Selective and sensitive quantitation of 18 steroids in human serum using Stellar mass spectrometer

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Abstract

Purpose: To demonstrate the highly sensitive and selective quantification of 18 serum steroids using the hybrid quadrupole – linear ion trap Stellar MS in multiple scan modes.

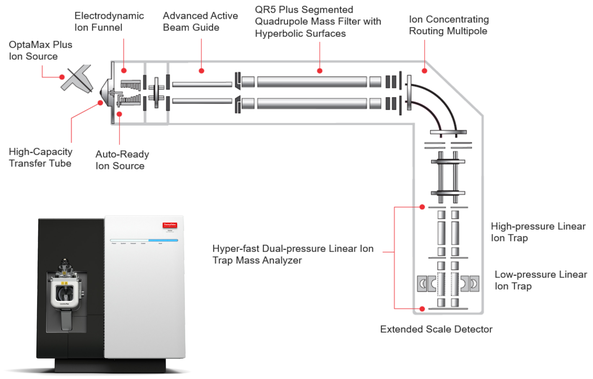
Methods: Steroid detections in HCD, CID, or MS3 was optimized, and the optimal scan modes were selected for the quantification.

Results: Stellar MS provides hardware and software improvements and optional alternative fragmentation modes of CID and MS3 that enhanced sensitivity and selectivity of steroids quantification.

Introduction

Successful biomarker quantitation in complex matrices requires sensitivity and specificity to ensure the measured signal is attributed to the targeted analytes. To reduce background interferences and provide greater specificity, alternative fragmentation mechanisms and/or MS3 fragmentation are an efficient solution. The Thermo Scientific™ Stellar™ mass spectrometer (MS) (**Figure 1**) is a hybrid quadrupole, dual-pressure linear ion trap MS that offers two types of orthogonal, yet complementary fragmentation modes with rapid MS2 and MS3 scans: beam-type high-energy collision-induced dissociation (HCD), which is a triple quadrupole (QqQ)-like fragmentation, and resonance-type collision-induced dissociation (CID).[1] This poster is the first to demonstrate the advantages of utilizing multiple collision activations and MS3 scans provided by Stellar MS to the selective and sensitive quantification of 18 steroids in serum.

Figure 1. Stellar MS diagram



Materials and methods

Sample preparation

Certified reference standards of steroids and their corresponding internal standards (IS) were purchased from Cerilliant. Thermo Scientific™ Optima™ LC-MS grade water, methanol, acetonitrile, acetic acid were purchased from Fisher Scientific. Bovine serum albumin (BSA) and NH_4F was purchased from Sigma-Aldrich. The serially diluted steroid standards and IS solution were added into 0.05% BSA to generate calibration solutions over 4-orders of magnitude concentration range. Calibration samples Cal-4, 5, 6, and 7 were used for the inter- and intra-day imprecision study. Commercial QC serum samples containing DHT were provided by collaborators. Steroids were extracted from 160 µL matrix solutions after protein precipitation in the presence of cold acetonitrile and acetic acid, and reconstituted in 100 µL 50% methanol, of which 5 µL was injected for LC-MS analysis.

LC-MS parameters and data analysis

Samples were analyzed on a Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to a Stellar MS. Steroid separation was achieved on a Kinetex ™ C18 column (2.1 x 150 mm, 2.6 µm) at 50° C. The LC gradient and extracted ion chromatograms of each steroid compound are shown in **Figure 2**. Stellar MS parameters are shown in **Table 1**. Data were acquired using Thermo Scientific™ Xcalibur™ software version 4.7 and processed using Thermo Scientific™ TraceFinder™ software version 5.2.

Figure 2. Representative EIC of the separation of all steroid compounds. The inserted table shows the LC flow gradient over a 16-minute run time.

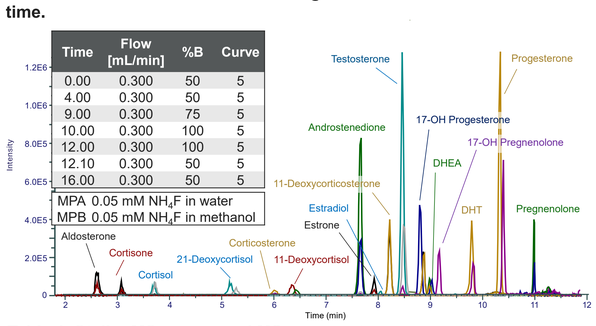
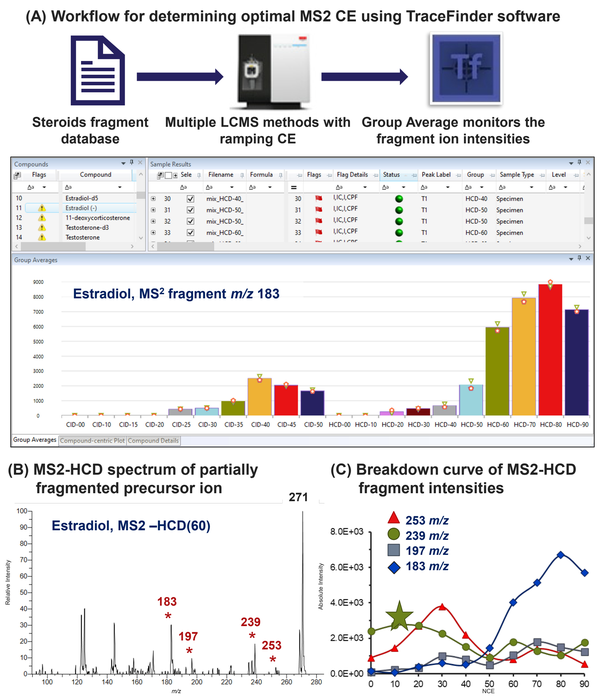


