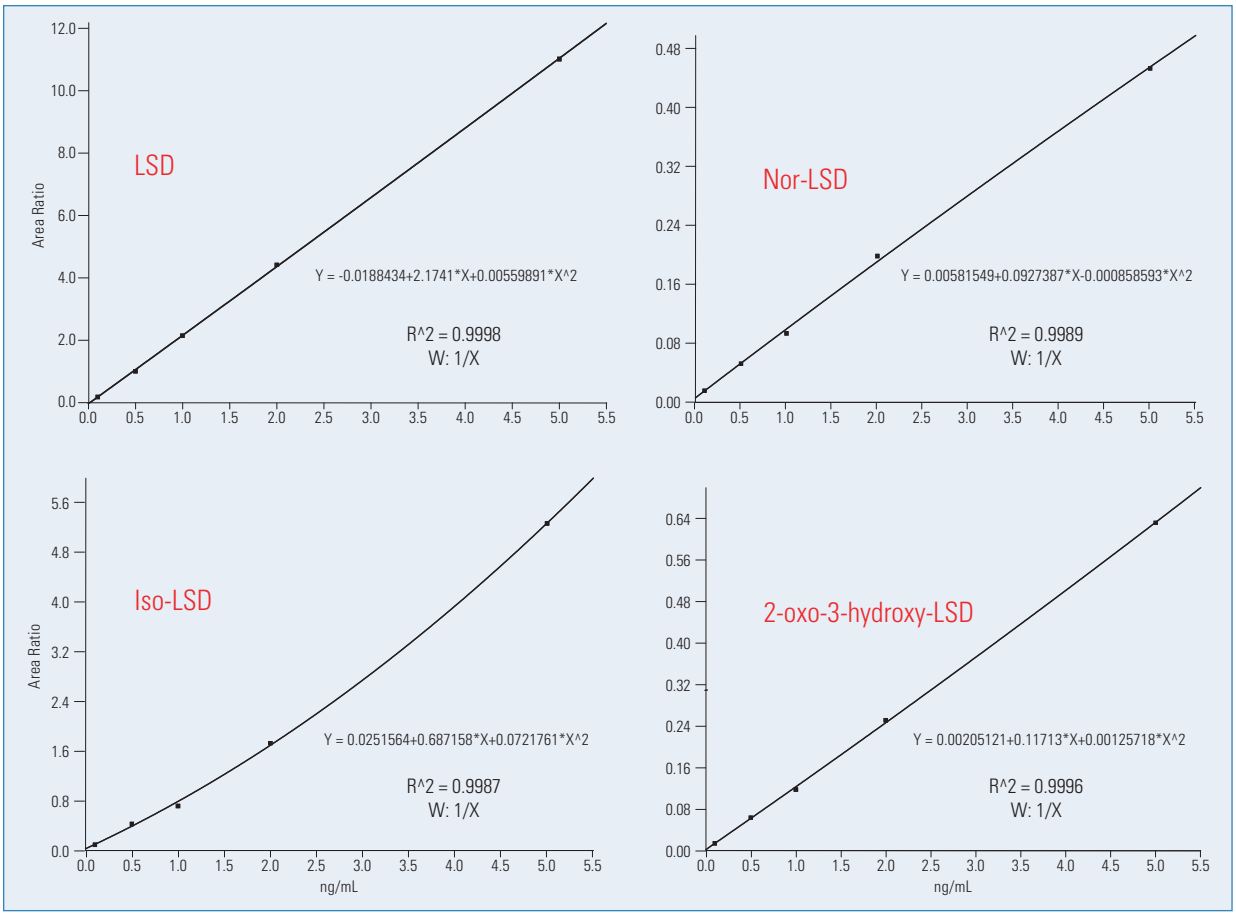

Figure 3: Representative calibration curves from standards spiked in urine

Compounds	Quantification transition	Collision energy	Confirmation transition	Tube lens voltage
LSD	324.0/223.1	30	324.01/207.1	50
Iso-LSD	324.0/223.1	30	324.01/207.1	50
Nor LSD	310.9/208.9	28	310.91/194.0	54
Nor-iso-LSD	310.9/208.9	28	310.91/194.0	54
2-oxo-3-hydroxy-LSD	356.0/236.6	30	356.01/222.0	36
d3-LSD	327.1/210.1	50	327.11/226.2	30

Results and Discussion

The LC-ESI/SRM chromatograms obtained for a blank urine spiked at 0.5 ng/mL are shown in Figure 2. As presented, LSD and iso-LSD are separated using the chromatographic conditions described previously. Identification of LSD is performed using two characteristic transitions and the retention time given by its deuterated internal standard.

Linearity

Calibration curves obtained for each compound spiked in urine samples are presented in Figure 3. Concentration ranges were comprised between 0.1 ng/mL and 5 ng/mL.

Conclusion

This application note described a sensitive, specific method developed for the quantitation of lysergide and metabolites in various biological matrices for forensic use.

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Analysis of Multiple Illicit Drugs, Methadone, and their Metabolites in Oral Fluid Using a Linear Ion Trap Mass Spectrometer

Min He, Gargi Choudhary, Diane Cho, Karen Salomon and Julian Phillips, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- LXQ
- Surveyor Plus
- Drugs of Abuse Forensics
- Hypersil GOLD Columns
- MS^3 Quantification

Introduction

Traditionally, the analysis of urine samples has been the major approach for the detecting of drugs of abuse.[1] However, a common risk for this type of analysis is adulteration or manipulation of the sample at the point of collection. As an alternative, the analysis of oral fluid provides an easy method of sample collection and has the advantage of providing a relatively clean matrix. Because of the reduced sample volume, this technique requires a high sensitivity and robust analytical method to make an attractive alternative to conventional methods.

In this report, a rapid and rugged LC-MS/MS method using the Thermo Scientific LXQ is described for analyzing a mixture of twenty drugs and their metabolites using intelligent automated mass spectrometry (INTAMS). The detection limits for the mixture of drugs and dynamic range are superior to results reported previously.[2] In addition, this method provides for the simultaneous identification and quantification of drugs and their metabolites.

Experimental Conditions

Sample Preparation:

Ten milliliters of oral fluid collected from a volunteer were protein precipitated using 30 mL acetonitrile. The sample was vortexed and then centrifuged at 5,000 rpm for 10 minutes. The supernatant was evaporated to dryness under nitrogen and reconstituted in 5 mL water. Table 1 provides a list of 20 drugs along with the parent and product ion masses. For quantification experiments, known amounts of a stock solution of the 20 drug mixture were spiked into the treated oral fluid to prepare the standards in concentrations ranging from 50 fg/µL to 1 ng/µL.

Compound	Parent ion *m/z*	Product ions *m/z*
EEE[a]	214.3	196.2
Normorphine	272.3	201.0
AEM[b]	182.3	150.1, 122.1
Morphine	286.3	229.1, 211.2
Norcodeine	286.3	243.3, 225.3, 215.0
Codeine	300.3	175.0, 225.3
6-Acetylmorphine	328.3	268.3, 193.2
m-Hydroxybenzoylecgonine	306.2	168.2
Benzoylnorecgonine	276.2	154.1
Benzoylecgonine	290.3	168.2
Acetylcodeine	342.3	282.3, 225.2
Heroin	370.3	310.2, 328.2, 268.3
Cocaine	304.3	182.1
Norcocaine	290.2	168.1, 136.2
Cocaethylene	318.3	196.2
Norcocaethylene	304.2	182.1, 136.1
Methadol	312.3	223.1, 249.2, 171.2
EDDP[c]	278.0	249.2
Propoxyphene	340.1	266.1
Methadone	310.9	266.2

Table 1: List of 20 drugs and metabolites with their respective parent and product ion masses. EEE: ecgonine ethyl ester; AEM: anhydroecgonine methyl ester; EDDP: 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium

HPLC:

LC System: Thermo Scientific Surveyor Plus

Column: Thermo Scientific Hypersil GOLD™ (20×2.1 mm, 1.9 µm particle size)

Mobile phase:

(A) water with 0.1% formic acid and 10 mM ammonium acetate

(B) acetonitrile with 0.1% formic acid

Flow rate: 400 µL/min

Injection volume: 10 µL

Gradient:

t (min)	A%	B%
0.00	95	5
0.10	95	5
1.00	85	15
4.20	50	50
4.21	95	5
7.00	95	5

Mass Spectrometer:

The LXQ linear ion trap mass spectrometer was operated in positive atmospheric pressure chemical ionization (APCI) mode. The corona discharge needle voltage was 4.5 kV and the vaporizer temperature was 400 °C. The capillary temperature was 220 °C and the sheath gas flow was 25 units. All scan events were acquired with one micro scan. No internal standard was used. The set up of the acquisition method using INTAMS is shown in Figure 1.

Results and Discussions

INTAMS data acquisition software was used for the simultaneous identification of 20 drugs in oral fluid. The extracted ion chromatogram is shown in Figure 2. INTAMS software enables the maximum number of scans to be acquired under a given chromatographic peak by obtaining MS/MS spectra on only the masses identified within a specified time window which helps facilitate a faster duty cycle.

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In addition, the excellent ion statistics and the fast cycle time of the LXQ linear ion trap mass spectrometer enabled the simultaneous quantification and identification of these analytes. Calibration curves based on MS/MS spectra were generated using the standards for the drug mixture spiked in oral fluid over a concentration range from 50 fg/µL to 1.0 ng/µL. Figure 3 shows calibration curves for 8 of the 20 compounds analyzed simultaneously. The R^2 values of these curves are better than 0.996 and they exhibit linear dynamic range over 3 to 4 orders of magnitude. The detection limits (LOD and LOQ) for each analyte in oral fluid are listed in Table 2 along with the linear dynamic ranges. Compared with data published previously[2], the LXQ linear ion trap provided up to 10 times lower detection limits and an increased linear dynamic range.

Further confirmatory information and higher specificity results were also easily generated by performing quantification based on MS^3 data. The use of MS^3 quantification is demonstrated for the ecogonine ethyl ester sample (EEE) which undergoes a neutral loss of water molecule upon ion activation. When spiked in oral fluid, interference from the matrix masked the analyte peak. This was overcome as shown in Figure 4. The signal-to-noise ratio (S/N) of the extracted ion chromatogram obtained from MS^3 data (top chromatogram) is dramatically higher than that obtained from the MS/MS data. The high quality of the MS^n spectra obtained using the LXQ also results in greater sensitivity over a wider linear dynamic range (Figure 4b and 4c).

The quantitative study was completed by analyzing two QC oral fluid samples, each containing a mixture of ten drugs. The results shown in Table 3 demonstrate a high level of quantification accuracy, with a deviation of less than 10% for all the analytes. In addition, excellent reproducibility was demonstrated with the %RSD being less than 9% for all the compounds within five injections.

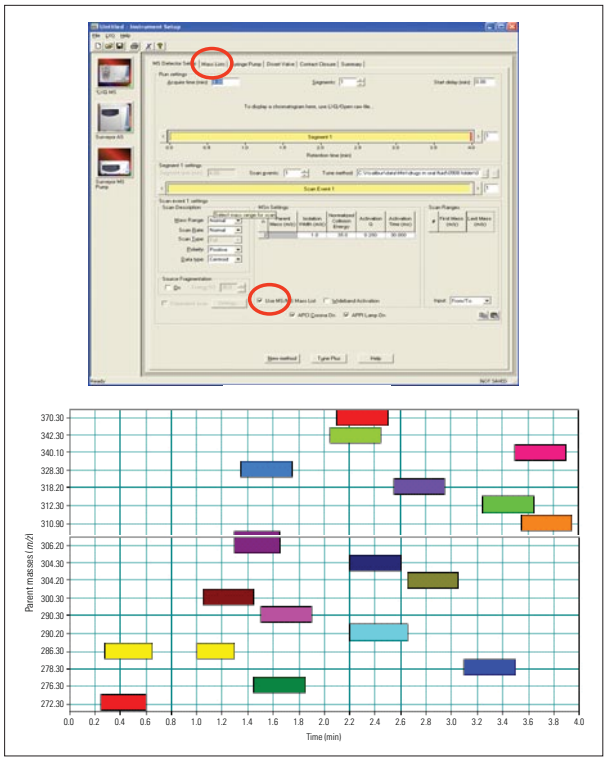

Figure 1: INTAMS (Intelligent Automated Mass Spectrometry) data acquisition software setup for simultaneous analysis of 20 compounds

Data Analysis

Mass Frontier™ software includes a number of tools for structure identification. The powerful search features and database management make it valuable for identifying drugs, metabolites and related compounds. A library of target drugs can be easily searched. As an example, the MS/MS spectrum obtained from 6-acetylmorphine in oral fluid was searched against an NIST library using Mass Frontier software. In addition to being the top hit (Figure 5), the chromatographic elution time and the mass of the precursor ion provide added degrees of confidence for identification.

