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Clinical research

Selective and sensitive measurement of 17 steroids in human serum using a Stellar mass spectrometer

Authors

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Keywords

Stellar mass spectrometer, hybrid quadrupole linear ion trap MS, targeted quantitative analysis, steroids, human serum, alternative fragmentation, HCD, CID, MS^3, improved sensitivity and selectivity

Application benefits

- Simultaneous quantification of a serum steroid panel using fast MS^2 and MS^3 scan event acquisition with alternative and complementary fragmentation capabilities for improved sensitivity and selectivity
- Expedited compound optimization using online LC-MS injection and Thermo Scientific™ TraceFinder™ software

Goal

To demonstrate the development of a method for sensitive quantification of 17 endogenous steroids in human serum, leveraging the unique selectivity of complementary fragmentation mechanisms and the rapid acquisition speed of the Thermo Scientific™ Stellar™ mass spectrometer.

Introduction

Steroidogenesis is the biological process by which the body synthesizes steroid hormones from cholesterol. It begins in the mitochondria with the conversion of cholesterol into pregnenolone, which serves as the precursor for several classes of steroids, including progestogens, corticosteroids, androgens, and estrogens (Figure 1). Steroidogenesis is vital for numerous physiological functions, including maintaining cell membrane structure, hormone production, cellular signaling, growth and development regulation, reproduction, and immune functions.[1] Disruptions in steroidogenesis can result in endocrine disorders such as congenital adrenal hyperplasia, Cushing's syndrome, and certain forms of hypertension.[1-3] Therefore, accurate clinical research laboratory tests require researchers to prioritize the right analytical testing methods to ensure reliable monitoring of steroid hormones.

Currently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the gold standard for quantifying steroids in serum due to its ability to measure multiple steroids simultaneously with high sensitivity and selectivity. However, quantifying such a steroid panel in human biofluids is challenged by the structural similarity among different steroids and their low concentrations in biological samples. Researchers are exploring LC-MS systems with sufficient sensitivity, specificity, selectivity, wide dynamic range, and excellent precision and accuracy for reliable concentration measurements.

In this technical note, we demonstrated the streamlined development of a method for the simultaneous measurement of 17 steroids in human serum using a Thermo Scientific™ Vanquish™ Horizon ultra-high performance liquid chromatography (UHPLC) coupled to the Stellar mass spectrometer (MS) (Figure 2). The Stellar MS is a hybrid quadrupole, dual-pressure linear ion trap MS that offers two types of orthogonal, yet complementary, fragmentation modes with rapid MS^2 and MS^3 scans: beam-type high-energy collision-induced dissociation (HCD), which is a triple quadrupole (QqQ)-like fragmentation, and resonance-type collision-induced dissociation (CID).[4] We utilized TraceFinder software to aid the compound optimization and demonstrate the advantages of utilizing multiple collision activations and MS^3 scans performed by the Stellar MS to the selective and sensitive quantification of 17 steroids in serum, highlighting its potential to improve the monitoring of clinical biomarkers and therapeutic drugs.

