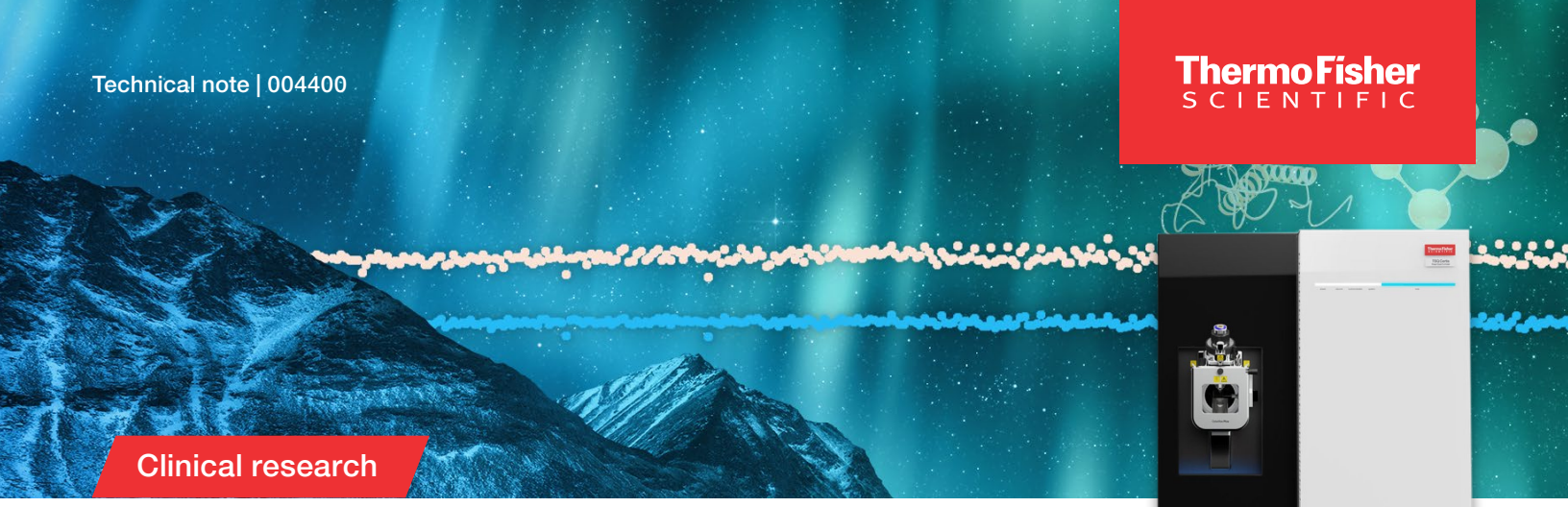
Technical note | 004400



Clinical research

Robust and sensitive quantification of 18 steroids using human serum with the TSQ Certis mass spectrometer

Authors

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Keywords

TSQ Certis mass spectrometer, Vanquish Horizon UHPLC, steroid analysis, human serum, robustness

Application benefits

- Simultaneous quantification of 18 steroids using serum with a 9-minute LC-MS method
- Robust MS responses over 311 injections for all 18 steroids

Goal

To establish a highly robust and sensitive LC–MS/MS method for the simultaneous quantification of 18 endogenous steroids using human serum with the Thermo Scientific™ Vanquish™ Horizon UHPLC System coupled to the Thermo Scientific™ TSQ Certis™ Triple Quadrupole Mass Spectrometer (MS). The 9-minute method demonstrated excellent sensitivity, selectivity, and linearity across broad concentration ranges. Its performance was further verified using commercial serum calibrators and QC samples containing six representative steroids, all of which achieved strong correlation ($R^2 > 0.99$) and accurate quantification. Method robustness was demonstrated over 311 consecutive injections, yielding highly consistent peak areas. Overall, the TSQ Certis MS exhibited the precision, stability, and throughput required for reliable steroid quantification in clinical research laboratory settings.

Introduction

Steroidogenesis is the biological process by which the body synthesizes steroid hormones from cholesterol. Accurate and reliable quantification of endogenous steroids is essential for biomonitoring key physiological functions in clinical research laboratories, including hormone regulation, growth and development, reproduction, and immune function.[1-3]

Traditional analytical techniques such as chemiluminescence immunoassay and radioimmunoassay are widely used for steroid detection; however, liquid chromatography–tandem mass spectrometry (LC–MS/MS) is now considered the gold standard due to its superior sensitivity, selectivity, and ability to measure multiple analytes simultaneously. Despite these advantages, steroid quantification in serum by LC–MS/MS presents several analytical challenges. Steroids are often present at very low concentrations, and the complexity of the serum matrix can interfere with accurate detection and quantification.