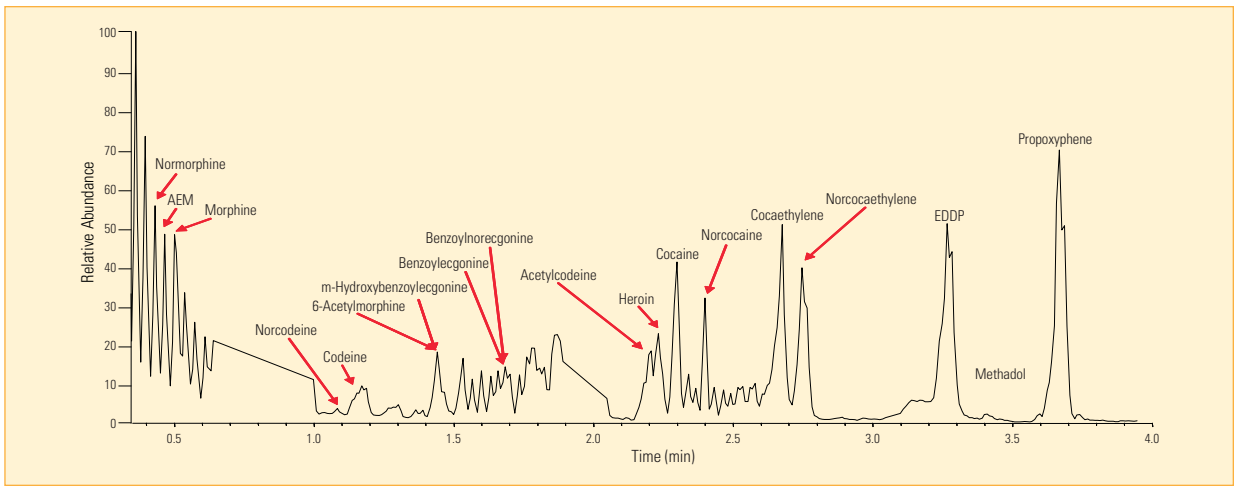

Figure 2: Chromatogram of the drugs and metabolites in oral fluid using LC-MS/MS with INTAMS data acquisition software

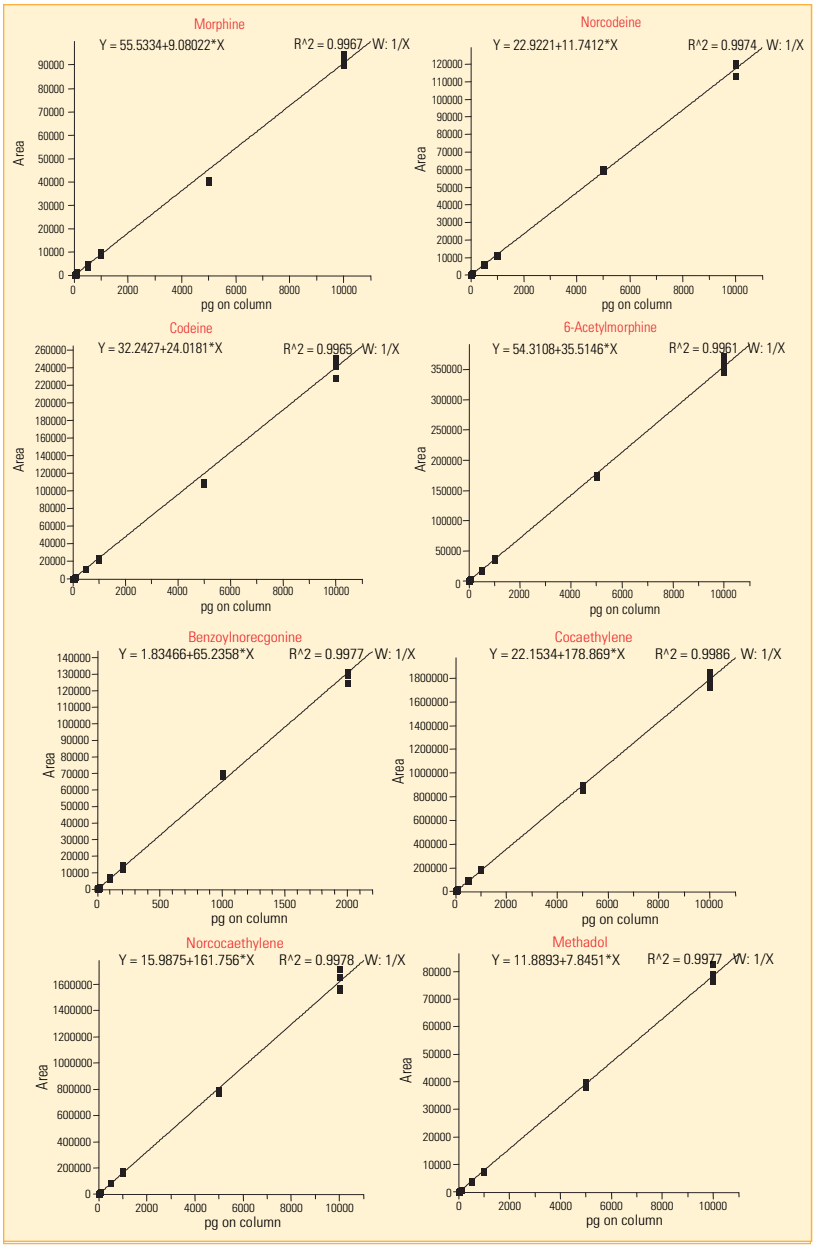

Figure 3: Representative calibration curves for eight drugs in oral fluid

Conclusions

Rigorous simultaneous characterization and quantification of a large number of drugs and their metabolites in a biological matrix can be performed in a fast and robust LC-MS/MS method using an LXQ linear ion trap mass spectrometer. The superior sensitivity and faster cycle time of the LXQ makes this possible in a single chromatographic run, resulting in high throughput analyses. High specificity quantification was done using MS^3 data which can reduce overall chemical noise even if there is a co-eluting isobaric interfering ion. Additional compound confirmation was obtained using Mass Frontier software, where a high match score to a library search provided enhanced confidence in the compound identification.

Acknowledgements

The authors would like to thank Dr. C. Murphy for her assistance and technical discussions. C. Yang and R. Chen are acknowledged for suggestion and advice.

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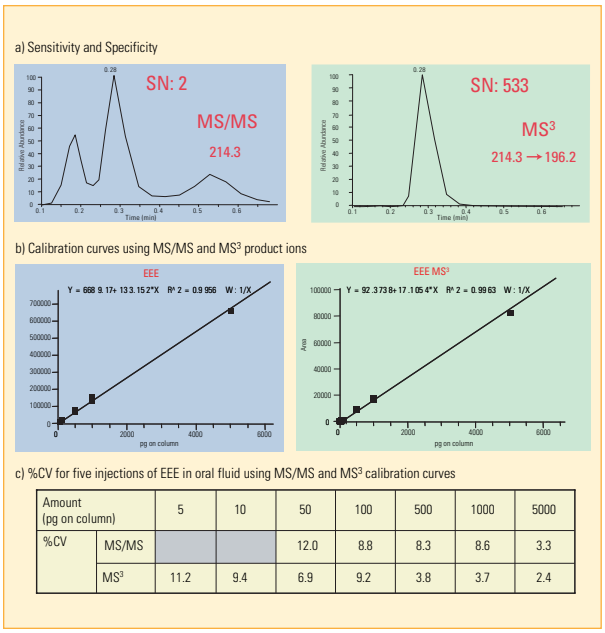
Compound	LOD (pg)	LOQ (pg)	Linear dynamic range (pg)
EEE	1	5	5-5000
Normorphine	5	10	10-10000
AEM	5	10	10-10000
Morphine	5	10	10-10000
Norcodeine	5	10	10-10000
Codeine	1	5	5-10000
6-Acetylmorphine	1	5	5-10000
m-Hydroxybenzoylecgonine	0.2	1	1-2000
Benzoylnorecgonine	0.2	1	1-2000
Benzoylecgonine	0.5	1	1-10000

Compound	LOD (pg)	LOQ (pg)	Linear dynamic range (pg)
Acetylcodeine	0.5	1	1-10000
Heroin	0.5	1	1-10000
Cocaine	0.5	1	1-10000
Norcocaine	0.5	1	1-10000
Cocaethylene	0.5	1	1-10000
Norcocaethylene	0.5	1	1-10000
Methadol	1	5	1-10000
EDDP	0.5	1	1-10000
Propoxyphene	1	5	5-10000
Methadone	0.5	1	1-10000

Table 2: LOD (limit of detection), LOQ (limit of quantification) and linear dynamic range for analysis of 20 drugs and metabolites in oral fluid using the LXQ linear ion trap mass spectrometer



Amount (pg on column)		5	10	50	100	500	1000	5000
%CV	MS/MS			12.0	8.8	8.3	8.6	3.3
	MS^3	11.2	9.4	6.9	9.2	3.8	3.7	2.4

Figure 4: Analysis of EEE (Ecgonine Ethyl Ester) in oral fluid using MS/MS and MS^3 spectra product ions

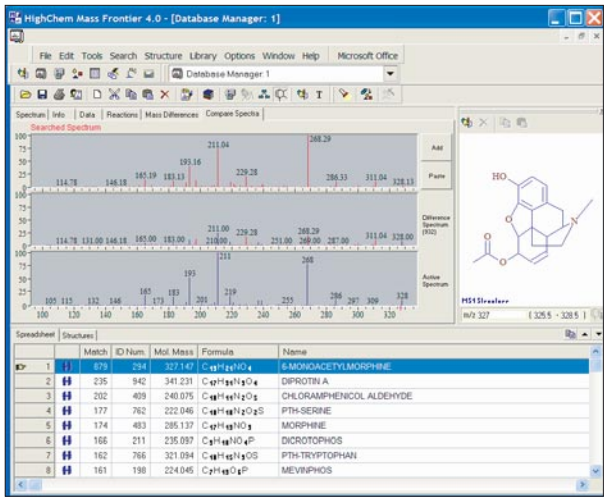


Figure 5: Library search results for 6-acetylmorphine using Mass Frontier software. High match score is highlighted

Compound	QC Sample I (5 injections) Conc (pg)	Calc. conc. (pg)	% Diff	% RSD	QC Sample II (5 injections) Conc (pg)	Calc. conc. (pg)	% Diff	% RSD
EEE[a]	200.0	183.2	-8.4	4.6	40.0	37.7	-5.7	5.6
Morphine	200.0	189.2	-5.4	7.6	40.0	40.4	1.0	8.9
Norcodeine	200.0	190.8	-4.6	5.5	40.0	40.1	0.3	7.8
6-Acetylmorphine	200.0	182.2	-8.9	8.1	40.0	41.0	2.6	8.4
Cocaethylene	133.3	120.1	-9.7	7.4	26.7	26.3	-1.5	1.6
Norcocaethylene	200.0	190.6	-4.7	5.5	40.0	42.0	4.9	7.4
Methadol	200.0	184.6	-7.7	9.6	40.0	37.6	-6.1	3.8
EDDP	133.3	121.4	-8.9	4.9	26.7	24.8	-7.1	4.4
Propoxyphene	200.0	190.4	-4.7	4.0	40.0	42.4	6.3	5.8
Methadone	133.3	122.5	-9.5	7.2	26.7	24.9	-6.8	3.9

Table 3: Quantification results for the analysis of unknown levels of drugs in oral fluid. [a] *based on MS^3 results*

Mass Spectrometers are general purpose laboratory instruments. They have not been cleared or approved by the United States Food and Drug Administration, the European IVD Directive or any other agency for diagnostic, clinical or other medical use.