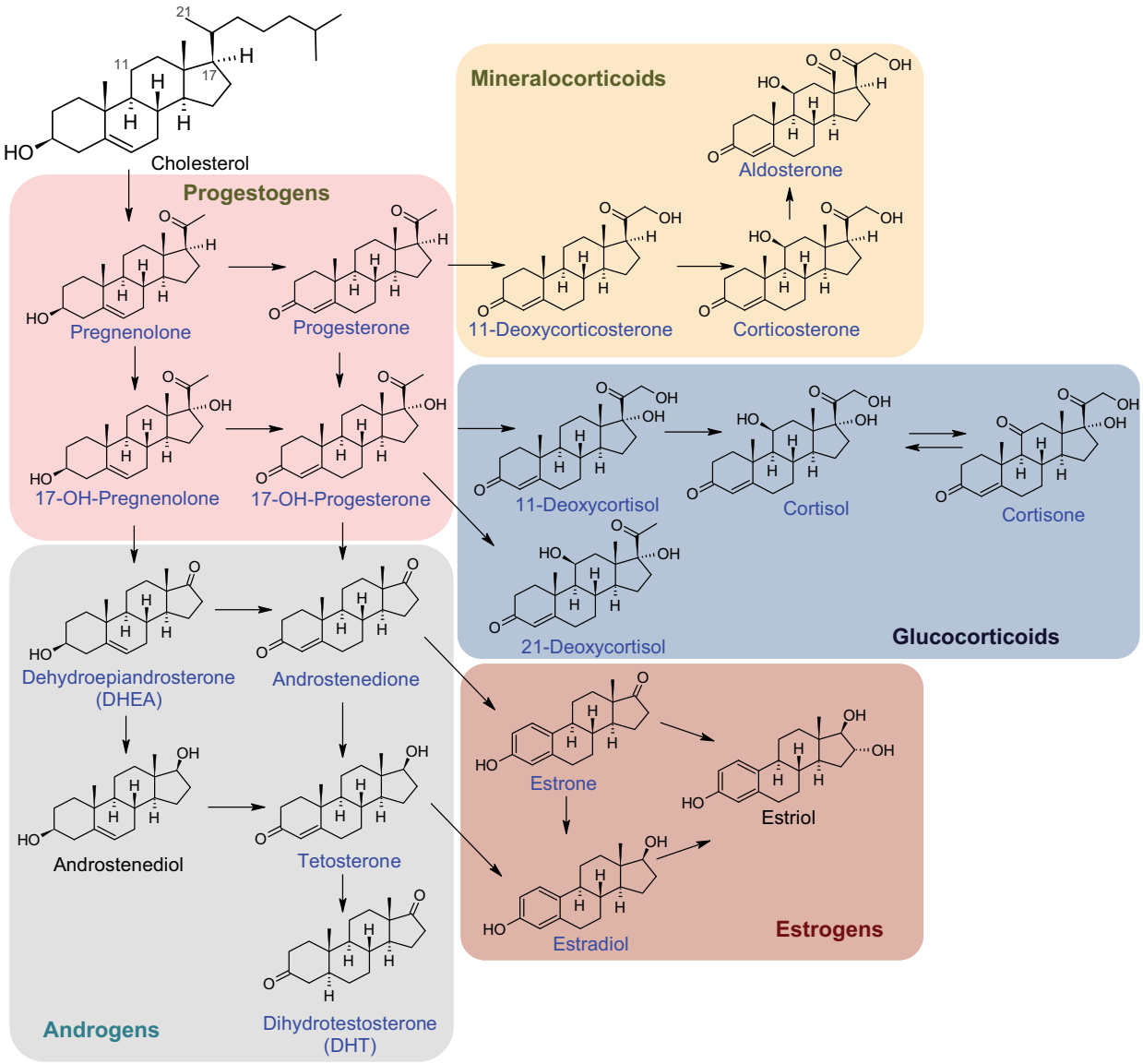

Figure 1. Steroidogenesis pathway. The compounds in blue are the ones detected in this technical note.