Table 1. Stellar MS source and MSn parameters.

Thermo Scientific OptaMax™ Plus ion source properties	
Spray Voltage (V)	(+) 1500 / (-) 2500
Sheath / Aux / Sweep Gas (Arb)	50 / 14 / 2
Ion Transfer Tube (ºC)	325
Vaporizer Temp (ºC)	525
Collision Gas Pressure (mTorr)	8
Chromatography Peak Width (s)	3
MS1 parameters	
Scan Rate (kDa/s)	125
Scan Range (*m/z*)	70 – 500
AGC Target	3e4
RF Lens (%)	30
Max Injection Time Mode	Auto
Polarity	Both
Source Fragmentation (V)	0
Targeted-MSn Parameters	
Isolation Window (*m/z*)	2
MS2 / MS3 Scan Rate (kDa/s)	125 / 67
MS2 / MS3 Scan Range	Auto
HCD / CID Collision Energy Type	Normalized
AGC Target	1e4
Max Injection Time Mode	Dynamic
Points per peak	7
Data Type	Centroid

Figure 3. Steroid CE optimization using estradiol as an example. (A) Workflow to determine optimal CE for CID and HCD MS2 fragmentation. (B) Identification of potential MS3 candidates. (C) Evaluation of optimal CE for each MS3 candidate. The selected product ion repeats the workflow in A.



Results

Compound Optimization – MS2 and MS3 Parameters

Due to the drastic chemical structure differences in small molecules, optimizing MS parameters is highly recommended to achieve ultimate sensitivity and selectivity. **Figure 3A** illustrates the process of optimizing the MS2 collision energy (CE) utilizing the Group function in TraceFinder. The fast scan speed of Stellar MS allows for the simultaneous optimization of 18 steroids and 16 IS in both MS2-HCD and MS2-CID scan modes without retention time (RT) scheduling. From this data, potential MS3 candidates are identified (**Figure 3B**) and the optimal CE is determined to maximize MS3 product ion intensity (**Figure 3C**). The ramped CE workflow is then repeated for MS3 fragmentation

Evaluation and Selection of Optimal Scan Modes

Each steroid was analyzed with the optimal CE in MS2-HCD, MS2-CID, and MS3 scan modes with a 5 min RT window (**Figure 4**). With 115 MS scans performed per cycle, each scan mode is evaluated within the same injection – ultimately saving time and resources during method optimization. The performance of each scan mode is best evaluated in complex matrices where co-eluting interferences are most likely (**Figure 5**).

Figure 4. TIC of 11-deoxycortisol in the MS2-HCD mode. The 6.415 to 6.440 min time range is zoomed-in to show that within one cycle (dynamic, ~1.2 sec), a total of 115 MS1, MS2, and MS3 scans were performed, highlighting the fast scan speed of Stellar MS.

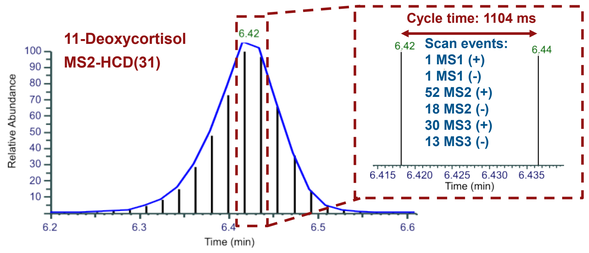


Figure 5. EIC of the quantifier (Quan) and qualifier (Qual) ions of DHT (0.6 pg injected on-column) from commercial QC samples detected by MS2-HCD (QqQ-like fragmentation, (A) or MS3 (B). (C) The MS3 fragmentation spectra of DHT (0.6 pg injected). (D) The proposed fragmentation mechanism of DHT.