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Rapid Quantitative and Confirmational Screening for Drugs in Race Horse Urine by ESI-LC-MS/MS and MS^3

Patrick Russell[1], Paul Steinberg[2], Mary L. Blackburn[2], Diane Cho[2], Robert Heather[2]
[1]University of Florida, Racing Laboratory, Gainesville, Florida; [2]Thermo Fisher Scientific, San Jose, California

Key Words
- LTQ™
- Confirmation
- Drug Screening
- MS^3
- Quantitation

Introduction

Drugs of abuse in the horse racing industry encompass a variety of chemical classes and are typically analyzed from a complex urine matrix. These factors render the rapid and effective diagnostic screening of these drugs at low levels difficult. Traditionally, quantitation has been performed by triple quadrupole mass spectrometry using reaction monitoring (SRM) mode. However, this method does not monitor structurally diagnostic fragmentation. Thus, a second step involving derivatization and GC/MS confirmation was required.

Goal

To develop a simple and fast, yet rugged LC/MS based method capable of simultaneous qualitative and quantitative analysis. We have evaluated the application of the Thermo Scientific LTQ linear ion trap mass spectrometer for providing low levels of detection, good reproducibility, and wide linear dynamic range required for reliable quantitation, with simultaneous structural confirmation using diagnostic full-scan MS/MS or MS^3 mass spectrometry.

Experimental Conditions

Sample Preparation

Standards and Unknowns: Standards of the compounds listed in Table 1 were prepared neat and in urine. Urine standards and unknowns were spiked, dried, and reconstituted with 90% water and 10% acetonitrile with 0.1% acetic acid. Typical Instrument Setup settings are shown in Figure 1.

HPLC

HPLC System: Thermo Scientific Surveyor™ LC system

Column: Thermo Scientific BETASIL™ C18, 3 µm, 100 × 2.1 mm

Flow Rate: 350 µL/min

Injection Volume: 10 µL (full loop)

Mobile Phase: (A) Water with 0.1% acetic acid
(B) Acetonitrile with 0.1% acetic acid

Gradient: 92% A to 90% B.

MS

Mass Spectrometry: Thermo Scientific LTQ linear ion trap mass spectrometer

API Source: Thermo Scientific Ion Max™ source with electrospray ionization (ESI) probe

Ion Transfer Capillary: 220°C; Sheath Gas: 30 units

Auxiliary Gas: 0 units; Sweep Gas: 20 units

Spray Voltage: 4.5 kV; Isolation Width: 3 amu

Normalized Collision Energy™: 28%

WideBand Activation™: Applied as needed (see Table 1)

Ion Polarity Mode: positive or negative (see Table 1)

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Results

Quantitation

Calibration curves were established using neat standards based on ion intensities from full-scan MS/MS chromatograms. Chromatograms for all the compounds listed in Table 1 were obtained in a single chromatographic run at each concentration. Figure 2 shows reconstructed ion chromatograms (RICs) from the analysis of the 50 pg/µL standards. The MS/MS spectra for all the drugs, with the exception of ketoprofen, are shown in Figure 3.

The MS/MS spectra were generated using a Normalized Collision Energy of 28%. The use of Normalized Collision Energy alleviates the necessity to optimize the collision energy for each compound as is necessary in traditional triple-quadrupole analysis, thus making this method extremely easy to set up and run. Compounds that underwent a non-specific water loss were additionally fragmented using WideBand Activation (see Table 1). This mode of fragmentation results in information-rich spectra enabling structural confirmation without requiring an additional MS^3 transition. The compound ketoprofen undergoes a neutral loss outside of the WideBand Activation window and was selected for an MS/MS to MS^3 comparison study and is discussed later.

Chromatographic and mass spectrometric methods were validated using the neat standards; subsequently the experiments were repeated using standards in horse urine. The RICs from these experiments are shown in Figure 4. Using the RICs, calibration curves were created for each of the compounds either neat (Figure 5) or in urine matrix (Figure 6). The calibration curves were linear over the three orders of magnitude assayed. In addition to demonstrating linearity, the quantitative results shown in Tables 2 and 3 demonstrate excellent reproducibility.

SEGMENT	RT	ID#	COMPOUND	*M/Z*	WB	RIC
1	3.40	416	Theobromine	181.0		137 + 163 + 181
	4.44	417	Theophylline	181.0		124 + 137
	4.56	152	Dyphylline	255.1		181
2	5.58	071	Caffeine	195.1		138
	5.71	089	Chlorothiazide	-293.9		214 + 215
	6.02	107	Cromolyn-Na	469.2	✔✔✔	245
	6.20	198	Hydroclorothiazide	-295.8		205 + 232 + 269
	6.49	311	Pemoline	177.0		106
3	7.20	614	Petoxifyline	279.1		181
4	8.95	117	Dexamethasone	393.1	✔	355 + 337 + 319
	9.60	481	Boldenone	287.1	✔	121 + 135 + 173
	10.16	499	Ketoprofen†	255 (209)		209 (105 + 194)
5	11.28	216	Indomethacin	358.0		139 + 174
	11.33	130	Diclofenac	295.9	✔	215 + 250
	11.93	175	Flufenamic Acid	282.1		264
	12.05	235	Meclofenamic Acid	295.9	✔	242 + 243

† *Ketoprofen was analyzed by both MS/MS and MS^3 for comparison study.*
4 *WB denotes use of wideband activation during MS/MS fragmentation.*

Table 1: List of target compounds; corresponding RT (retention time), segment (method segment see Figure 1), *m/z* denotes isolation mass and Ion Polarity, WB–WideBand Activation, RIC–masses used in generation of Reconstructed Ion Chromatograms for quantitation.

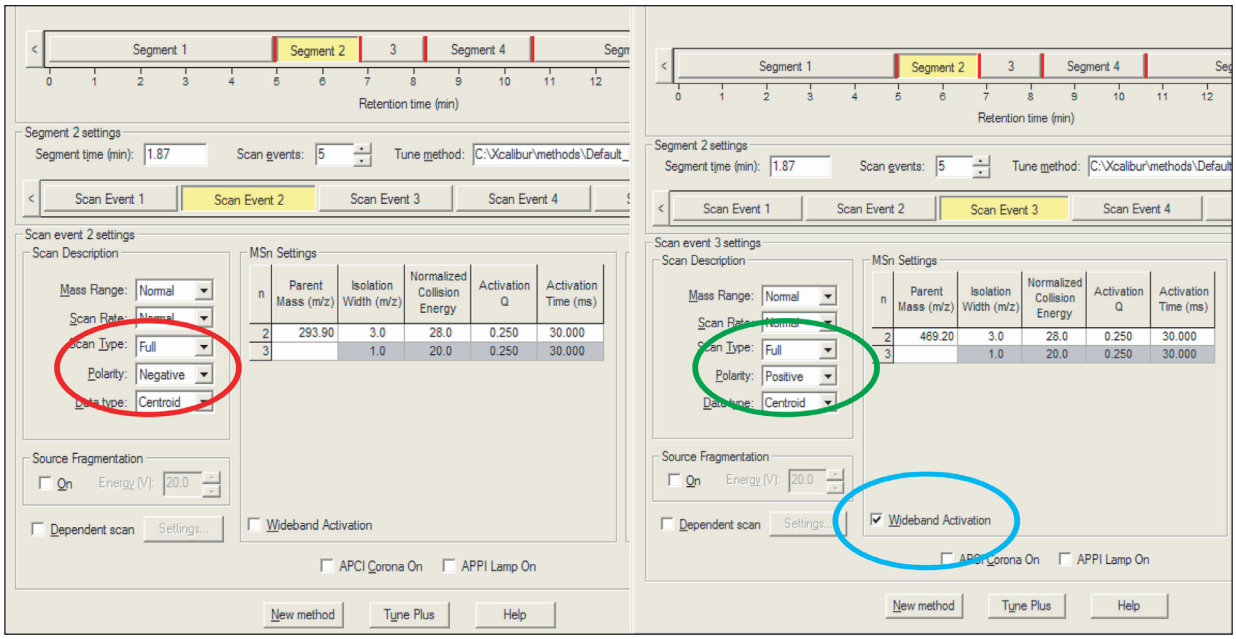


Figure 1: Instrument Setup settings for chlorothiazide (segment 2, scan event 2) and cromolyn-Na (segment 2, scan event 3)

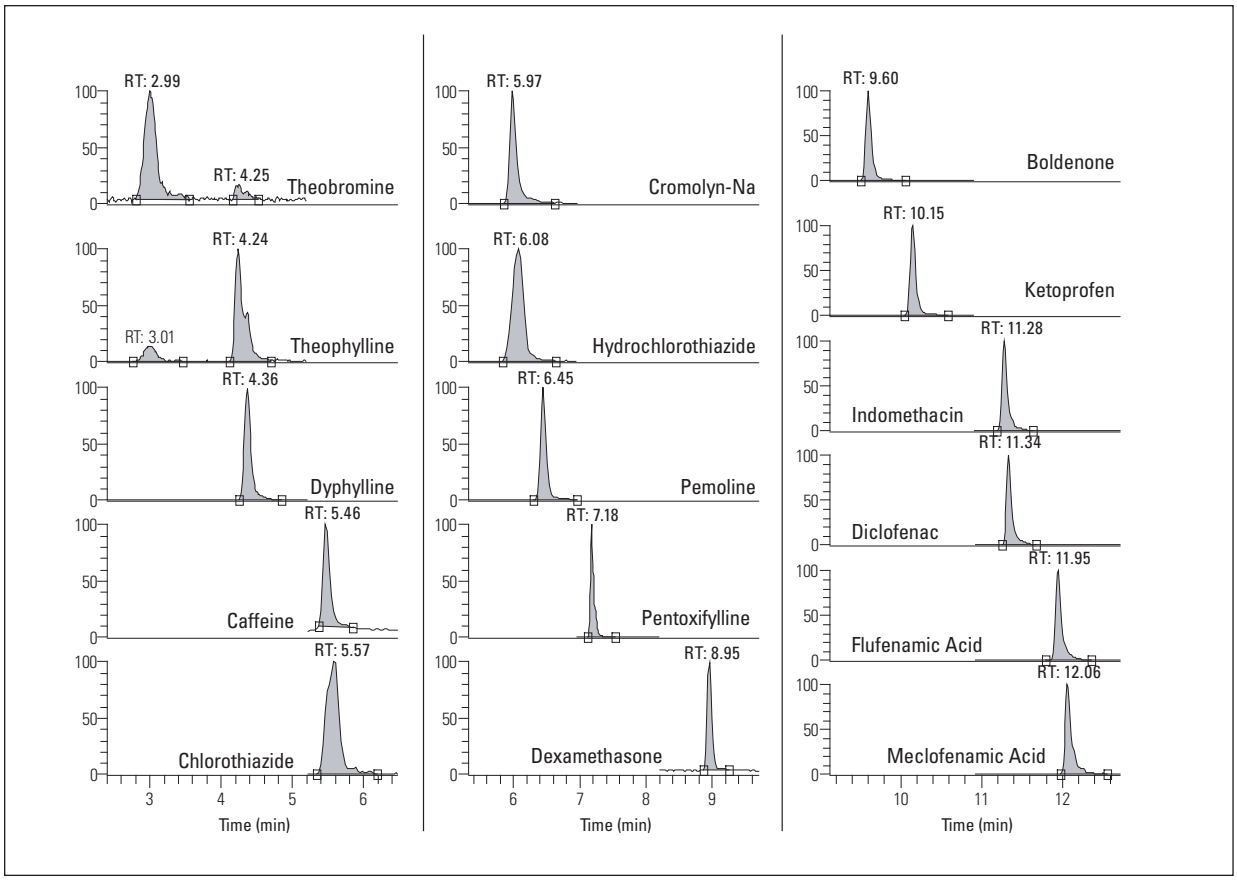


Figure 2: Reconstructed ion chromatograms, using the product ions listed in Table 1 (RIC column), from the analysis of 10 µL of the 50 pg/µL standards in solvent