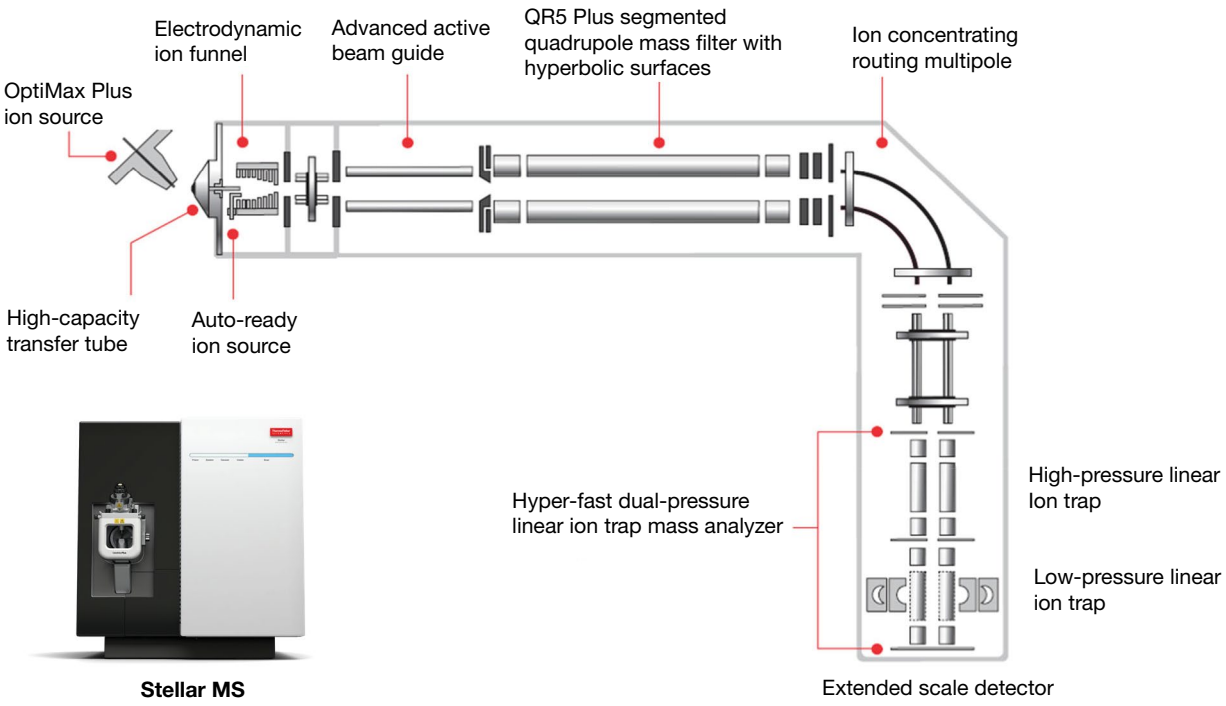

Figure 2. Thermo Scientific Stellar MS diagram

Experimental

Materials

Certified reference standards of 17 synthetic steroids, including 4 progestogens, 4 androgens, 2 estrogens, 3 mineralocorticoids, and 4 glucocorticoids, and their corresponding internal standards (IS) were purchased from Cerilliant (Round Rock, TX). Fisher Chemical™ Optima™ LC/MS grade water, methanol, acetonitrile, formic acid, acetic acid, and HPLC-grade isopropanol were used. Bovine serum albumin (BSA) and ammonium fluoride (NH_4F) (≥99.99% trace metals basis) were purchased from Sigma-Aldrich (St. Louis, MO). The steroid standards were serially diluted in 50% water/methanol to generate Calibration Working Solutions over four orders of magnitude concentration range. For the calibration curve, 10 µL of the Calibration Working Solution and 10 µL IS solution were added to 160 µL of 0.05% BSA (w/v). The majority of the steroids can be reliably detected at the calibration levels 4 or 5 (~0.2 ng/mL), thus Cal-4, 5, 6, and 7 were used to demonstrate the inter-day and intra-day imprecision study. Commercial quality control (QC) samples containing testosterone, androstenedione, and dihydrotestosterone (DHT) in human serum were acquired from a collaborator in the German Sport University Cologne and analyzed to highlight the improved selectivity provided by the MS^3 scans in the Stellar MS.

Sample preparation

The calibration samples were acidified with 20 µL acetic acid, precipitated with 400 µL of ice-cold acetonitrile, vortexed, and centrifuged at 21,000 ×g for 10 min at 4 °C. The supernatant of 500 µL was transferred to a new polypropylene tube, dried to complete dryness, reconstituted in 100 µL 50% water/methanol, vortexed thoroughly, and centrifuged again at 21,000 ×g for 10 min at room temperature. The top 90 µL was carefully transferred to the glass LC-MS vial with insert, of which 5 µL was injected for LC-MS analysis. The commercial QC samples were prepared by the German Sport University Cologne with minor modifications.

Liquid chromatography – mass spectrometry

Samples were analyzed on a Vanquish Horizon UHPLC system coupled to the Stellar mass spectrometer in the targeted-MS^2 (tMS^2) and targeted-MS^3 (tMS^3) scan modes. The mobile phase, analytical column, and LC gradient are specified in Table 1. The Stellar MS was equipped with the Thermo Scientific™ OptaMax™ Plus ion source and heated electrospray ionization (HESI) sprayer. Multiple fragmentation schemes were evaluated and optimized on the Stellar MS. The source and scan properties are listed in Table 2.

Table 1. Chromatographic conditions used to separate steroids

UHPLC conditions			
Gradient	Time (min)	% B	Flow (µL/min)
	0.0	50	300
	4.0	50	300
	9.0	75	300
	10.0	100	300
	12.0	100	300
	12.1	50	300
	16.0	50	300
Column	Kinetex™ C18, 2.1 × 150 mm, 2.6 µm @ 50 °C		
Mobile phase A	0.05 mM NH_4F in water		
Mobile phase B	0.05 mM NH_4F in methanol		
Needle wash	50% isopropanol/acetonitrile		

Table 2. Ion source and global parameters for Stellar MS

Stellar MS parameters	
OptaMax Plus ion source properties	
Spray voltage (V)	(+) 1,500 / (-) 2,500
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
MS^1 parameters	
Scan rate (kDa/s)	125
Scan range (*m/z*)	70–500
AGC target	3e4
RF lens (%)	30
Max. injection time mode	Auto
Data type	Centroid
Polarity	Both
Source fragmentation (V)	0
Targeted-MS^n parameters	
Isolation window (*m/z*)	2
MS^2 / MS^3 scan rate (kDa/s)	125 / 67
MS^2 / MS^3 scan range	Auto
HCD / CID collision energy type	Normalized
AGC target	1e4
Max. injection time mode	Dynamic
Points per peak	7
Data type	Centroid

Data analysis

Data was acquired in TraceFinder software (v 5.2 Clinical) and processed for compound optimization and quantitative performance, including detection limit, quantitation limit and linearity.