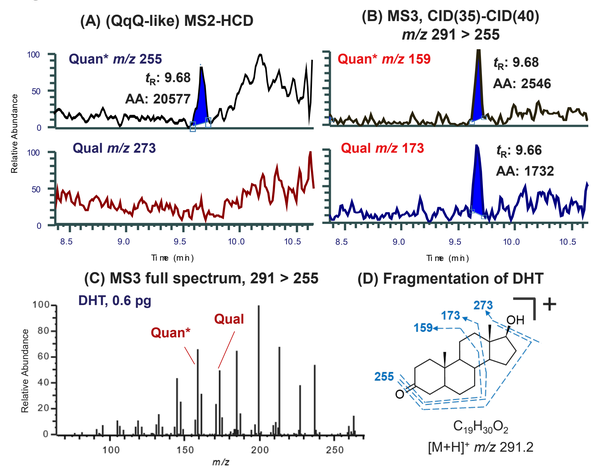
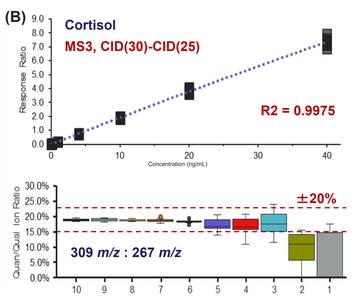


Figure 6. Determination of LOQ. (A) Calibration curve concentrations after sample extraction and the amount injected on-column. (B) An example CAL curve of cortisol (MS3) and the ion ratio stability across all calibrators (n=9).

(A)

Curve	Conc. ng/mL	On-column, pg
Cal-10	40	200
Cal-9	20	100
Cal-8	10	50
Cal-7	4	20
Cal-6	1	5
Cal-5	0.2	1
Cal-4	0.1	0.5
Cal-3	0.04	0.2
Cal-2	0.02	0.1
Cal-1	0.01	0.05

(B)



Evaluation and selection of optimal scan modes (continued)

Figure 5 illustrates such challenges using serum dihydrotestosterone (DHT) as an example. The Quan/Qual ion ratio calculation of DHT suffers from high background at the detection limit using MS2-HCD (QqQ-like fragmentation, **Figure 5A**), whereas Stellar MS provided an alternative MS3 fragmentation that drastically reduced the background of the qualifier ions (*m/z* 173, **Figure 5B**), therefore improving the sensitivity and selectivity of the measurement. Additionally, the PRM capabilities of the Stellar MS allows for post-acquisition selection of optimal product ions in all scan modes.

Quantification of steroids

Linear calibration curves were built with reference standards following the concentrations listed in **Figure 6A**. 17-OH-pregnenolone, DHEA, and pregnenolone were prepared at 10x of the listed levels, and DHT, estradiol, and estrone were 2x. LOQ was determined using a weighting factor of 1/x with R2 values greater than 0.99, |%Diff| < 30%, %RSD and ion ratio < 20% (**Figure 6B**). **Table 2** summarizes the LOQ, inter-day imprecision results, and the %RSD of the IS using the optimal scan mode.

Table 2. The steroids LOQ and scan mode with the optimal CE. The reproducibility of the measurement is represented by the inter-day imprecision %RSD of the analytes (3 days, N=27) in CAL-5 and internal standard %RSD in CAL-4, 5, 6, and 7 samples.

Compound name	LOQ (pg on-column)	Scan mode (CE)	Cal-5 Inter-day %RSD	IS %RSD (N = 108)
Aldosterone (-)	0.50	CID(30)-CID(25)	10.8%	10.2%
Aldosterone (+)	0.10	CID(40)	9.0%	6.9%
Cortisone	0.20	HCD(40)	11.5%	8.2%
Cortisol	0.10	HCD(30)	10.1%	8.7%
21-Deoxycortisol	0.20	HCD(20)	9.0%	7.0%
Corticosterone	0.50	CID(29)	10.8%	8.7%
11-Deoxycortisol	0.10	HCD(31)	10.4%	8.7%
Androstenedione	0.10	CID(40)	9.8%	9.4%
Estrone (-)	1.00	HCD(20)-HCD(60)	18.1%	7.2%
Estradiol (-)	1.00	HCD(10)-HCD(80)	17.5%	8.0%
11-Deoxycorticosterone	0.10	HCD(40)	7.2%	9.7%
Testosterone	0.05	HCD(40)	7.7%	8.3%
17-OH-Progesterone	0.10	HCD(40)	10.6%	9.7%
DHEA (-H2O)	2.00	CID(30)-CID(40)	7.3%	5.4%
17-OH-Pregnenolone (-)	2.00	CID(30)	16.4%	7.5%
DHT	0.40	CID(35)-CID(40)	7.7%	6.6%
Progesterone	0.05	HCD(40)	6.1%	7.9%
Pregnenolone (-H2O)	2.00	HCD(30)	12.6%	7.2%

Conclusions

Sensitive and selective quantification of steroids in serum using the Stellar MS was demonstrated. The 5-min RT scheduling could be shortened to accommodate more numbers of analytes in the method. Overall, Stellar MS showed fast scan speed, alternative fragmentation, novel source design, and software features that enabled high-throughput, sensitive, and selective biomarker quantifications required by clinical research.

References

1.Remes, P. M., et. al., J. Proteome Res. 2024, 5476.

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General Laboratory Equipment – Not For Diagnostic Procedures.

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