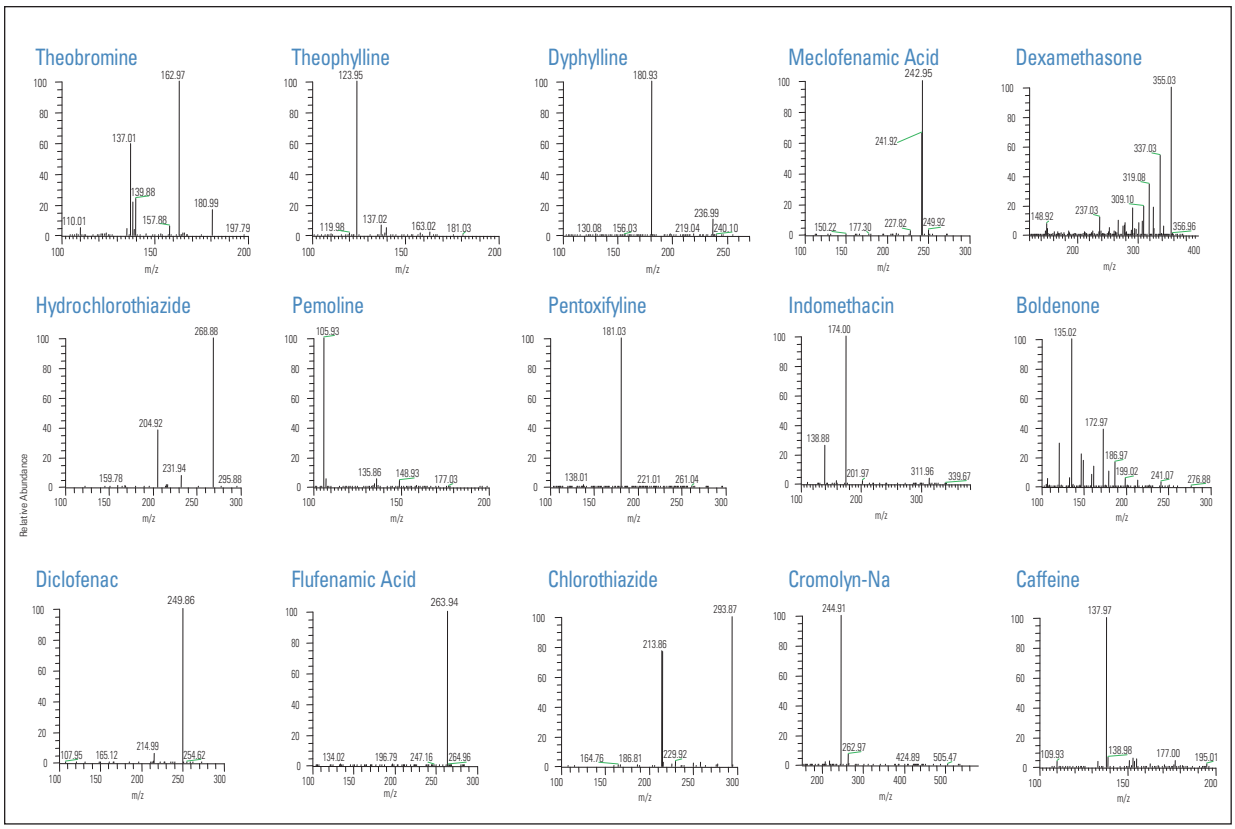


Figure 3: Full-scan MS/MS spectra corresponding to compounds depicted in Figure 2

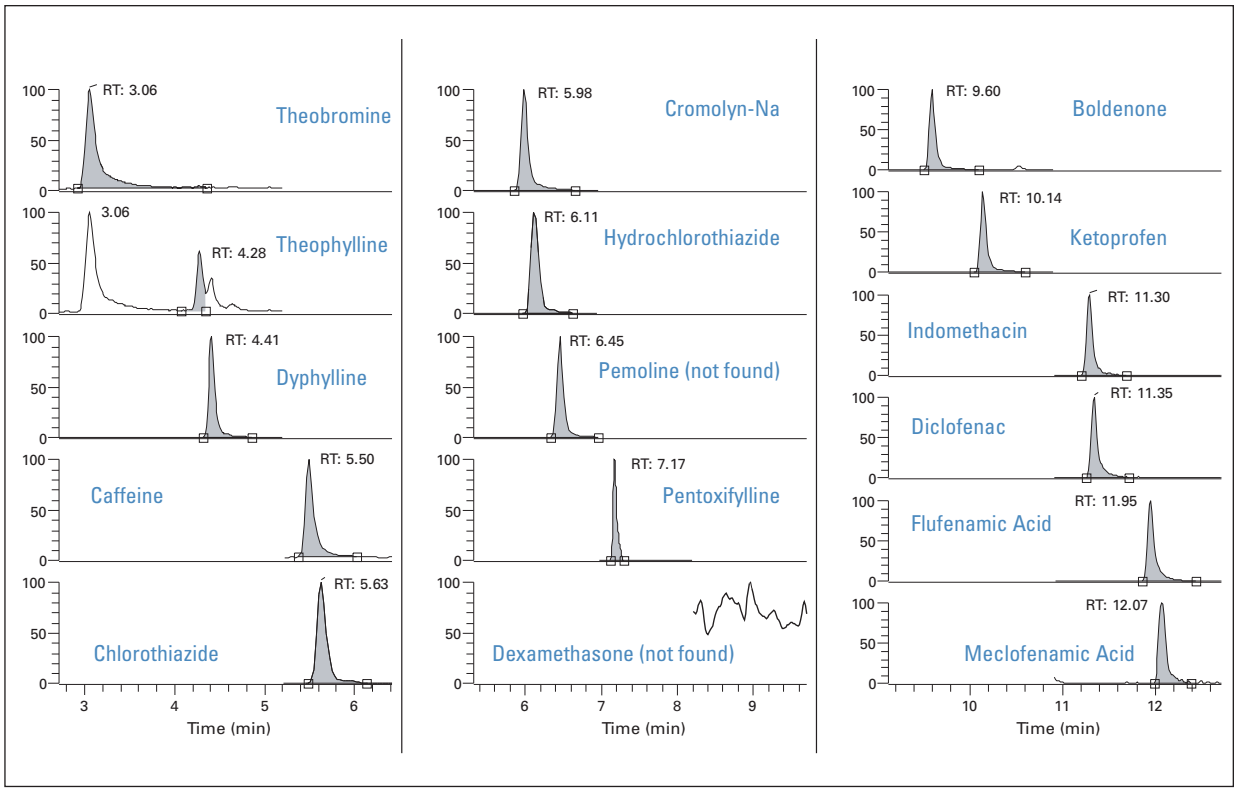


Figure 4: Reconstructed ion chromatograms, using the product ions listed in Table 1 (RIC column), from the analysis of a 10 µL injection of the 50 pg/µL standard in horse urine

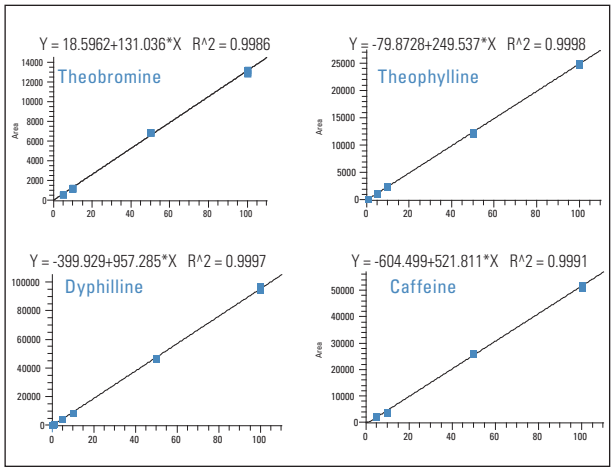


Figure 5: Representative calibration curves from standards prepared in solvent

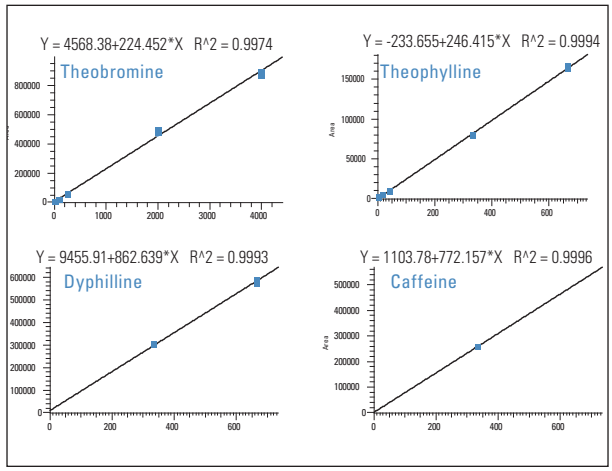


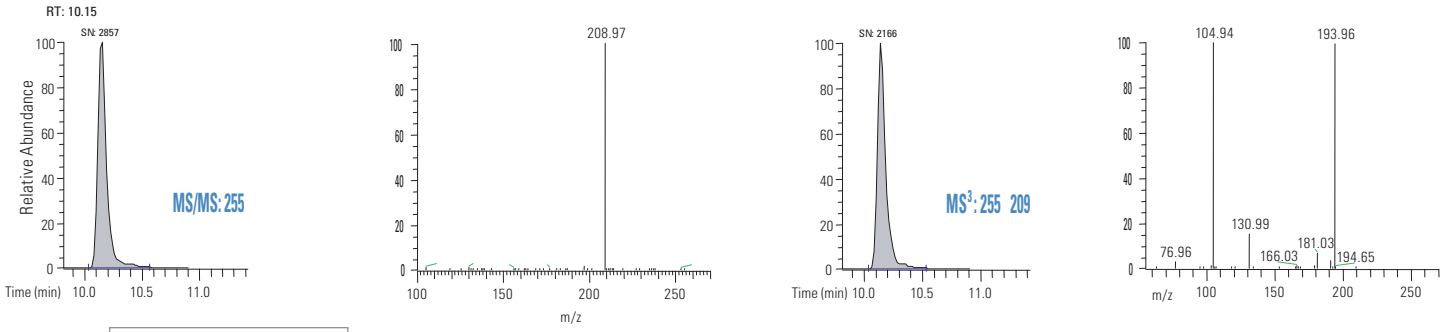
Figure 6: Representative calibration curves from standards prepared in horse urine

The %RSD for three replicate injections is less than 10% for all neat standards at the 1 pg/µL level and higher (see Table 2). The results for standards in urine were also excellent. The %RSD, five replicate injections, for the lowest level assayed in urine was less than 10% for most analytes (see Table 3), and commonly less than 3% for mid- and high-concentration samples. To complete the quantitative study, two QC urine samples were analyzed. The results shown in Table 4 demonstrate a high level of quantitation accuracy, with a deviation of less than 10% for most analytes. In addition, excellent reproducibility was demonstrated with the %RSD being less than 8% for all but two compounds (see Table 4).

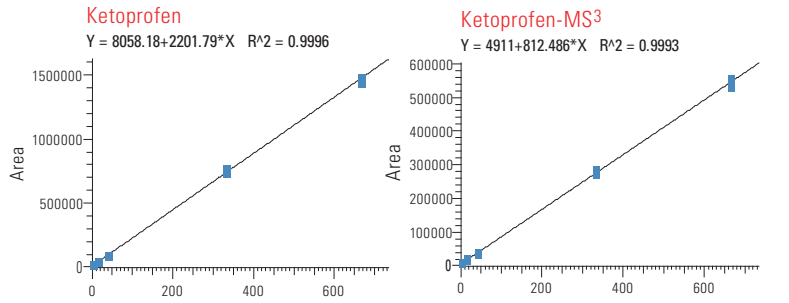
Ketoprofen – MS/MS vs. MS³: Ketoprofen undergoes a neutral loss of a 46 amu fragment in MS/MS mode due to the loss of the carboxyl group (see Ketoprofen structure). This is outside of the mass window for WideBand Activation and thus, an MS³ experiment was performed to generate additional diagnostic ions without sacrificing sensitivity or reproducibility. To demonstrate this, standards and two urine QC samples were analyzed in both MS/MS and MS³ mode, with results shown in Figure 7. There is no loss of sensitivity, accuracy, or reproducibility in obtaining this additional information. The %RSD from the MS/MS and MS³ data are virtually identical. While the sensitivity remains unchanged, the accuracy in the analysis of the unknowns is actually improved in the MS³ experiments (see Figure 7).