In this technical note, we describe a robust and sensitive LC–MS/MS method for the simultaneous measurement of 18 steroids using human serum on a Vanquish Horizon UHPLC system coupled to the TSQ Certis MS, equipped with the Thermo Scientific™ OptaMax™ Plus Ion Source (Figure 1). The advanced hardware and software capabilities of the TSQ Certis MS provide the robustness, selectivity, and sensitivity required for accurate steroid quantification using serum, highlighting its potential for improving the biomarkers and biomonitoring in clinical research labs.

Figure 1. Vanquish Horizon UHPLC coupled to TSQ Certis MS.

Experimental

Materials

Certified reference standards of 18 synthetic steroids and their corresponding internal standards (IS) were purchased from Cerilliant (Round Rock, TX). The analytes included:

- Progestogens: pregnenolone (Preg), progesterone (Prog), 17-hydroxypregnenolone (17-OH-Preg), and 17-hydroxyprogesterone (17-OH-Prog)
- Androgens: dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androstenedione, testosterone, and dihydrotestosterone (DHT)
- Estrogens: estradiol and estrone
- Mineralocorticoids: aldosterone, 11-deoxycorticosterone (11-DCC), and corticosterone
- Glucocorticoids: 11-deoxycortisol (11-DOC), 21-deoxycortisol (21-DOC), cortisol, and cortisone

Thermo Scientific™ UHPLC-MS grade water, methanol, acetonitrile; Fisher Chemical™ Optima™ LC/MS grade formic acid and acetic acid; and HPLC-grade isopropanol were obtained from the Fisher Scientific™ channel (Waltham, MA). Bovine serum albumin (BSA), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$, ACS grade), and ammonium fluoride (NH_4F, ≥99.99%, trace metals basis) were purchased from Sigma-Aldrich (St. Louis, MO).

Steroid standards were serially diluted in methanol to generate calibration working solutions spanning four orders of magnitude in concentration (Table 1). For calibration curve preparation, 10 µL of the calibration working solution and 10 µL of IS solution were added to 160 µL of 0.05% BSA in phosphate-buffered saline (PBS, w/v).

Commercial calibrator samples (6PLUS1™ Multilevel Serum Calibrator Set Steroid Panel 2, Chromsystem, Munich, Germany) and serum control samples (UTAK, Valencia, CA) were obtained from collaborators.

Sample preparation

Calibration samples were precipitated with 20 µL of $ZnSO_4$ and 700 µL of ice-cold acetonitrile, vortexed, and centrifuged at 21,000 × g for 10 minutes at 4 °C. The 750 µL supernatant was transferred to a new polypropylene tube, dried completely, reconstituted in 100 µL of 50% water/methanol, vortexed, and centrifuged again at 21,000 × g for 10 minutes at 4 °C. The upper 90 µL was carefully transferred to a glass LC–MS vial with an insert, and 5 µL was injected for LC–MS analysis.

Commercial calibrator and control samples were prepared by collaborators using solid-phase extraction (SPE; method not disclosed).

Liquid chromatography – mass spectrometry

Samples were analyzed on a Vanquish Horizon UHPLC system coupled to the TSQ Certis MS operated in selected reaction monitoring (SRM) mode. The heated electrospray ionization (HESI) probe on the equipped OptaMax Plus HESI source was kept at the position: "center" (left-right) / "HESI" (in-out) / LM (up-down). The mobile phase composition, analytical column details, LC gradient, and overlaid extracted ion chromatograms (EICs) are shown in Figure 2. Source and scan parameters are summarized in Tables 2 and 3.

Data analysis

All data were acquired and processed using Thermo Scientific™ TraceFinder™ Software (v 5.2 Clinical).

Table 1. Concentrations of the steroids in the calibration samples.

Curve	Aldosterone, Cortisone, Cortisol, 21-DOC, Corticosterone, 11-DOC, Androstenedione, 11-DCC, Testosterone, 17-OH-Prog, Prog		Estrone, Estradiol, DHT		DHEAS, DHEA, 17-OH-Preg, Preg	
	Spiked BSA calibration solution conc. (ng/mL)	Conc. in LC-MS vial (ng/mL)	Spiked BSA calibration solution conc. (ng/mL)	Conc. in LC-MS vial (ng/mL)	Spiked BSA calibration solution conc. (ng/mL)	Conc. in LC-MS vial (ng/mL)
Cal-11	480.000	40.000	960.000	80.000	4800.000	400.000
Cal-10	48.000	4.000	96.000	8.000	480.000	40.000
Cal-9	12.000	1.000	24.000	2.000	120.000	10.000
Cal-8	2.400	0.200	4.800	0.400	24.000	2.000
Cal-7	1.200	0.100	2.400	0.200	12.000	1.000
Cal-6	0.480	0.040	0.960	0.080	4.800	0.400
Cal-5	0.240	0.020	0.480	0.040	2.400	0.200
Cal-4	0.120	0.010	0.240	0.020	1.200	0.100
Cal-3	0.048	0.004	0.096	0.008	0.480	0.040
Cal-2	0.024	0.002	0.048	0.004	0.240	0.020
Cal-1	0.012	0.001	0.024	0.002	0.120	0.010
IS	20.000	1.600	40.000	3.200	200.000	16.000