Results and discussion

Compound optimization

Given the significant variations in the chemical structures of small molecules, it is highly recommended to optimize MS parameters to achieve maximum sensitivity and selectivity for their quantitative analysis in complex matrices using LC-MS/MS methods. The Stellar MS offers several beneficial and differentiating features compared to a conventional QqQ MS commonly employed for targeted quantitative analysis for small molecules, including the rapid acquisition rate with MS^2 up to 140 Hz and MS^3 up to 40 Hz, fast polarity switching of 5 milliseconds, and the capability of acquiring the full fragmentation spectra of the analyte precursor.[4] The resonance-type CID fragmentation and MS^3 scan provide alternative, yet complimentary capabilities to the QqQ-like HCD and could improve the detection sensitivity and selectivity. TraceFinder software offers an automated feature to facilitate online optimization of MS parameters in a panel of analytes. Figure 3 illustrates the optimization process using the MS^2 normalized collision energy (NCE) for HCD and CID for testosterone. Other MS parameters, such as RF lens and vaporizer temperature, can be similarly optimized using TraceFinder software. The targeted product ions can be determined from literature, MS fragmentation spectra library repositories, such as Thermo Scientific™ mzCloud™ (https://www.mzcloud.org/) spectral library and MassBank of North America (https://mona.fiehnlab.ucdavis.edu/), or a survey MS^n scan.

In this technical note, up to four most intense fragment ions of each steroid and its corresponding IS were selected from the published quantification method using QqQ MS,[5] in addition to the online LC-MS injection of the synthetic standards with NCE 30% in both HCD and CID MS^2 scans. Our empirical data indicated that NCE 30% offers efficient fragmentations to many small molecules in both HCD and CID MS^2, thus, it was chosen for the survey scan. A series of instrument methods were built for the steroid panel with varying NCE settings: 0 to 90% NCE for HCD (10% interval) and 0 to 50% NCE for CID (5% internal). The same steroid mixture was injected in duplicate for each method, and the average peak areas of the target fragment ions were determined in TraceFinder software from the Group Average. Figure 3 uses testosterone product ion *m/z* 97 and estradiol product *m/z* 183 to illustrate the peak area changes with ramping NCE settings. The optimal NCE values and potential MS^3 candidates can be selected from the resulting “breakdown curves.” The fast tandem MS scan speed of the Stellar MS allows for the simultaneous optimization of steroids and corresponding IS in both MS^2-HCD and MS^2-CID scan modes without retention time (RT) scheduling.