Robustness

To assess the ruggedness of the method, a 166 pg/µL standard in horse urine was assayed over 100 consecutive injections. The results are displayed in Figure 9. The mean and coefficient of variation (%CV) for four compounds: theobromine, caffeine, pentoxyphylline, and ketoprofen were determined to be less than 4% for all four compounds.



SAMPLE	KETOPROFEN AREA	KETOPROFEN CAL. CONC.	KETOPROFEN %RSD	KETOPROFEN - MS³ AREA	KETOPROFEN - MS³ CAL. CONC.	KETOPROFEN - MS³ %RSD
3.3 pg/µL	20350	6.073	3.75%	8205	3.874	4.05%
	19635	5.751		8012	3.636	
	20281	6.042		8201	3.87	
	20046	5.936		7928	3.531	
	19185	5.548		8137	3.79	
16.6 pg/µL	45386	17.362	3.86%	17551	15.425	2.82%
	43015	16.293		18007	15.988	
	44934	17.158		17120	14.892	
	42855	16.221		17380	15.214	
	42111	15.885		17830	15.769	
41.6 pg/µL	90353	37.637	2.23%	37454	40.022	2.09%
	87917	36.539		36469	38.805	
	87285	36.254		37773	40.417	
	91348	38.086		36210	38.485	
	87594	36.393		37289	39.818	
33.3 pg/µL	762536	340.72	1.67%	279903	339.663	1.97%
	760825	339.948		278910	338.436	
	755161	337.394		270466	328.	
	762369	340.644		282912	343.382	
	733111	327.452		284341	345.147	
650 pg/µL	1447518	649.573	1.72%	554232	678.703	2.86%
	1482572	665.378		519271	635.495	
	1505908	675.901		551821	675.723	
	1505854	675.876		552697	676.806	
	1466808	658.27		533792	653.441	



	Unknown 1 Conc. (pg/µL)	Unknown 1 Cal. Conc.	Unknown 1 Difference	Unknown 1 %RSD	Unknown 2 Conc. (pg/µL)	Unknown 2 Cal. Conc.	Unknown 2 Difference	Unknown 2 %RSD
Ketoprofen	41.6	38.0	91.4%	1.22%	104.1	104.6	100.5%	1.53%
Ketoprofen - MS³	41.6	41.7	100.2%	1.26%	104.1	106.1	101.9%	2.05%

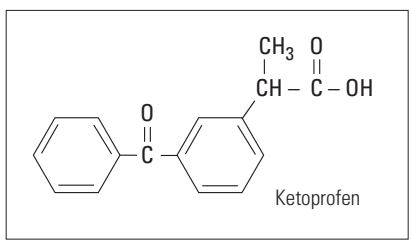


Figure 7: Comparison of MS/MS to MS³ quantitation of Ketoprofen

	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA
		0.1 pg/µL			0.5 pg/µL		
Theobromine							
Theophylline							234
Dyphylline	72	0.49	0.52%	427	1	2.40%	784
Caffeine							
Chlorothiazine				75	0	24.53%	149
Cromolyn-Na	176	0.25	42.33%	456	1	1.28%	917
Hydroclorothiazide							255
Pemoline	102	0.34	18.81%	400	1	11.86%	780
Petoxifyline	793	-0.32	3.79%	4308	0	1.86%	8319
Dexamethasone				479	0	2.45%	1017
Boldenone	219	-0.01	14.44%	1261	0	1.97%	2471
Ketoprofen	315	0.60	7.64%	1191	1	9.43%	2381
Indomethacin				103	1	11.11%	212
Diclofenac	64	0.30	11.55%	281	1	8.84%	475
Flufenamic Acid	310	0.22	13.97%	892	1	2.52%	1780
Meclofenamic Acid	14	0.18	17.51%	61	1	41.26%	113

Table 2: Quantitation results for standards prepared in solvent

	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD
	3.3 pg/µL			16.6 pg/µL			41.6 pg/µL			333.3 pg/µL		
Theophylline (A)	1129	6	10.48%	2540	17	5.53%	5185	38	2.82%	45170	334	2.11%
Dyphylline (A)	9832	5	1.94%	22461	17	2.73%	44917	39	0.98%	322434	334	2.24%
Caffeine (A)	6330	6	6.86%	15401	18	4.94%	30016	37	2.14%	258749	336	1.20%
Chlorothiazine (A)	1798	7	5.99%	3834	17	5.48%	7967	37	0.94%	70426	334	1.24%
Hydroclorothiazide (A)	2487	7	3.07%	5684	18	2.05%	11276	38	2.02%	92748	331	1.39%
Pentoxifylline (A)	122524	-2	-6.01%	296023	16	3.35%	605430	49	2.36%	3152583	332	0.97%
Boldenone (A)	13426	7	2.77%	33942	19	1.55%	58628	35	2.21%	593649	334	1.24%
Ketoprofen (A)	19899	6	3.75%	43660	17	3.86%	88899	37	2.23%	754801	337	1.67%
Ketoprofen – MS^3 (A)	8097	4	4.05%	17578	15	2.82%	37039	40	2.09%	279306	339	1.97%
Indomethacin (A)	1087	2	28.26%	2382	13	6.78%	6442	48	4.05%	35792	332	2.61%
Diclofenac (A)	2577	3	2.37%	5355	13	5.46%	14471	46	4.62%	78597	332	2.94%
Meclofenamic Acid (A)	290	7	10.78%	447	10	22.75%	2130	44	2.92%	6086	122	16.84%
	6.6 pg/µL			33.4 pg/µL			83.3 pg/µL			333.3 pg/µL		
Cromolyn-Na (B)	11298	9	2.70%	30183	27	1.08%	88171	83	0.80%	698392	675	2.40%
Flufenamic Acid (B)	3442	15	3.59%	7763	21	1.84%	55407	88	2.44%	135790	200	13.92%
	20 pg/µL			100 pg/µL			250 pg/µL			2000 pg/µL		
Theobromine (C)	6293	51	1.86%	17288	92	3.23%	51615	222	1.58%	471898	2009	0.77%

Table 3: Quantitation results for standards prepared in horse urine

AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD
1.0 pg/µL			5.0 pg/µL			10 pg/µL			50 pg/µL			100 pg/µL	
		625	5	0.47%	1222	9	2.55%	6856	52	0.41%	12993	99	1.43%
1	1.79%	1208	5	2.58%	2408	10	2.52%	12207	49	1.36%	24967	100	0.66%
1	3.10%	4359	5	0.91%	8593	9	0.72%	46667	49	1.47%	95782	100	1.13%
		2330	6	5.03%	3912	9	3.15%	26131	51	0.85%	51308	99	1.07%
1	22.14%	645	4	13.31%	1294	9	4.88%	7711	53	2.66%	14231	98	1.10%
1	5.66%	4672	5	7.11%	8889	9	2.63%	49369	51	3.40%	96148	100	1.12%
1	7.88%	1170	5	11.66%	2663	10	2.83%	13545	50	1.19%	26856	100	2.72%
1	3.65%	3774	5	2.79%	8117	10	1.35%	42321	50	0.62%	84890	100	1.79%
1	1.13%	44808	5	0.56%	93532	10	1.05%	474442	53	2.08%	877603	98	1.77%
1	5.52%	5758	5	1.14%	11952	10	0.47%	60483	51	2.69%	118294	100	1.68%
1	5.08%	12325	5	2.25%	23958	10	1.07%	120812	50	1.33%	238560	100	1.68%
1	7.15%	11906	5	1.22%	22945	10	3.13%	119082	48	1.17%	253903	101	0.46%
1	2.78%	212	1	2.78%	2259	10	2.80%	11565	50	1.77%	23189	100	0.18%
1	8.40%	2382	5	1.20%	4712	10	3.18%	24920	50	1.14%	50161	100	2.06%
1	6.92%	8546	5	0.39%	17468	10	2.60%	90104	50	0.36%	178996	100	1.28%
1	7.84%	641	5	7.97%	1446	10	12.83%	7294	51	1.39%	14337	100	0.21%

AVERAGE AREA	AVERAGE CALC. CONC	%RSD
650 pg/µL		
96129	667	1.31%
575760	667	3.71%
511382	666	1.21%
143677	666	0.54%
186723	668	1.64%
5840616	667	0.95%
1301762	666	1.22%
1481732	665	1.72%
542362	664	2.86%
61378	667	1.74%
122821	668	2.18%
7065	142	10.74%
1350 pg/µL		
88842	84	2.10%
170496	249	10.77%
4000 pg/µL		
825323	3998	1.85%

	QC Sample				QC Sample 2			
	Conc. (pg/µL)	Cal. Conc.	Difference	%RSD	Conc. (pg/µL)	Cal. Conc.	Difference	%RSD
Theobromine (C)	250.0	231.7	92.7%	1.72%	625.0	615.0	98.4%	1.84%
Theophylline (A)	41.6	38.6	92.7%	1.96%	104.1	103.5	99.4%	2.58%
Dyphylline (A)	41.6	41.3	99.3%	2.02%	104.1	115.5	110.9%	3.38%
Caffeine (A)	41.6	42.4	101.9%	3.30%	104.1	109.6	105.3%	3.05%
Chlorothiazine (A)	41.6	43.0	103.3%	2.64%	104.1	114.4	109.9%	1.65%
Cromolyn-Na (B)	83.3	83.9	100.7%	2.10%	210.0	193.9	92.4%	1.77%
Hydroclorothiazide (A)	41.6	41.8	100.5%	2.64%	104.1	113.1	108.6%	2.27%
Pentoxifylline (A)	41.6	44.5	106.9%	2.23%	104.1	126.5	121.5%	1.43%
Boldenone (A)	41.6	38.8	93.4%	1.04%	104.1	102.4	98.4%	2.63%
Ketoprofen (A)	41.6	38.0	91.4%	1.22%	104.1	104.6	100.5%	1.53%
Ketoprofen – MS^3 (A)	41.6	41.7	100.2%	1.26%	104.1	106.1	101.9%	2.05%
Indomethacin (A)	41.6	49.7	119.5%	5.78%	104.1	116.4	111.8%	1.62%
Diclofenac (A)	41.6	48.4	116.3%	5.89%	104.1	124.1	119.2%	7.94%
Flufenamic Acid (B)	83.3	60.7	72.9%	2.21%	210.0	141.6	67.4%	19.10%
Meclofenamic Acid (A)	41.6	33.3	80.1%	6.74%	104.1	89.8	86.3%	21.76%

Table 4: Quantitation results for the analysis of unknown levels of drugs in horse urine

Conclusions

Positive and negative ion detection of co-eluting drugs was accomplished in a single chromatographic run using automated polarity switching. Drugs that underwent a neutral water loss were further fragmented using WideBand Activation to provide a diagnostically rich MS/MS spectrum for structural confirmation. The compound ketoprofen underwent a prominent, non-specific neutral loss of formic acid and was further analyzed using an MS^3 transition. Full-scan MS^n data was reprocessed to quantify all 16 compounds by reconstructed ion chromatograms (RICs), or post-acquisition MRM, and provided results comparable to triple quadrupole SRM quantitation. It is possible to achieve the low % RSD required in quantitation due to the fast cycle time of the Thermo Scientific LTQ. In the case of non-specific neutral molecule losses, MS^3 experiments generated diagnostic spectra for confirmational purposes while providing quantitative results comparable to the MS/MS data. Results of the ruggedness study demonstrate no appreciable loss of sensitivity or reproducibility across 100 replicate urine injections. Thus, using the Thermo Scientific LTQ two-dimensional linear ion trap, we have demonstrated the development of a simple, rapid, and rugged method capable of confirmational screening and simultaneous quantitation of drugs in horse urine using both full-scan LC/MS/MS and MS^3 spectra.