UHPLC conditions	
Column	Thermo Scientifc™ Hypersil GOLD™ C18 2.1 × 100 mm, 1.9 µm
Mobile phase A	0.2 mM NH_4F in water
Mobile phase B	Methanol
Needle wash	50:50 Isopropanol:Acetonitrile
Flow rate	0.3 mL/min
Inj. volume	5.0 µL

Time	Flow [mL/min]	%B
0.0	0.3	50
2.0	0.3	50
7.0	0.3	100
8.0	0.3	100
8.1	0.3	50
9.0	0.3	50

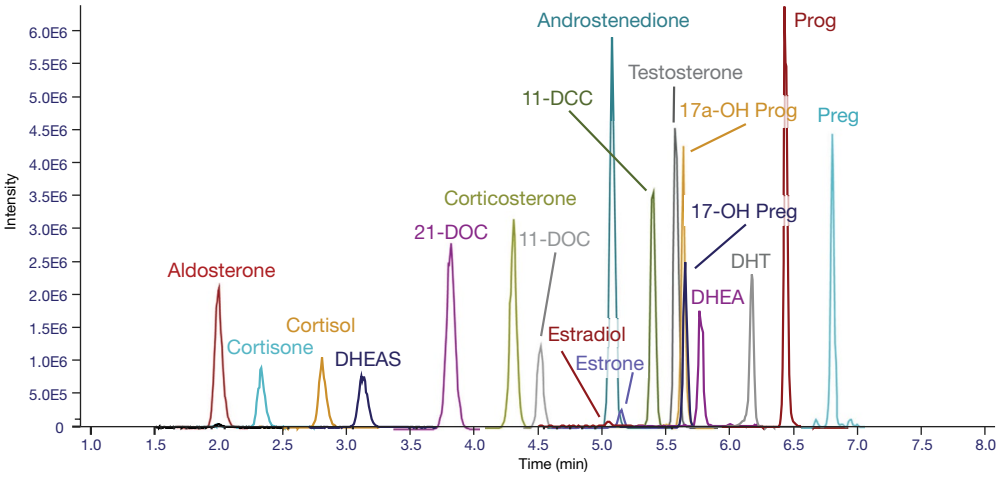

Figure 2. The LC conditions, gradient, and representative extracted ion chromatogram (EIC) overlay of steroid separation in BSA matrix.

Table 2. HESI source and SRM global parameters.

HESI source	
Spray voltage (V)	1,500 (+) / 2,500 (-)
Sheath gas (Arb)	50
Aux gas (Arb)	14
Sweep gas (Arb)	2
Ion transfer tube temp. (°C)	325
Vaporizer temp. (°C)	525

SRM parameters	
Cycle time (s)	0.4
Collision gas pressure (mTorr)	2.0
Q1 resolution (FWHM)	0.7
Q3 resolution (FWHM)	1.2

Table 3. SRM parameters.