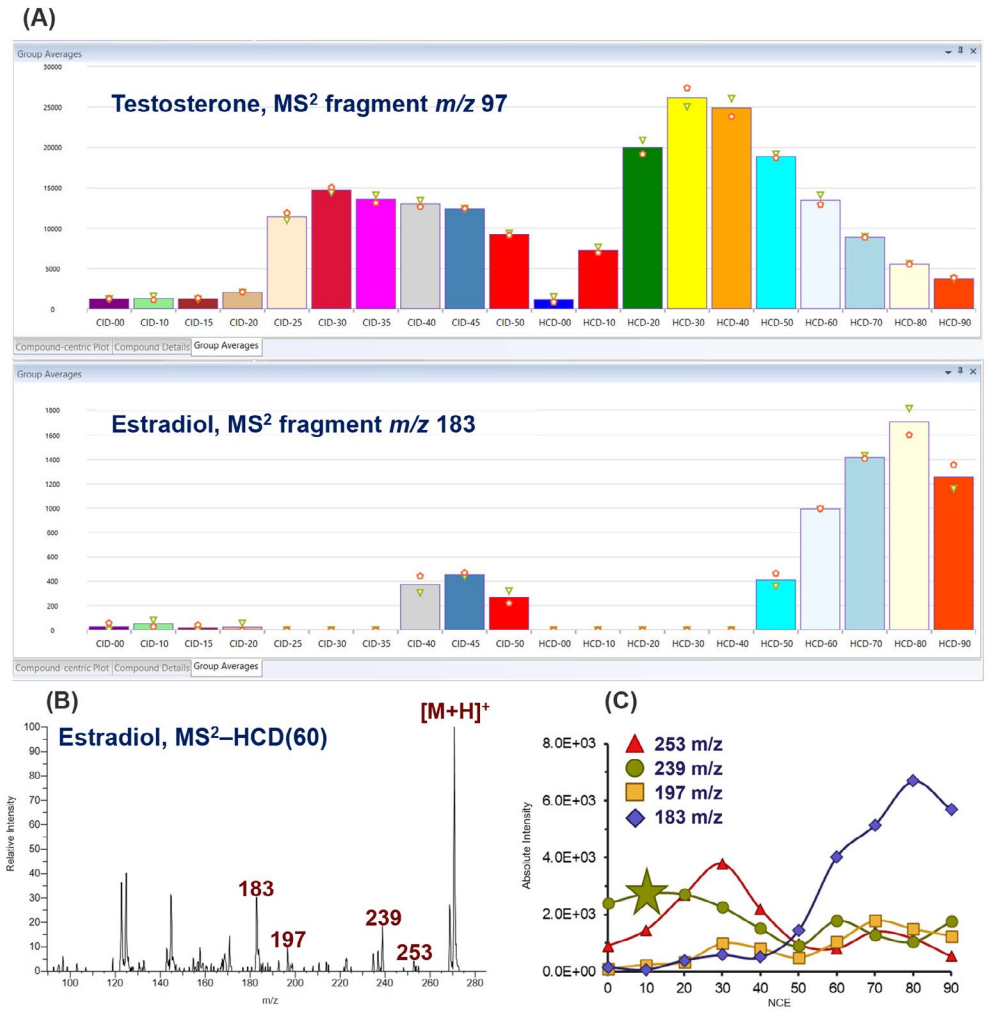

Figure 3. The determination of the optimal NCEs for steroids in CID and HCD MS² scan events in TraceFinder software using (A) testosterone fragment ion *m/z* 97 and estradiol fragment *m/z* 183 as examples; (B) MS²-HCD spectrum of partially fragmented estradiol precursor ion; (C) overlaid breakdown curves of estradiol MS²-HCD fragment intensities. The potential MS³ candidates for estradiol are highlighted with an asterisk.

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-minute retention time window (Figure 4 and Table 3). The "Dynamic" setting in the Max Injection Time Mode adjusts the maximum injection time value on the fly to be as large as possible and respect the Chromatography Peak Width and the Points Per Peak sampling rate requirements specified by the user in the Instrument Method Setup (Table 2).[4] As previously noted, the Stellar MS system demonstrates exceptional speed, achieving acquisition rates of up to 140 Hz. Figure 5 shows an extracted ion chromatogram of 11-deoxycortisol, exemplifying this rapid acquisition capability. While monitoring 115 different scan events in one cycle, we were able to measure 14 points at the base across a 15.5 second peak width, corresponding to an overall acquisition speed of 104 Hz. The performance of each scan mode is best evaluated in complex matrices where co-eluting interferences are most likely. Figure 6 illustrates such challenges using serum dihydrotestosterone (DHT) from a commercial quality control sample as an example. The quantifier-to-qualifier (Quan/Qual) ion ratio calculation of DHT suffers from high background at the detection limit using MS²-HCD (QqQ-like fragmentation, Figure 6A) using the Qual ion *m/z* 273 as an example. In contrast, the Stellar MS provided alternative MS³ fragmentation that drastically reduced the background of the qualifier ions (*m/z* 173, Figure 6B), therefore improving the sensitivity and selectivity of the measurement. Additionally, the recording of the full fragmentation spectra in the Stellar MS allows for post-acquisition selection of optimal product ions in all scan modes.

