Delivering the Right Results

Anti-Doping Control Using Comprehensive Multidimensional Gas Chromatography Time-of-Flight Mass Spectrometry (GCxGC-TOFMS) for Enhanced Detection of Anabolic Steroids in Urine

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OVERVIEW

- Demonstrate the enhanced detection and increased peak capacity available with GCxGC-TOFMS for Anti-Doping control screening.
- Show how comprehensive two-dimensional chromatography resolves analytes that would otherwise totally coelute by one-dimensional chromatography.
- Illustrate deconvolution of fast acquisition non-skewed time-of-flight mass spectra (TOFMS) for trace level anabolic steroids in heavy sample matrices.
- Show data results that exceed the World Anti-Doping Agency (WADA) cut-off guidelines.
- Show linearity results indicating the quantitative capabilities of GCxGC-TOFMS for Anti-doping control screening.

INTRODUCTION

This research presents the application of GCxGC-TOFMS for the analysis of androgenic anabolic steroids in urine. Steroid screening analysis in urine is complex and labor intensive requiring sensitive mass spectrometric instrumentation and optimized chromatographic separations. GCxGC provides increased peak capacity and enhanced chromatographic resolution. The fast acquisition of TOFMS, up to 500 Hz, successfully acquires the data density needed to fully characterize low levels of steroids in extremely complex sample matrices such as urine. The data rich files are processed with deconvolution algorithms which deliver qualitative identification as well as multiple compound quantification in a single run. The results show LOD values at or below 2 ppb (2 ng/mL) for five anabolic steroids with a quantitative calibration linearity of 99% or greater.

GCxGC-TOFMS was used to evaluate five trimethylsilyl derivatized anabolic steroid standards spiked in urine. A five point calibration from 2 to 100 ng/mL was generated using the spiked urine standards. Methyl-testosterone was used as an internal standard (ISTD). Sample preparation followed a well established acid hydrolysis procedure followed by extraction and trimethylsilyl derivatization. Subsequent sample analysis was conducted utilizing a GCxGC separation followed by TOFMS detection at an acquisition rate of 100 spectra per second.

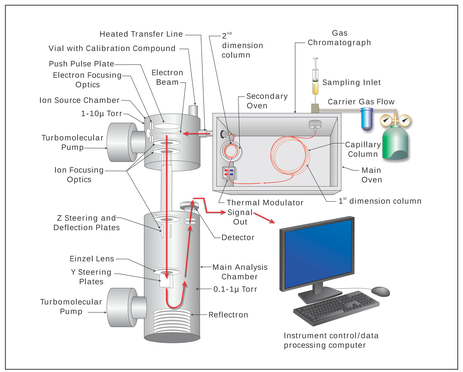

Figure 1. Pegasus® 4D GCxGC-TOFMS Schematic.

EXPERIMENTAL WORKFLOW

Steroid Standards were prepared, derivatized, and spiked into 2 mL aliquots of urine at the concentrations of 2, 10, 20, 50, and 100 ng/mL.

Steroid Target Analytes

- 19-Norandrosterone (metabolite of Nandrolone)
- Boldenone (anabolic steroid developed for veterinary use)
- 17a-Methylandrostan-3a, 17ß-diol (metabolite of Methyltestosterone)
- 3-Hydroxy tosterone (used as an internal standard—ISTD)

Sample Acid Hydrolysis/Extraction/Derivatization Procedure

- Sample preparation in 2 mL urine
- Add Sodium phosphate buffer to pH 6
- Add ISTD Methyltestosterone at 200 ng/mL
- Hydrolyze with ß-Glucuronidase for 1 Hr at 50ºC
- Add Potassium carbonate solution to pH 9 and shake for 5 min @ 3000 rpm
- Extract with 5 mL Methyl-tert-butyl-ether (MTBE)
- Transfer organic phase to a clean glass vial
- Evaporate to dryness with nitrogen and derivatize with 100 µL MSTFA-NH4I-Ethanethiol for 30 minutes at 60ºC

GCxGC-TOFMS Analysis Parameters

- Gas Chromatograph: Agilent 7890 equipped with a LECO dual stage quad jet thermal modulator and Gerstel MPS2 autosampler
- Primary Column: 30 m x 0.25 mm id. x 0.25 µm film thickness Rxi-5ms (Restek Corp., Bellefonte, PA)
- Secondary Column: 1.20 m x 0.10 mm id. x 0.10 µm film thickness BPX-50 (SGE Analytical Science, Austin, TX)
- Carrier Gas: Helium set @ 1.5 mL/min
- Injection Mode: splitless
- Injection Volume: 3 µL
- Inlet Temperature: 280°C
- Primary Column Temperature Program: Initial temperature set @ 140ºC for 0.2min. ramped @ 20°C/min. to 170ºC then ramped @ 5°C/min. to 260°C for 2.0 min., then ramped @ 10°C/min. to 315°C for 12 min.
- Secondary Column Temperature Program: Initial temperature set @ 145ºC for 0.2 min. ramped @ 20°C/min. to 175ºC then ramped @ 5°C/min. to 265°C for 2.0 min., then ramped @ 10°C/min. to 320°C for 12 min.
- Total run time: 39.2 min.