Compound name	Start time (min)	End time (min)	Polarity	Precursor *m/z*	Quantifier *m/z*	CE	Qualifier *m/z*	CE	Dwell time (ms)	RF lens (V)	In-source fragmentation (V)
Aldosterone	1.0	3.0	Pos	361.2	343.1	18.0	315.1	20.0	8.7	68	0
[$^{13}C_3$]-Aldosterone	1.0	3.0	Pos	364.2	346.1	18.0	318.2	20.0	8.7	68	0
Cortisone	1.3	3.3	Pos	361.2	163.1	23.7	343.0	16.4	8.7	61	0
[$^{13}C_3$]-Cortisone	1.3	3.3	Pos	364.2	166.1	23.7	346.1	16.4	8.7	61	0
Cortisol	1.8	3.8	Pos	363.2	121.0	24.4	309.1	16.9	8.7	50	0
[$^{2}H_4$]-Cortisol	1.8	3.8	Pos	367.2	121.0	24.4	313.2	17.0	8.7	50	0
DHEAS	1.7	4.7	Neg	367.2	97.0	32.5	80.0	55.0	5.6	74	0
[$^{2}H_5$]-DHEAS	1.7	4.7	Neg	372.2	98.0	32.5	81.0	55.0	5.6	74	0
21-DOC	2.8	4.8	Pos	347.2	311.1	15.1	269.0	19.0	5.1	57	0
[$^{2}H_8$]-21-DOC	2.8	4.8	Pos	355.3	319.2	15.1	275.1	19.0	5.1	57	0
Corticosterone	3.3	5.3	Pos	347.2	120.9	25.0	145.1	30.1	5.1	49	0
[$^{13}C_3$]-Corticosterone	3.3	5.3	Pos	350.2	124.2	25.0	148.0	30.1	5.1	49	0
11-DOC	3.5	5.5	Pos	347.2	96.9	25.1	109.0	27.0	5.1	56	0
[$^{2}H_5$]-11-DOC	3.5	5.5	Pos	352.3	100.2	25.1	113.2	27.0	5.1	56	0
Androstenedione	4.1	6.1	Pos	287.2	96.9	21.7	109.0	23.9	5.0	53	0
[$^{13}C_3$]-Androstenedione	4.1	6.1	Pos	290.2	100.0	21.7	112.0	23.9	5.0	53	0
Estrone	4.2	6.2	Neg	269.2	145.1	37.7	183.0	36.9	5.0	76	0
[$^{13}C_3$]-Estrone	4.2	6.2	Neg	272.2	148.1	37.7	186.0	36.9	5.0	76	0
Estradiol	4.1	6.1	Neg	271.2	183.0	40.1	145.0	39.6	5.0	95	0
[$^{2}H_5$]-Estradiol	4.1	6.1	Neg	276.2	187.2	40.1	147.1	39.6	5.0	95	0
11-DCC**	4.4	6.4	Pos	331.2	97.0	20.0	109.0	24.0	5.0	57	0
Testosterone	4.6	6.6	Pos	289.2	97.1	22.0	109.1	25.0	5.0	56	0
[$^{2}H_3$]-Testosterone	4.6	6.6	Pos	292.2	97.0	22.0	109.0	25.0	5.0	56	0
17-OH-Prog	4.6	6.6	Pos	331.2	97.2	25.0	109.1	28.0	5.0	56	0
[$^{2}H_8$]-17-OH-Prog	4.6	6.6	Pos	339.3	100.1	25.0	113.1	28.0	5.0	56	0
DHEA*	4.8	6.8	Pos	271.2	253.2	12.0	213.1	19.0	5.0	46	0
[$^{2}H_5$]-DHEA*	4.8	6.8	Pos	276.3	258.2	12.0	218.2	19.0	5.0	46	0
17-OH-Preg***	4.7	6.7	Neg	331.2	287.3	25.0	313.2	25.0	5.0	55	0
DHT	5.2	7.2	Pos	291.1	255.1	15.0	159.2	22.0	5.0	44	20.4
[$^{2}H_3$]-DHT	5.2	7.2	Pos	294.3	258.2	15.0	159.2	22.0	5.0	44	20.4
Prog	5.4	7.4	Pos	315.2	96.9	22.0	109.0	24.9	5.0	57	0
[$^{2}H_9$]-Prog	5.4	7.4	Pos	324.3	100.2	22.0	113.2	24.9	5.0	57	0
Preg*	5.8	7.8	Pos	299.2	281.2	13.0	159.1	22.0	5.0	50	0
[$^{13}C_2$,$^{2}H_2$]-Preg*	5.8	7.8	Pos	303.3	285.1	13.0	161.1	22.0	5.0	50	0

*DHEA and Preg precursors are M–H_2O ions

**11-DCC uses [$^{13}C_3$]-androstenedione as the IS

***17-OH-Preg uses [$^{2}H_8$]-17-OH-Prog as the IS

Results and discussion

MS parameter optimization

SRM parameters, including collision energy, RF lens voltage, and in-source fragmentation voltage, were optimized via direct infusion of synthetic steroid standards mixed through a T-connector with 50% mobile phase B at 0.3 mL/min. The $[M–H_2O+H]^+$ ions were selected as precursor ions for DHEA and Preg due to their higher signal intensity.

The OptaMax Plus HESI source on the TSQ Certis MS provided stable spray performance over a broad vaporizer temperature range. Figure 3 shows the effect of vaporizer temperature (300–600 °C, in 50 °C increments) on steroid MS responses. Up to a two-fold increase in relative peak area was observed for most steroids (Figure 3A–C), while DHT, DHEA, and Preg exhibited significant signal enhancements (6- to 14-fold; Figure 3D). A vaporizer temperature of 525 °C was selected as the optimal global parameter for subsequent experiments.