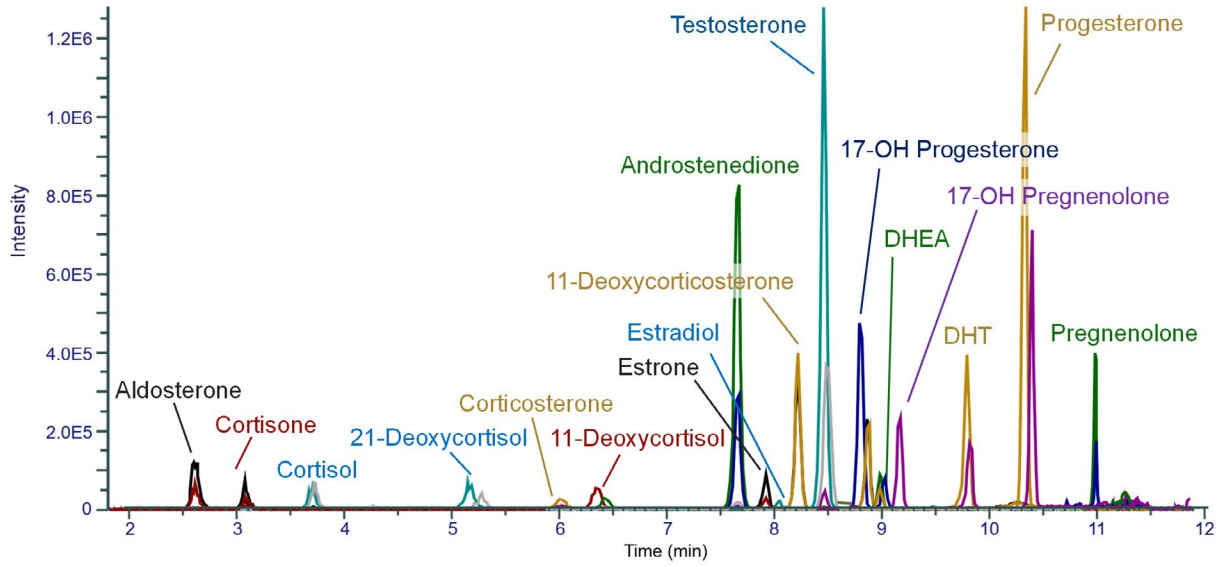

Figure 4. Representative overlaid extracted ion chromatogram (EIC) of the steroids in a calibration sample

Figure 5. EIC 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 s), a total of 115 MS^1, MS^2, and MS^3 scans were performed, highlighting the fast scan speed of the Stellar MS.

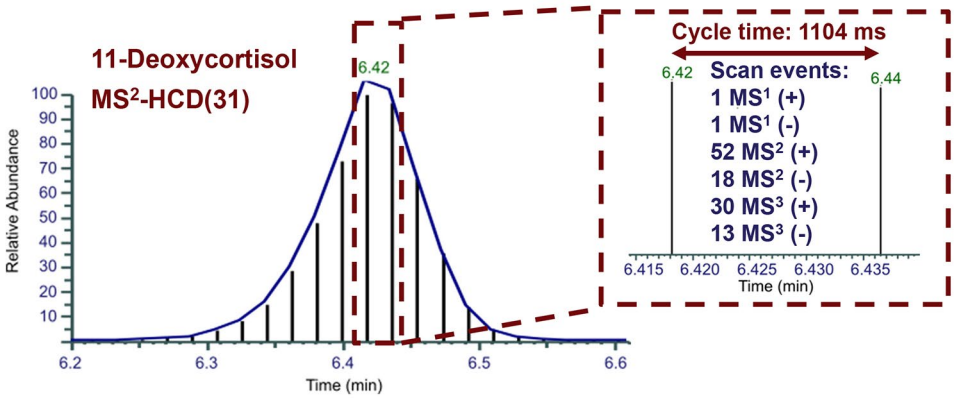

Figure 6. EIC of the quantifier (Quan) and qualifier (Qual) ions of DHT (0.6 pg injected on-column) from commercial QC samples. (A) Detected by MS^2-HCD (QqQ-like fragmentation) or (B) MS^3 in the Stellar MS. (C) The MS^3 fragmentation spectra of DHT (0.6 pg injected). (D) The proposed fragmentation mechanism of DHT.