GCxGC parameters

- Column Temperature Offset: 5°C
- Modulator Temperature Offset: 20°C
- Modulation Period: 4.0 s Hot pulse time: 0.8s
- Cool Time Between Stages: 1.20 s

Mass Spectrometer: LECO Pegasus 4D

- Mass Range: 45 – 750 m/z
- Acquisition Rate: 100 spectra/s
- Ion Source Temperature: 230°C
- Detector Voltage: 1950 V
- Electron Energy: -70 eV

GCxGC-TOFMS RESULTS

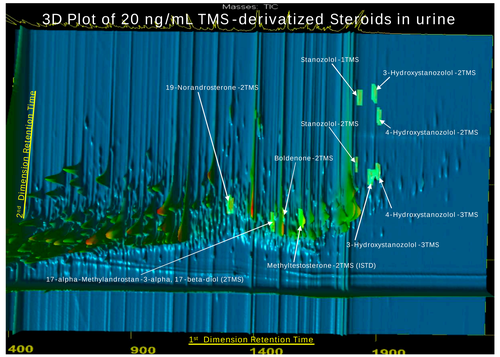

Figure 2. The three-dimensional surface plot total ion chromatogram in Figure 2 shows the complexity of the urine matrix as well as the peak resolution of the labeled derivatized steroids.

QUANTITATIVE CALIBRATION LINEARITY

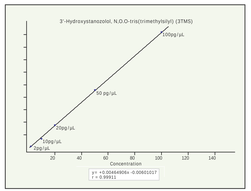

Figure 3. In Figure 3 above, the five point calibration curve for the tri-TMS derivative of 3-Hydroxystanozolol is illustrated from 2 – 100 ppb. The correlation coefficient shown in Figure 3 shows linearity greater than 99.9%.

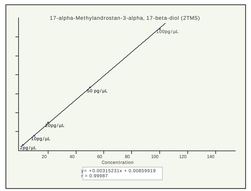

Figure 4. In Figure 4 above, the five point calibration curve for the di-TMS derivative of 17-alpha-Methylandrostan-3-alpha,17-beta-diol is illustrated from 2 – 100 ppb. The correlation coefficient shown in Figure 4 shows linearity greater than 99.9%.

Name	Absolute R.T. (sec , sec)	Min Conc.	Max Conc.	Type	Curve Order	Equation	Correlation Coefficients
19-Norandrosterone, 3-trimethylsilyl ether, 17-trimethylsilyl enol ether	1292 , 1.840	1	150	Analyte	1	y=+0.00590503x + 0.0106961	0.99924
17-alpha-Methylandrostan-3-alpha, 17-beta-diol (2TMS)	1464 , 1.630	1	150	Analyte	1	y=+0.00315231x + 0.00859919	0.99987
Boldenone (2TMS)	1500 , 1.680	1	150	Analyte	1	y=+0.0174845x + 0.0131281	0.99927
Methyltestosterone 2TMS	1580 , 1.520			Internal Standard	1	NA	NA
3'-Hydroxystanozolol, N, O, O-tris(trimethylsilyl) derivative	1864 , 2.200	1	150	Analyte	1	y=+0.00464806x - 0.00801017	0.99911
4-Hydroxystanozolol (3TMS)	1884 , 2.170	1	150	Analyte	1	y=+0.00353856x - 0.00390789	0.99961

Table 1. In Table 1 above, all of the steroids used in this research are shown with correlation coeffient linearity values of greater than 99.9%.

GCxGC ENHANCED PEAK CAPACITY

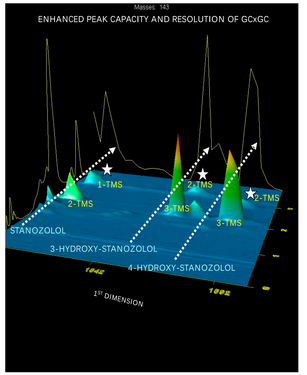

Figure 5. This three-dimensional surface plot chromatogram above shows a zoomed-in portion of the 20 ng/mL steroid GCxGC-TOFMS analysis where Stanozolol, 3-Hydroxystanozolol, and 4-Hydroxystanozolol elute. This illustration is an excellent example of how the increased peak capacity of comprehensive two-dimensional chromatography can resolve components in the second dimension that would otherwise coelute in the first dimension. The Stanozolol and Hydroxystanozolols derivatize inconsistently with either 1, 2, or 3 trimethylsilyl groups. Each of the compounds, Stanozolol, 3-Hydroxystanozolol, and 4-Hydroxystanozolol elute in the same 1st dimension retention time modulation periods, shown by the white arrows in the figure above. However the same compounds that are derivatized with fewer TMS groups elute with later 2nd dimension retention times respectively, designated by the stars in Figure 5. Note that these fully and partially derivatized steroids are completely resolved in GCxGC which would otherwise coelute completely by one-dimensional chromatography.