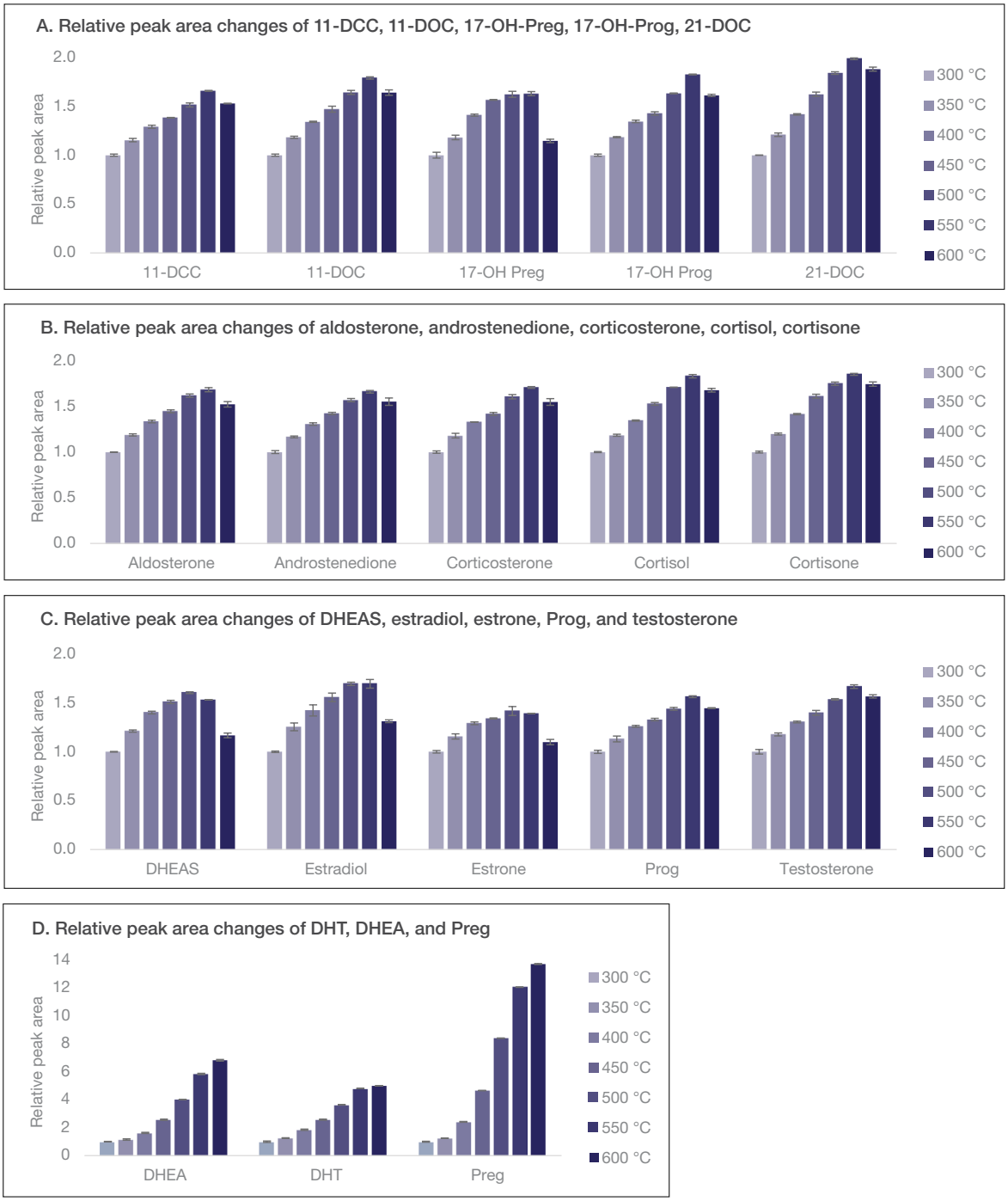

Figure 3. HESI source vaporizer temperature optimization. (A to C) MS responses increased up to 2x for most steroids when the vaporizer temperature changed from 300 °C to 600 °C. (D) Significant response increases were observed in DHT and water loss precursors of DHEA and Preg with increasing vaporizer temperatures.

Quantification of steroids

Calibration curves for steroids in 0.05% BSA were generated using linear regression with a 1/x weighting factor. All calibration curves exhibited coefficients of determination (R^2) greater than 0.99 across up to four orders of magnitude in concentration. Limits of quantification (LOQs) were defined as the lowest concentration that reached |% Diff|, %RSD (of the calculated amount), %CV (of the peak areas) and relative ion ratio <20% (N = 4) (Table 4). Estrone, DHT, and testosterone were selected as representative examples to demonstrate the calibration curves and corresponding EICs of quantifier and qualifier ions at their LOQ levels (Figure 4).

The developed method was then applied to the commercial serum steroid calibrator set (6PLUS1 Multilevel Serum Calibrator Set, Chromsystems Instruments & Chemicals GmbH, Germany), and quality control (QC) samples (Steroid Serum Control, UTAK Laboratories Inc, Valencia, CA) prepared via SPE. The calibrator samples contained 11-DCC, 17-OH-Prog, androstenedione, DHEA, DHT, estradiol, Prog, and testosterone, while QC samples included 17-OH-Prog, androstenedione, DHEA, DHT, and testosterone. Calibration curves were externally constructed based on peak areas.