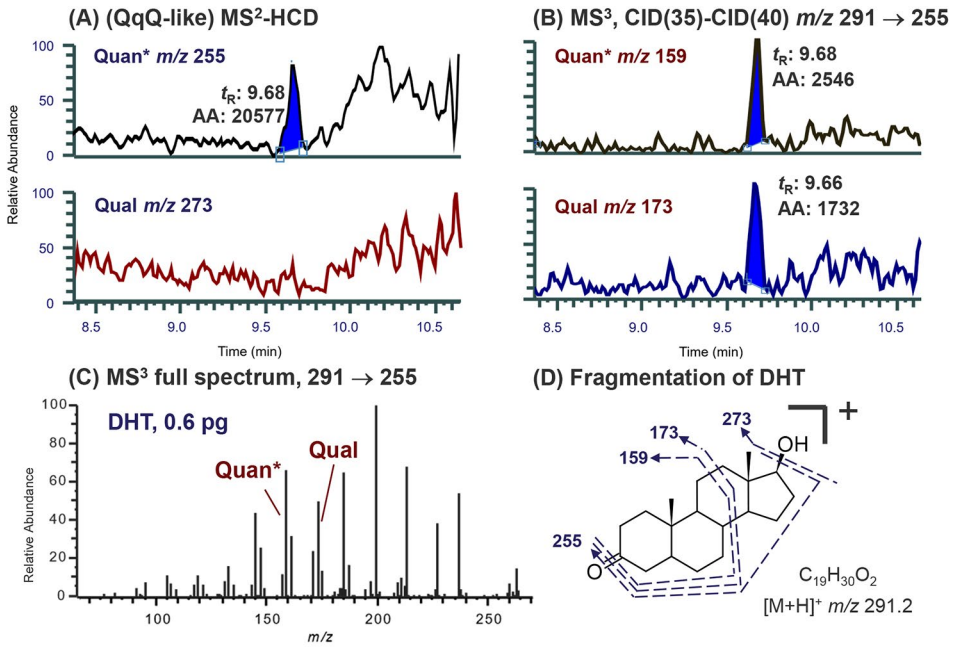

Table 3. Retention time, precursor ion *m/z*, and optimal parameters for MS^2 and MS^3 scan events

Compounds	Polarity	Retention time (min)	$[M+H]^+$ *m/z*	HCD (NCE)	CID (NCE)	MS^3 (NCE)	IS	$[M+H]^+$ *m/z*
Aldosterone	Pos	2.7	361.2	20	40	CID(30)-CID(30)	$[^{13}C_3]$-Aldosterone	364.2
Aldosterone	Neg	2.7	359.2	30	30	CID(30)-CID(25)	$[^{13}C_3]$-Aldosterone	362.2
Cortisone	Pos	3.1	361.2	40	40	CID(40)-CID(25)	$[^{13}C_3]$-Cortisone	364.2
Cortisol	Pos	3.7	363.2	30	30	CID(30)-CID(25)	$[^2H_4]$-Cortisol	367.2
21-Deoxycortisol	Pos	5.2	347.2	20	30	CID(25)-CID(30)	$[^2H_8]$-21-Deoxycortisol	355.2
Corticosterone	Pos	5.9	347.2	30	30	CID(30)-CID(30)	$[^{13}C_3]$-Corticosterone	350.2
11-Deoxycortisol	Pos	6.3	347.2	30	30	CID(35)-CID(25)	$[^2H_5]$-11-Deoxycortisol	352.2
Androstenedione	Pos	7.7	287.2	30	40	CID(30)-CID(30)	$[^{13}C_3]$-Androstenedione	290.2
Estrone	Neg	7.9	269.2	70	50**	HCD(20)-HCD(60)	$[^{13}C_3]$-Estrone	272.2
Estradiol	Neg	8.1	271.2	80	50**	HCD(10)-HCD(80)	$[^2H_5]$-Estradiol	276.2
11-Deoxycorticosterone	Pos	8.2	331.2	30	30	CID(30)-CID(30)		
Testosterone	Pos	8.5	289.2	40	40	CID(30)-CID(30)	$[^2H_3]$-Testosterone	292.2
17-OH-Progesterone	Pos	8.9	331.2	40	40	CID(30)-CID(25)	$[^2H_8]$-17-OH Progesterone	339.2
DHEA*	Pos	9.1	271.2	20	30	CID(30)-CID(40)	$[^2H_5]$-DHEA*	276.2
17-OH-Pregnenolone	Neg	9.2	331.2	30	30	CID(30)-CID(30)		
DHT	Pos	9.8	291.2	20†	30†	CID(35)-CID(40)†	$[^2H_3]$-DHT	294.2
Progesterone	Pos	10.5	315.2	40	40	CID(30)-CID(30)	$[^2H_9]$-Progesterone	324.2
Pregnenolone*	Pos	11.0	299.2	30	30	CID(30)-CID(30)	$[^{13}C_2,^2H_2]$-Pregnenolone*	303.2

*Precursor is M-H_2O

**Act Q 0.3

†Source fragmentation 26

Table 4. 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 in CAL-4, 5, 6, and 7 samples and %RSD of the IS peak areas (N = 108).