TOFMS TRUE SIGNAL DECONVOLUTION®

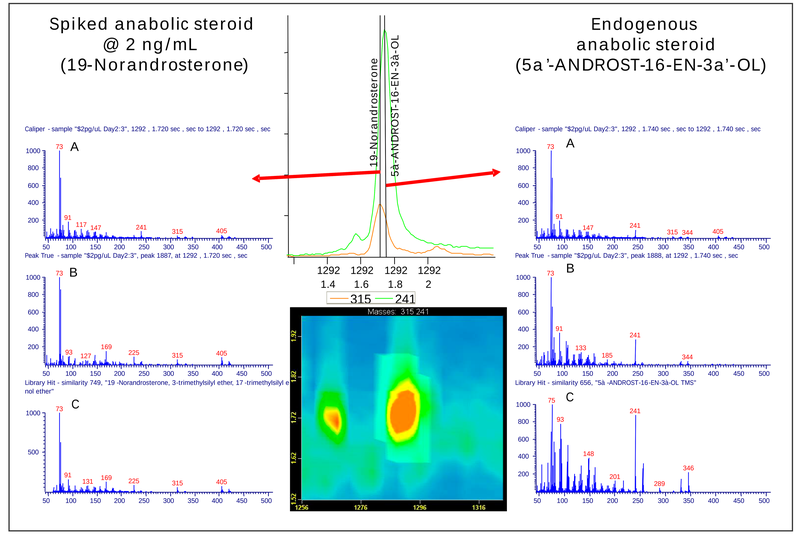

Figure 6. The figure above demonstrates a deconvolution example for the trace level concentration of the derivatized anabolic steroid (19-Norandrosterone) at 2 ppb. It is coeluted and buried under the endogenous anabolic steroid peak, 5a'-ANDROST-16-EN-3a'-OL. The 1st dimension chromatogram at the top-center of the figure shows the extracted ion traces for the unique masses of each peak at m/z 315 and m/z 241. The separation of the two peak apexes for these ions is ~20 milliseconds. The chromatogram at the bottom of Figure 6 shows the two-dimensional contour plot of this highlighted peak which encompasses both of these compounds. The Caliper or (total ion mass spectra) before deconvolution is illustrated by the two mass spectra labeled A. Notice that both Caliper mass spectra for the separate peaks look very similar. The mass spectra labeled B for each peak is the Peak True deconvoluted mass spectra. Notice that both of these deconvoluted mass spectra are very different from each other. The mass spectra labeled C is the library search matches for both Peak True deconvoluted mass spectra. This illustration clearly shows the deconvolution of a trace anabolic steroid even when it is buried in and masked under heavy sample matrix.

ACHIEVING WADA CUT-OFF LIMITS

Table 2. Steroids in Urine 2 ng/mL (2ppb) Standard with Quantitative S/N Ratios

Name	Quant S/N	Area	Concentration	1st Dimension Time (s)	2nd Dimension Time (s)	Quant Mass	Similarity
19-Norandrosterone, 3-trimethylsilyl ether, 17-trimethylsilyl enol ether	1353.20	387757	2 ng/ml	1292	1.72	315	749
17-alpha-Methylandrostan-3-alpha, 17-beta-diol (2TMS)	1064.50	271595	2 ng/ml	1464	1.50	143	857
Boldenone (2TMS)	870.86	1041076	2 ng/ml	1500	1.58	208	744
Methyltestosterone 2TMS (ISTD)	30031.00	23916831	200 ng/ml	1576	1.54	301	908
3'-Hydroxystanozolol, N, O, O-tris(trimethylsilyl) deriv.	93.92	80321	2 ng/ml	1860	2.06	254	619
4-Hydroxystanozolol (3TMS)	85.12	127836	2 ng/ml	1884	2.16	143	739

Table 2. The table above shows the five steroid standards and the internal standard results at the low level 2 ng/mL concentration. This is the World Anti-Doping Agency (WADA) cut-off limit guidelines set for the minimum required performance levels (MRPL). The column highlighted in red lists the quantitative signal to noise ratio for each steroid. S/N ratios for all five steroids range from 85.12 to 1353.2 at the 2 ppb concentration. The low level cut-off MRPL guidelines are achieved and exceed the limits set by WADA.

CONCLUSIONS

This application demonstrates that steroid screening is improved significantly by GCxGC-TOFMS compared to one-dimensional chromatography using GCMS. Excellent calibration linearity for quantitation was achieved for all analytes at greater than 99.9%. Limits of detection at part per trillion levels were calculated from acquired data for all five steroids. This research demonstrates the ability of GCxGC-TOFMS to provide enhanced analyte detectability, deconvolution of trace level concentrations of steroids, and exceptional quantitation capabilities for the analysis of illegal anabolic steroid screening in urine. The results of this experimentation show that significantly increased analytical performance was accomplished using comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-TOFMS) for anti-doping screening control. In conclusion this application shows favorable and practical applicability for the positive identification of anabolic steroids in urine at or below the lowest allowable concentration limits established in the guidelines set forth by the World Anti-Doping Agency (WADA).

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