Table 4. LOQ values of steroids (pg on column). LOQ is defined as the lowest concentration with $R^2 > 0.99$, |% Diff|, %RSD (of the calculated amount), %CV (of the peak areas) and relative ion ratio <20% (N = 4).

Compound name	LOQ (pg o.c.)	%RSD at LOQ	%CV at LOQ	Linear range (pg o.c.)	R^2
Aldosterone	0.10	10.43	6.60	0.10–20	0.9994
Cortisone	0.10	8.21	6.77	0.10–200	0.9996
Cortisol	0.05	9.30	7.13	0.05–200	0.9998
DHEAS	0.10	11.70	5.13	0.10–200	0.9989
21-DOC	0.10	5.61	5.95	0.10–200	0.9991
Corticosterone	0.05	11.83	8.40	0.05–200	0.9997
11-DOC	0.02	11.67	8.59	0.02–20	0.9987
Androstenedione	0.02	10.38	8.14	0.02–20	0.9984
Estrone	0.20	4.20	3.24	0.20–400	0.9997
Estradiol	0.40	8.30	8.50	0.40–400	0.9994
11-DCC	0.02	5.89	4.49	0.02–200	0.9996
Testosterone	0.01	7.59	4.88	0.01–20	0.9984
17-OH-Prog	0.05	8.46	7.40	0.05–200	0.9998
DHEA	5.00	8.83	6.78	5.00–200	0.9940
17-OH-Preg	1.00	4.88	7.66	1.00–2,000	0.9990
DHT	0.20	8.66	7.37	0.20–40	0.9981
Prog	0.05	7.07	6.01	0.05–20	0.9976
Preg	5.00	3.44	0.89	5.00–200	0.9976