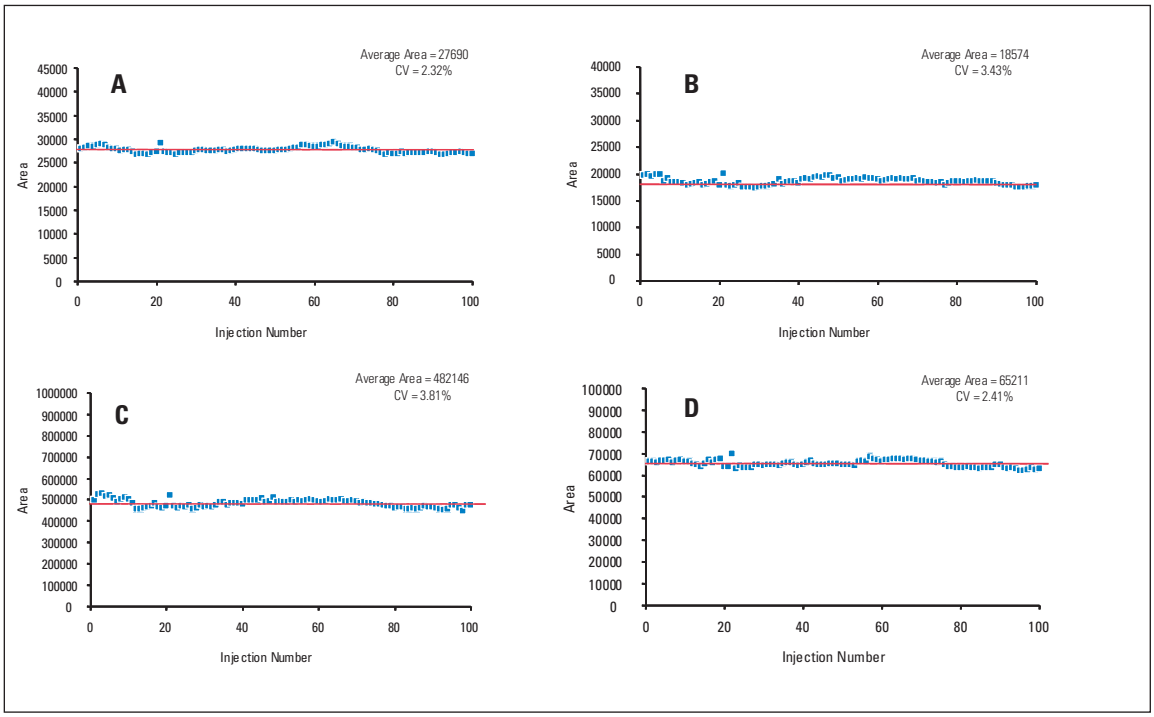


Figure 8: Ruggedness and reproducibility for 100 consecutive injections of a 166 pg/µL standard of theobromine, caffeine, pentoxyphylline, and ketoprofen in urine

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MS/MS as an LC Detector for the Screening of Drugs and Their Metabolites in Race Horse Urine

Gargi Choudhary and Diane Cho, Thermo Fisher Scientific, San Jose, CA
Wayne Skinner and Scott Stanley, University of California–Davis

Key Words

- LCQ Advantage MAX™
- Surveyor™
- Data Dependent™
- Drug Screening
- Metabolite ID
- Toxicology

Introduction

Imipramine is a tricyclic antidepressant drug that is not a Drug Enforcement Administration controlled substance but has been classified by the Association of Racing Commissioners International Inc. as a class two drug in horses. Desipramine is a major metabolite of imipramine. These two analytes were analyzed on-line by LC–PDA MS/MS from extracts of horse urine. The urine sample was first treated with β-glucuronidase to hydrolyze glucuronide conjugates of imipramine and desipramine. This was followed by solid phase extraction. The concentration of imipramine and desipramine in the sample was determined by the internal standard method using the peak area ratio and linear regression analysis.

This application note presents a rapid method for quantitation of imipramine and desipramine in horse urine. It illustrates the advantages of MS/MS detection in terms of specificity, sensitivity and unambiguous identification, for the analysis of drugs and their metabolites.

Goal

1) Develop a rapid method to identify and quantitate tricyclic antidepressant imipramine and its major metabolite desipramine in horse urine.
2) Demonstrate the advantages of using MS/MS to identify and confirm the detection of imipramine and its metabolites.
3) Determine presence and structure of minor metabolites using Data Dependent LC-MS/MS analysis.

Experimental Conditions HPLC

LC system: Thermo Scientific Surveyor MS Pump, Surveyor Autosampler and Surveyor PDA Detector

Mobile phase: A: water containing 0.2% formic acid
B: Acetonitrile containing 0.2% formic acid

Column: 50 ! 2.1 mm, 5 µm Thermo Scientific Hypersil™ C18 Column

Injection volume: 1 µL

Flow rate: 200 µL/min

Gradient:

Time (min)	% A	%B
0	98	2
0.2	98	2
8	25	75
9	10	90
10	10	90
10.01	98	2
15	98	2

Thermo SCIENTIFIC

Mass Spectrometer

Mass spectrometer: Thermo Scientific LCQ Advantage MAX
Ionization mode: Positive electrospray ionization (ESI)
Capillary temperature: 275 °C
Spray voltage: 4.5 kV
Sheath gas: 30 units
Sweep gas: 8 units

Analyte	*MH^+*	*Isolation Width*	*Collision Energy %*	*Scan Range*	*Quantifying MS/MS Product Ions*
Imipramine	281.2	1.5	30	75-285	86
Desipramine	267.2	1.5	30	70-290	236
Clomipramine (internal standard)	315.2	4	35	85-320	270

Table 1: MS parameters for imipramine, desipramine, and clomipramine (internal standard)

Standards

Calibration standards were prepared as follows:

Calibration level	*Volume of Imipramine and Desipramine working standard solution (µL)*	*Volume of Clomipramine working standard solution (µL)*	*Equivalent to Imipramine in the urine (ng/mL)*	*Equivalent to Clomipramine in the urine (ng/mL)*
C1	1:1 Dilution of C2	10	15.6	500
C2	1:1 Dilution of C3	10	31.3	500
C3	1:1 Dilution of C2	10	62.5	500
C4	1:1 Dilution of C2	10	125	500
C5	1:1 Dilution of C2	10	250	500
C6	1:1 Dilution of C2	10	500	500
C7	1:1 Dilution of C2	10	1000	500
C8	1:1 Dilution of C2	10	2000	500
C9	1:1 Dilution of C2	10	4000	500
C10	160	10	8000	500

Imipramine, desipramine and clomipramine working standard solutions were 50 ng/mL

Samples and Internal Standard

Imipramine was administered to the horse and a urine sample drawn after 0, 2, 4, 8 and 24 hours, post dose. One mL of the urine sample was spiked with 10 µL of 50 ng/µL clomipramine internal standard.

Sample Preparation

The calibration standard and urine samples were treated with β-glucuronidase to hydrolyze glucuronide conjugates of desipramine and imipramine, followed by solid phase extraction.

Results and Discussions

LC-UV-MS/MS analysis of imipramine and desipramine

Figures 1 and 2 show the analysis of tricyclic antidepressant imipramine, its major metabolite desipramine, and the internal standard clomipramine by LC with MS/MS and UV detection, respectively. Figure 1 shows base peak and extracted ion chromatograms for the three analytes along with MS and MS/MS spectra. The MS and MS/MS spectra help in unambiguous identification of these analytes and represent the high specificity that can be obtained from such an analysis. Further, the MS/MS spectra can be stored in a library and used for rapid confirmation of the drug and its metabolite. Figure 2 shows total spectra obtained from a PDA detector as well as UV trace at 254 nm and 280 nm. The position of elution of the three compounds had to be determined by sequential injections of individual analytes. As illustrated in Figure 2, the UV spectra for these compounds appear almost identical, making their unambiguous identification difficult.

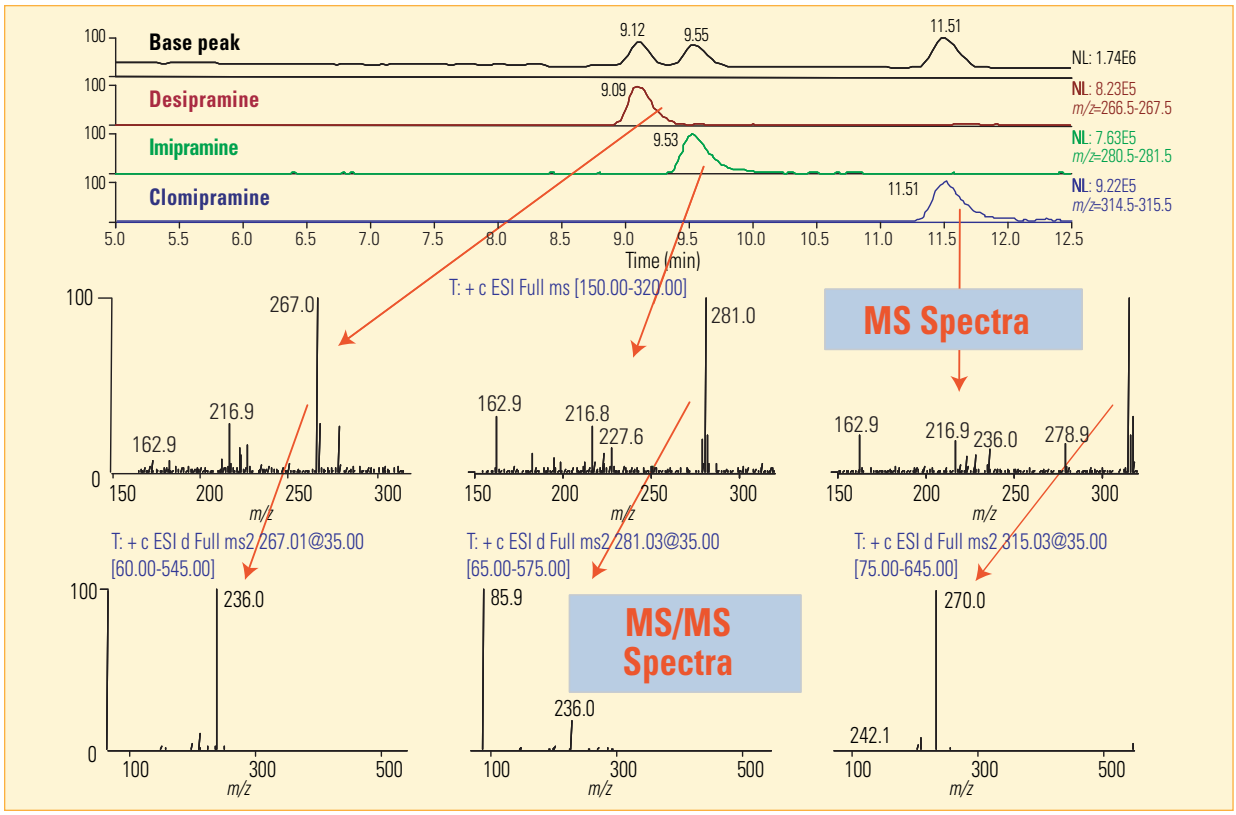


Figure 1: LC-MS/MS analysis of imipramine, desipramine and clomipramine (internal standard)