Compound name	LOQ (pg on-column)	Scan mode (CE)	Inter-day %RSD (N = 27)				IS %RSD
			Cal-4	Cal-5	Cal-6	Cal-7	
Aldosterone (-)	1.00	CID(30)-CID(25)	--	10.8%	14.0%	7.3%	10.2%
Aldosterone (+)	0.10	CID(40)	9.7%	9.0%	4.6%	3.8%	6.9%
Cortisone	0.20	HCD(40)	12.9%	11.5%	8.4%	7.9%	8.2%
Cortisol	0.10	HCD(30)	14.0%	10.1%	9.3%	8.9%	8.7%
21-Deoxycortisol	0.20	HCD(20)	11.8%	9.0%	9.2%	6.4%	7.0%
Corticosterone	0.50	CID(29)	14.5%	10.8%	7.4%	6.8%	8.7%
11-Deoxycortisol	0.10	HCD(31)	10.5%	10.4%	8.1%	6.6%	8.7%
Androstenedione	0.10	CID(40)	12.3%	9.8%	10.1%	6.2%	9.4%
Estrone (-)	1.00	HCD(20)-HCD(60)	24.2%	18.1%	7.3%	4.8%	7.2%
Estradiol (-)	1.00	HCD(10)-HCD(80)	23.0%	17.5%	18.0%	7.3%	8.0%
11-Deoxycorticosterone	0.10	HCD(40)	10.8%	7.2%	6.7%	5.0%	9.7%
Testosterone	0.05	HCD(40)	11.4%	7.7%	8.0%	6.0%	8.3%
17-OH-Progesterone	0.10	HCD(40)	9.2%	10.6%	8.1%	8.2%	9.7%
DHEA (-H_2O)	2.00	CID(30)-CID(40)	6.9%	7.3%	6.3%	4.3%	5.4%
17-OH-Pregnenolone (-)	2.00	CID(30)	20.3%	16.4%	8.3%	5.0%	7.5%
DHT	0.40	CID(35)-CID(40)	9.5%	7.7%	4.8%	3.9%	6.6%
Progesterone	0.05	HCD(40)	7.8%	6.1%	7.9%	7.3%	7.9%
Pregnenolone (-H_2O)	2.00	HCD(30)	12.6%	12.6%	6.8%	5.2%	7.2%

Quantification of steroids

Linear calibration curves were built with reference standards following the concentrations listed in Figure 7A. 17-OH-pregnenolone, DHEA, and pregnenolone were prepared at 10x the listed levels, and DHT, estradiol, and estrone were 2x. Limit of quantification (LOQ) using the described method was determined using a weighting factor of 1/x with R^2 values greater than 0.99, |%Diff| < 30%, %RSD and ion ratio < 20% (Figure 7B). Table 4 summarizes the LOQ, inter-day imprecision results, and the %RSD of the IS using the optimal scan mode. The developed method demonstrates high reproducibility, with inter-day imprecision (%RSD) below 20% at three steroid levels and internal standard %RSD across 108 injections remaining below 15%.

(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)

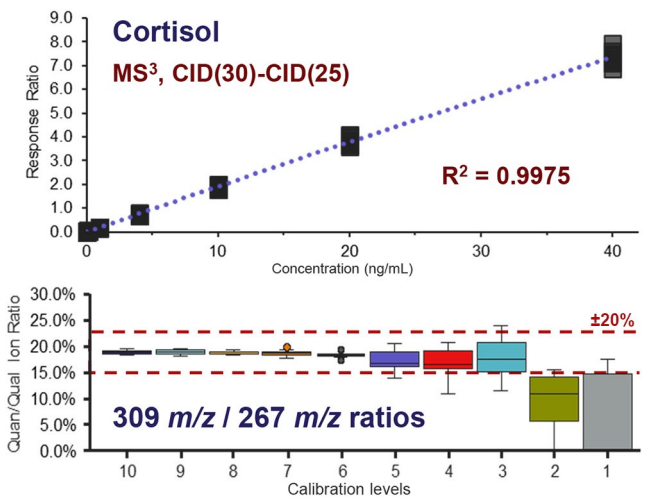

Figure 7. Determination of LOQ. (A) Calibration curve concentrations after sample extraction and the amount injected on-column. (B) An example CAL curve of cortisol (MS^3) and the ion ratio stability across all calibrators (N = 9).

Conclusion and future work

Sensitive and selective measurement of steroids in serum using the Stellar MS was demonstrated with a highly multiplexed method. The experimental results show method optimization can be performed using UHPLC separation at extremely low levels to streamline final method development in the presence of matrix. Leveraging the instrument flexibility of the Stellar MS, both beam-type and resonance-type dissociation techniques at the MS^2 and MS^3 levels were evaluated for the set of analytes across the spiking ranges, resulting in determination of the best parameters for the entire set of steroid hormones.

Future work will leverage the information obtained in the current work to extend LOQ performance for the set of steroid hormones. We demonstrated that the Stellar MS could perform fast and sensitive tMS^2 and tMS^3 in one method using HCD and CID with more than seven data points per LC peak at the lower spiking levels with UHPLC peak widths of 6 to 12 seconds. Additional method improvement could be achieved using narrower scheduled RT windows from the 5-minute setting in this work to less than 1 minute or by selecting only one scan event per analyte. Collectively, these steps will increase the injection time and further improve the data quality.

Overall, the 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.

Acknowledgements

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