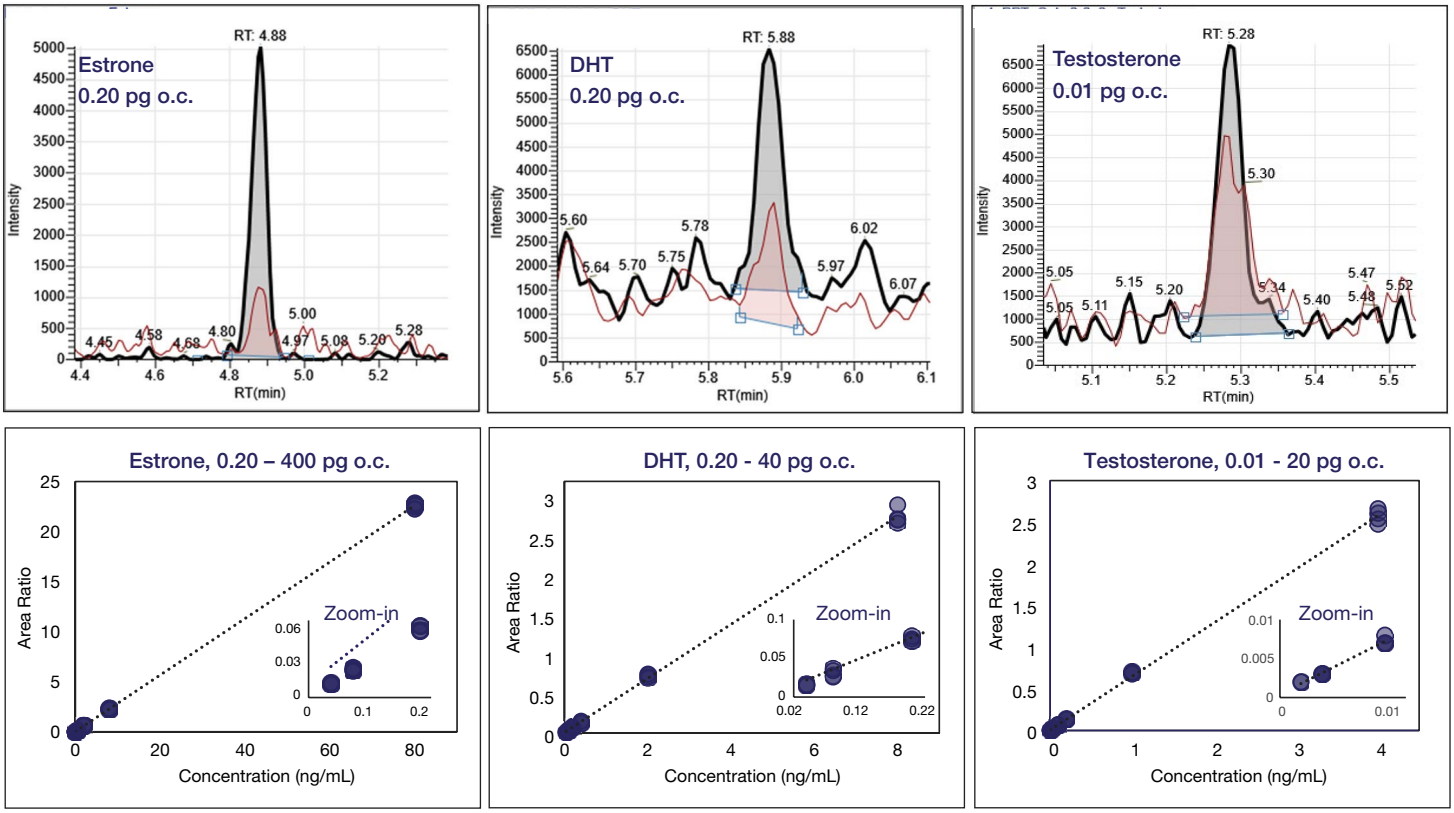

Figure 4. Calibration results of estrone, DHT, and testosterone in 0.05% BSA. (Top row) Quantifier (black trace) and qualifier (red trace) EIC overlay at the LOQ level, and (bottom row) their calibration curves and zoom-in to the lowest three calibration points. o.c. = on column.

All steroids demonstrated excellent linearity across their respective calibration ranges, with R^2 values exceeding 0.99. Expected concentrations and R^2 values for the calibrator set are summarized in Table 5. DHEA and estradiol were chosen as representative compounds to demonstrate the calibration curves and EICs of the quantifier ions in the STD 1 sample (Figure 5). Steroids in three QC samples, QC-L, QC-M, and QC-H, were quantified using calibration curves derived from the commercial calibrators. Measured steroid concentrations were within 30% of expected values, with %RSD values below 14% (Table 6).

Robustness

Excellent robustness of the method was demonstrated by the peak area changes of the IS in 311 injections of the calibration samples prepared using 0.05% BSA (Figure 6). For all steroids, the peak area %RSD values were below 17%, confirming the excellent quantitative stability and robustness of the system described.

Table 5. Expected concentrations of steroids from 6PLUS1 Multilevel Serum Calibrator Set using external calibration (N = 3).

Analyte	STD 1 (ng/mL)	STD 2 (ng/mL)	STD 3 (ng/mL)	STD 4 (ng/mL)	STD 5 (ng/mL)	STD 6 (ng/mL)	R^2
11-DCC	0.049	0.098	0.149	0.291	0.728	2.950	0.9998
17-OH Prog	0.089	0.484	0.978	1.930	3.930	22.500	0.9994
Androstenedione	0.200	0.409	0.820	1.470	4.830	14.800	0.9995
DHEA	0.886	4.940	9.460	14.100	27.900	55.900	0.9961
DHT	0.057	0.110	0.257	0.497	0.985	1.490	0.9945
Estradiol	0.039	0.101	0.261	0.516	1.540	5.240	0.9938
Progesterone	0.141	0.726	1.970	4.850	9.610	24.400	0.9992
Testosterone	0.055	0.252	0.984	2.880	5.760	11.600	0.9994

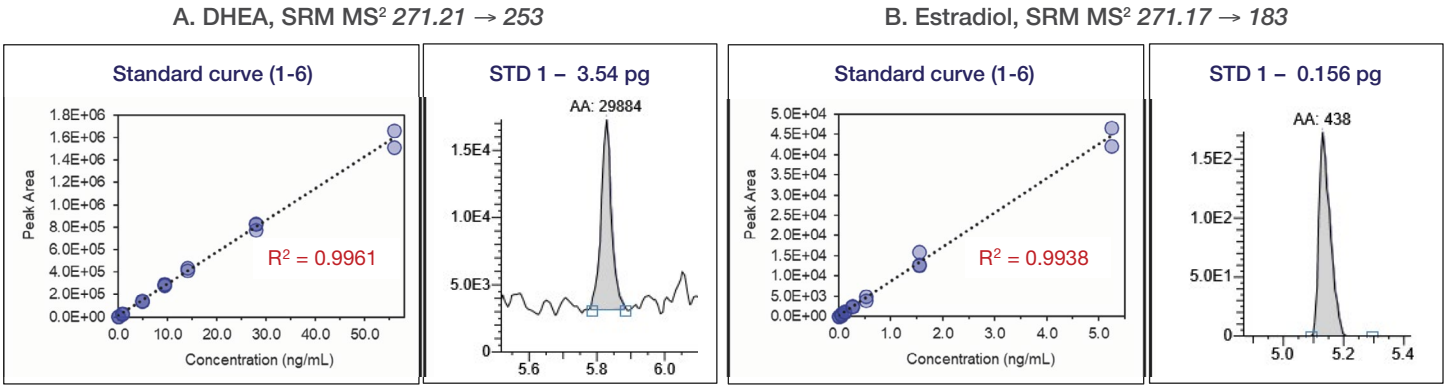

Figure 5. Calibration curves of DHEA and estradiol from 6PLUS1 Multilevel Serum Calibrator Set.