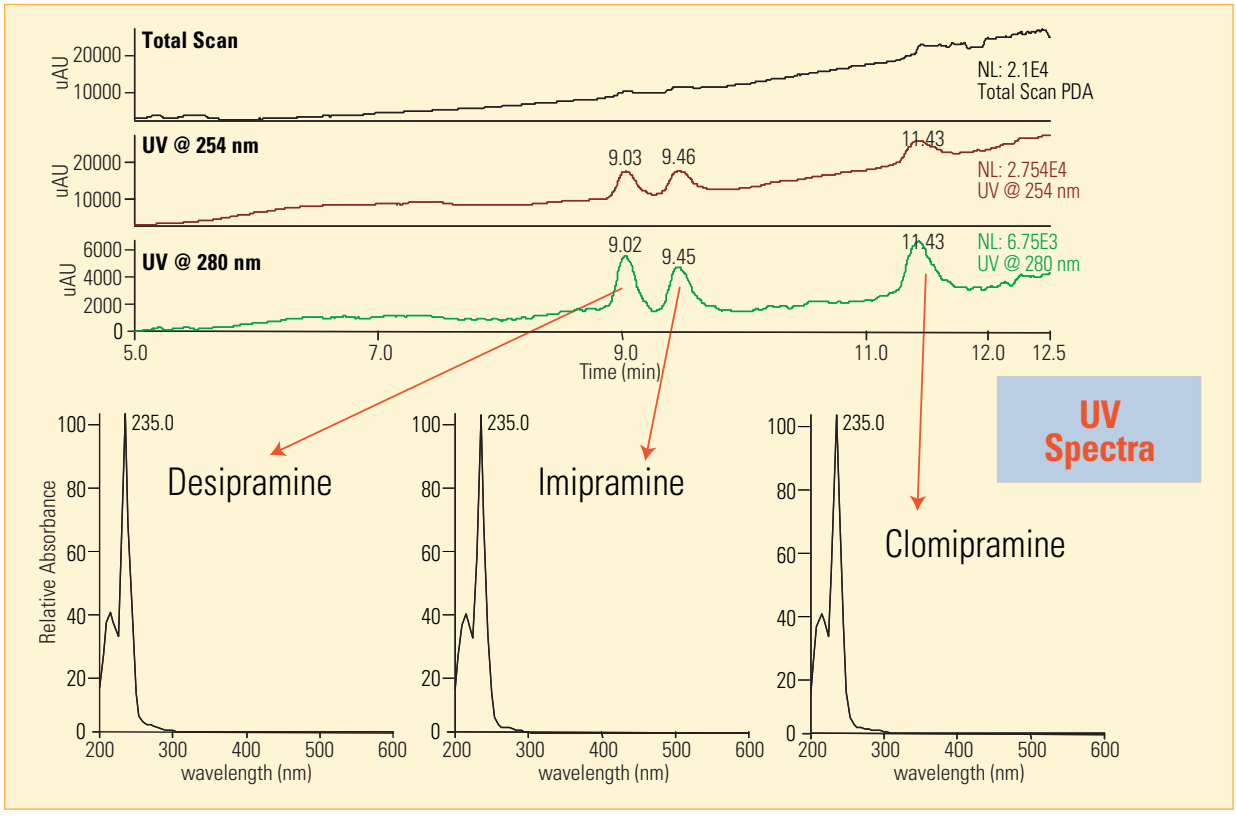


Figure 2: LC-UV analysis of imipramine, desipramine and clomipramine (internal standard)

Figure 3 shows chromatograms obtained for the analysis of imipramine, desipramine and clomipramine (IS) with MS and UV detection at levels of 5 and 0.5 ng on-column. At 0.5 ng on-column, both imipramine and desipramine could be easily identified when MS was used as a detector whereas these analytes were hardly visible in the UV trace. The concentration of clomipramine is the same at both these levels. This illustrates the excellent sensitivity that can be obtained during analysis by LC-MS/MS.

Quantitation of imipramine and desipramine in horse urine

Figures 4 and 5 show calibration curves obtained for imipramine and its major metabolite desipramine in horse urine with clomipramine used as an internal standard. The coefficient of correlation is 0.9896 for calibration curve of imipramine and 0.9836 for the calibration curve of desipramine. The % CV values are less than 7% for the imipramine calibration curve and 15% for the

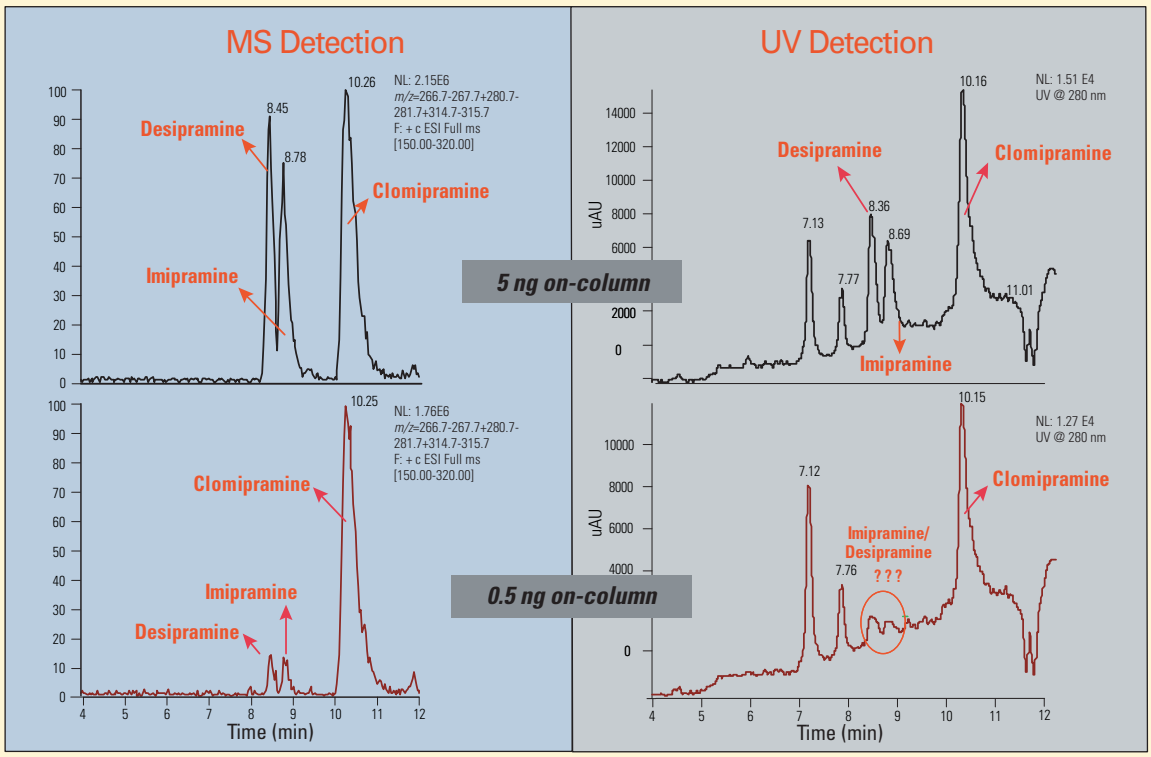
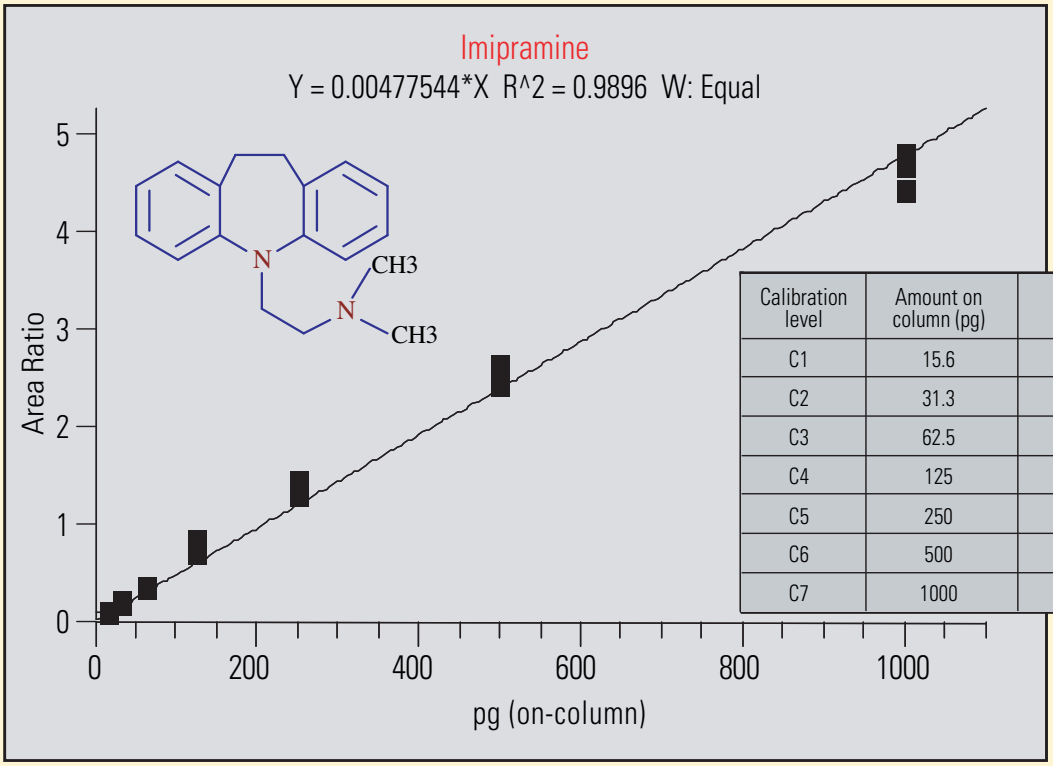
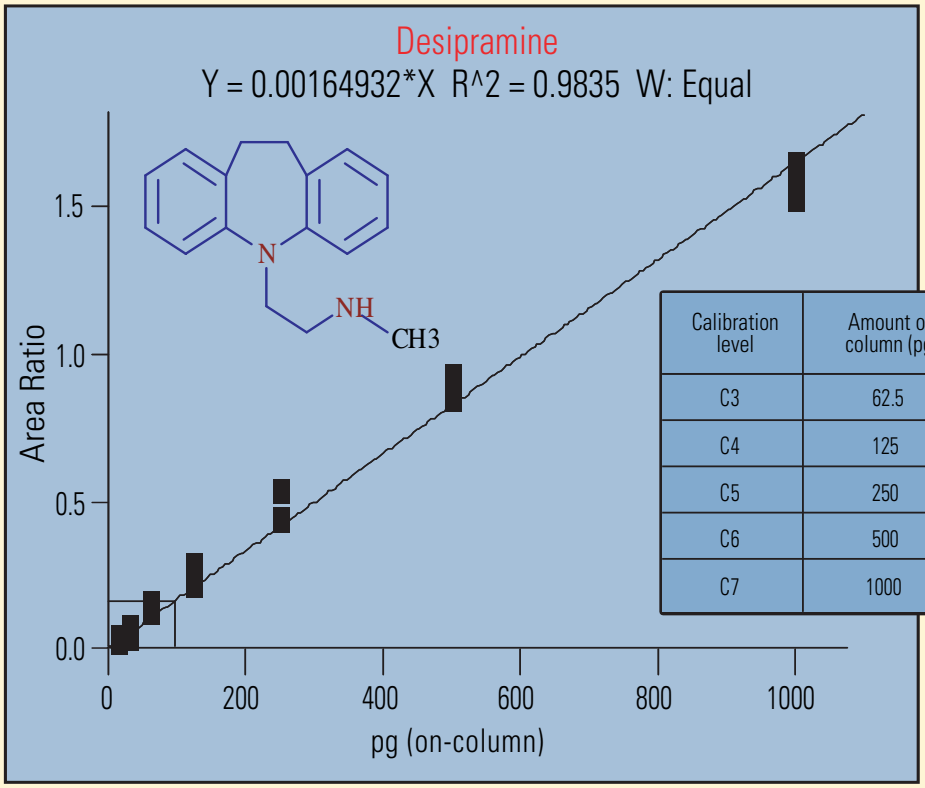


Figure 3: Comparison of MS and UV detection at two different concentrations



Calibration level	Amount on column (pg)	% CV
C1	15.6	4.25
C2	31.3	1.25
C3	62.5	2.28
C4	125	7.34
C5	250	5.32
C6	500	3.65
C7	1000	4.00

Figure 4: Calibration curve for imipramine in horse urine



Calibration level	Amount on column (pg)	% CV
C3	62.5	14.96
C4	125	14.30
C5	250	11.24
C6	500	4.73
C7	1000	4.02

Figure 5: Calibration curve for desipramine in horse urine

desipramine calibration curve. Figure 6 shows analysis of imipramine and desipramine in horse urine sample drawn two hours post-administration of the drug. The amount of imipramine and its major metabolite desipramine was determined using the calibration curves shown in Figures 4 and 5. Table 2 shows the amount of these two analytes as determined in horse urine. For the sample drawn two hours post-administration of the drug, the amount of imipramine and desipramine was determined to be 28 and 1567 ng/mL, respectively. The amount of desipramine determined at this time is above the upper limit of quantitation for the calibration curve shown in Figure 5.

Time (hr)	Imipramine (ng/mL)	Desipramine (ng/mL)
0+	17.56	20.85*
2	28.12	1567.16**
4	4.25*	189.06
8	6.75*	96.56
24	6.11*	13.11*

Table 2: Determination of imipramine and desipramine in horse urine for samples drawn at different times post injection of the drug (*below lower limit of quantitation, **above upper limit of quantitation)

Identification of metabolites of imipramine

A urine sample from the race horse obtained two hours after administration of the drug was also analyzed by Data Dependent LC-MS/MS, with MS/MS on the top two most intense ions to determine the presence of other metabolites. Figure 7 shows the workflow for such an analysis. The extracted ion chromatograms in Figure 8 show the presence of four additional metabolites: desmethyl desipramine, OH desipramine, OH-imipramine, and N-Oxide of imipramine, as well as their MS/MS fragmentation pattern. As indicated by the two peaks in the extracted ion chromatogram for *m/z* 297.2, imipramine is metabolized to two metabolites that have the same *m/z*. In this case, the MS/MS fragmentation pattern enables unambiguous distinction between the two metabolites.

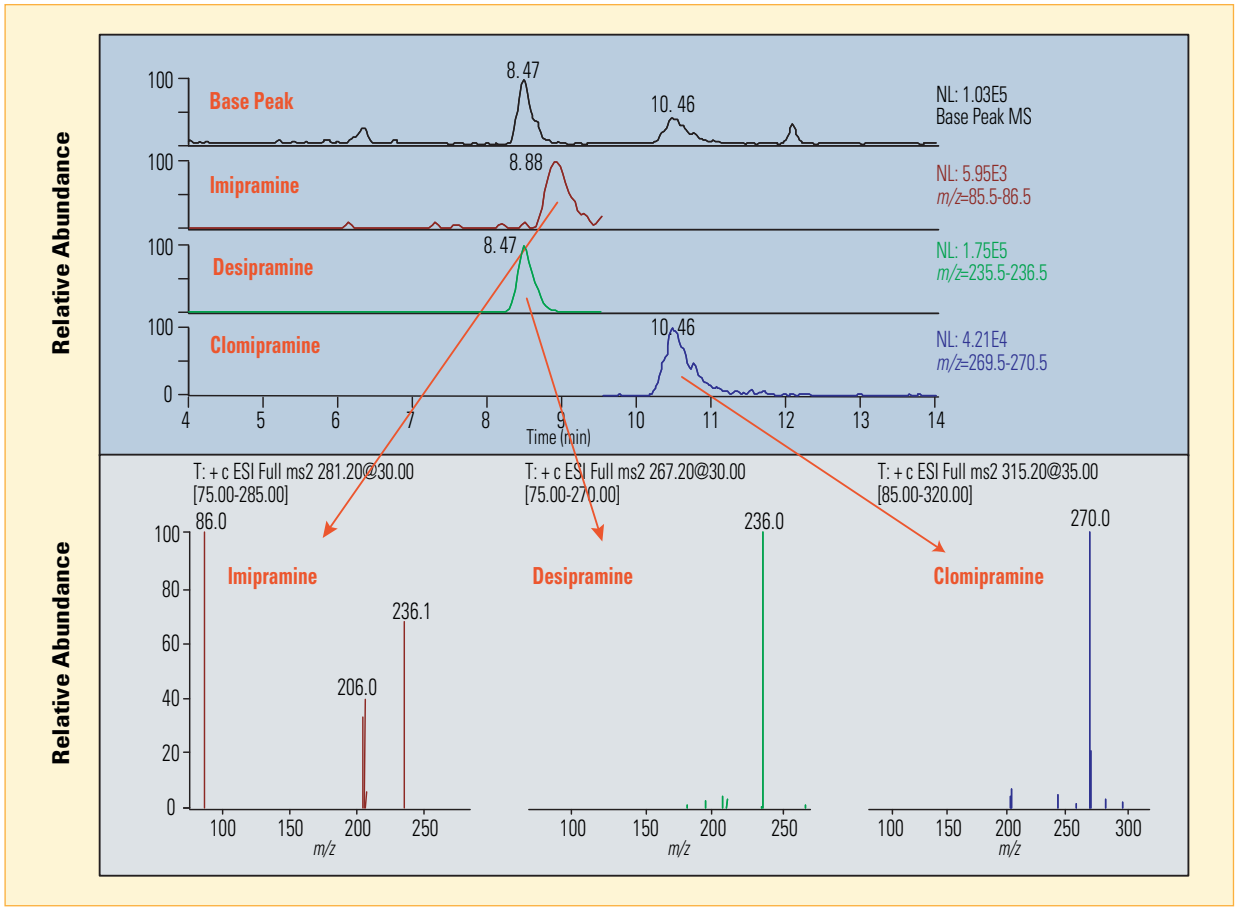


Figure 6: Analysis of imipramine and desipramine in horse urine for sample drawn two hour post-administration of drug

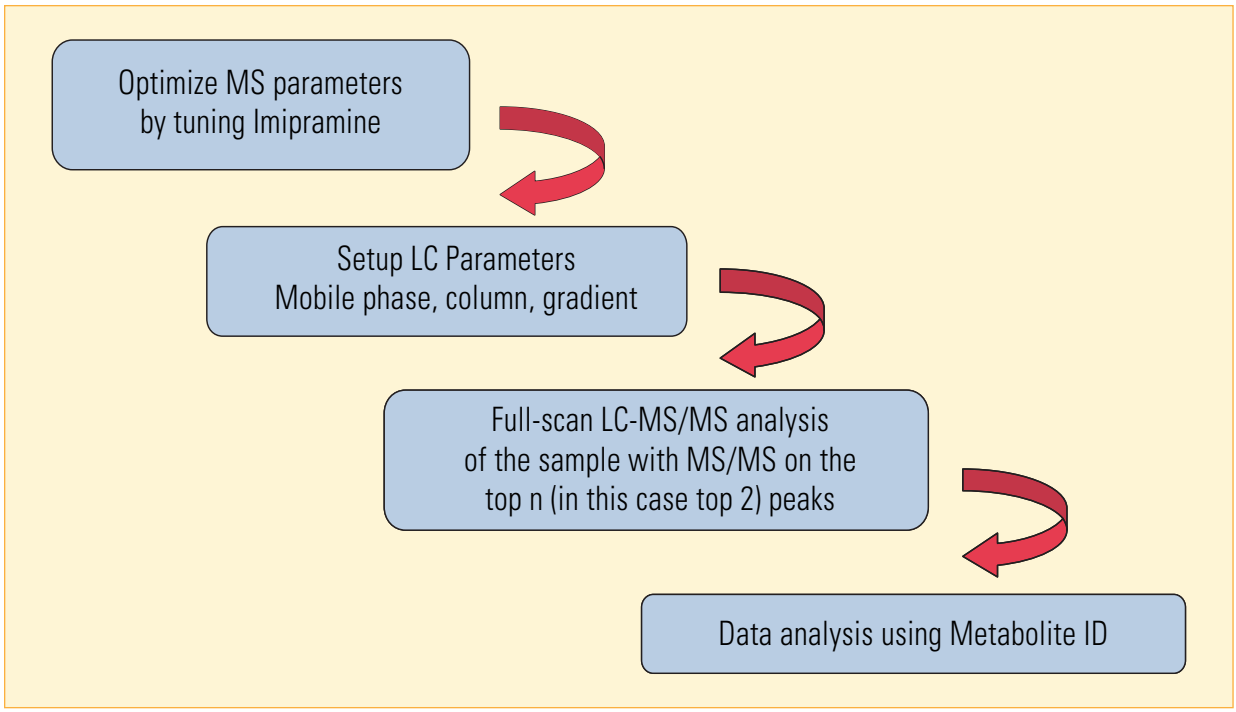


Figure 7: Workflow for the identification of imipramine and its metabolite in horse urine

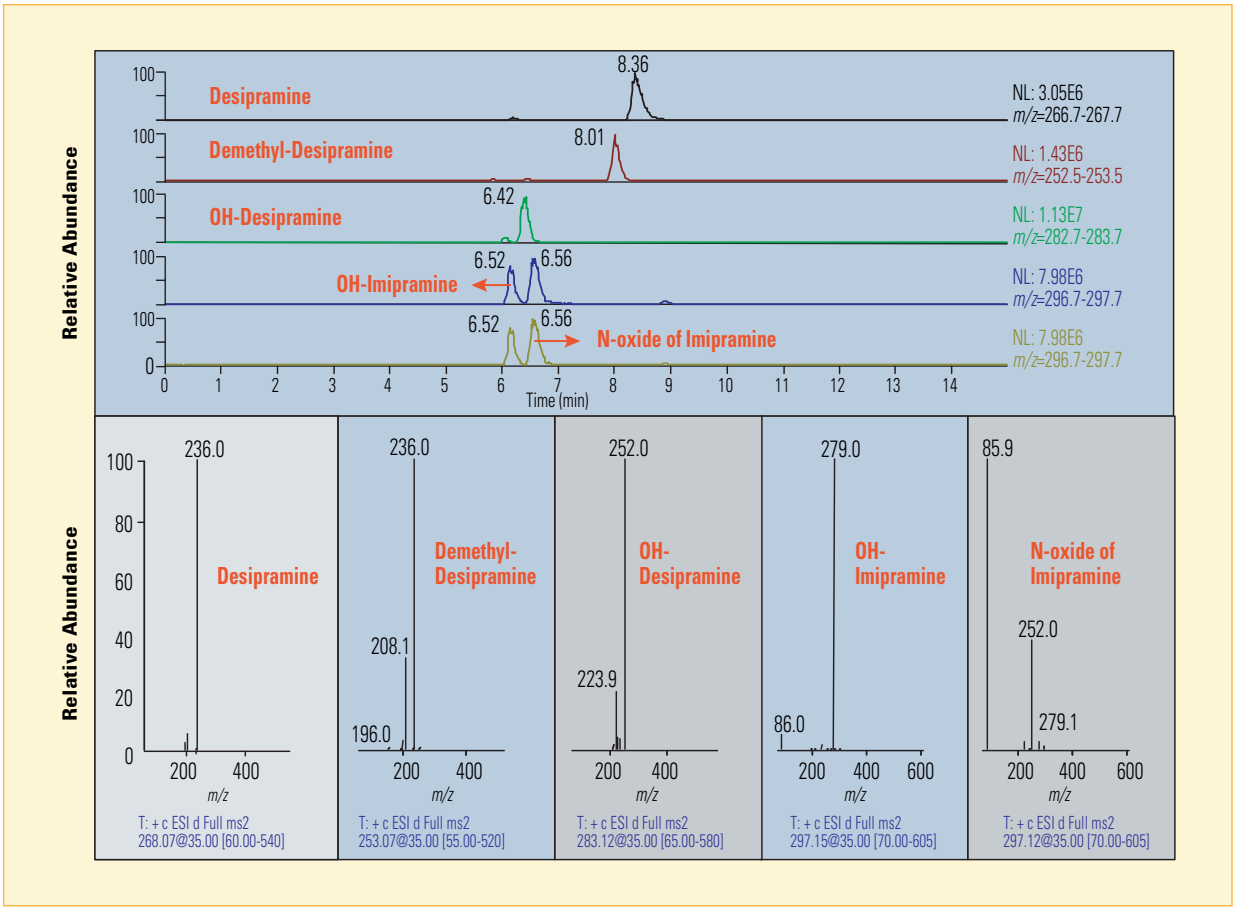


Figure 8: Data Dependent LC-MS/MS analysis of metabolites of imipramine

Conclusions

Full scan MS/MS analysis using a Thermo Scientific LCQ Advantage MAX ion trap mass spectrometer provides the selectivity and sensitivity necessary to support ADME/Tox studies of imipramine in horse urine. Analysis of drugs and their metabolites in complex biological samples using MS/MS detection enables unambiguous identification of these analytes. Data Dependent LC-MS/MS analysis facilitates presence and structural determination of several co-eluting minor metabolites. MS/MS information is invaluable in the identification of metabolites with the same *m/z* (e.g., OH-imipramine and N-oxide of imipramine).

Acknowledgements

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