Table 6. Expected concentrations, %Diff, and %RSD (N = 6) of steroids detected in the Steroid Serum Control samples using the calibration curves from the 6PLUS1 Multilevel Serum Calibrator Set.

QC analyte	QC-L			QC-M			QC-H		
	Expected conc. (ng/mL)	%Diff	%RSD	Expected conc. (ng/mL)	%Diff	%RSD	Expected conc. (ng/mL)	%Diff	%RSD
17-OH-Prog	0.745	+13.0%	4.3%	1.490	-4.7%	6.8%	5.250	-5.3%	5.6%
Androstenedione	0.610	+30.0%	5.7%	1.220	-3.2%	5.1%	5.580	-3.6%	4.8%
DHEA	5.800	+24.0%	3.9%	11.600	-0.9%	6.4%	24.900	-6.2%	4.3%
DHT	0.206	+29.0%	14.0%	0.412	-6.2%	11.0%	0.816	-6.2%	8.5%
Testosterone	0.745	+14.0%	4.3%	1.490	-4.9%	5.0%	4.750	-7.6%	6.3%

Compound name	Peak area %RSD
[$^{13}C_3$]-Aldosterone	16.21
[$^{13}C_3$]-Cortisone	3.63
[$^{2}H_4$]-Cortisol	4.50
[$^{2}H_5$]-DHEAS	3.82
[$^{2}H_8$]-21-DOC	4.43
[$^{13}C_3$]-Corticosterone	5.04
[$^{2}H_5$]-11-DOC	4.57
[$^{13}C_3$]-Androstenedione	7.15
[$^{13}C_3$]-Estrone	4.95
[$^{2}H_5$]-Estradiol	4.93
[$^{2}H_3$]-Testosterone	6.21
[$^{2}H_8$]-17-OH-Prog	7.10
[$^{2}H_5$]-DHEA	10.21
[$^{2}H_3$]-DHT	7.10
[$^{2}H_9$]-Prog	6.70
[$^{13}C_2$,$^{2}H_2$]-Preg	10.79

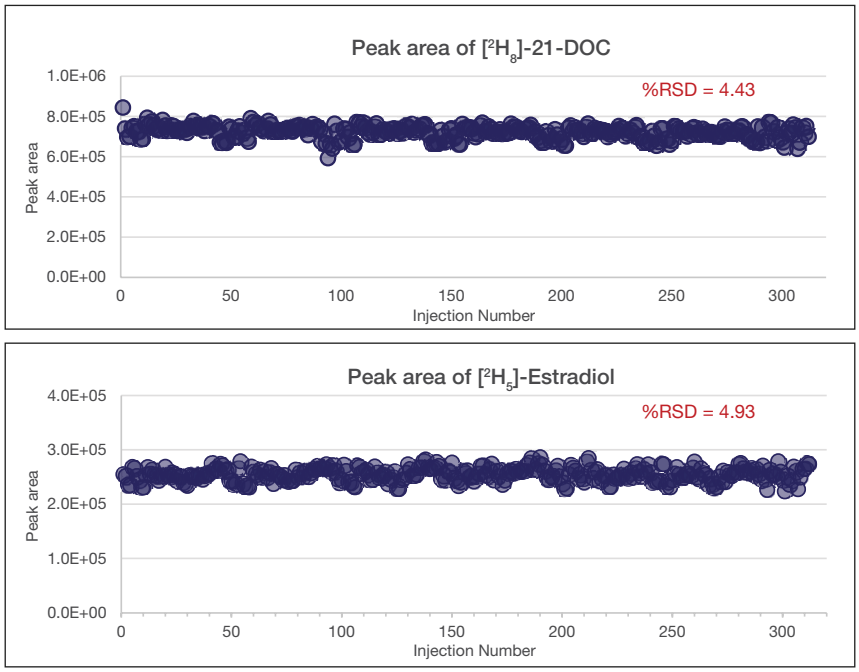

Figure 6. The robustness of the method was reflected via the %RSD of the IS peak areas over 311 injections of the steroid calibration samples prepared using 0.05% BSA. [$^{2}H_8$]-21-DOC and [$^{2}H_5$]-Estradiol were selected to demonstrate the peak area changes.

Conclusion and future work

A robust and sensitive LC–MS/MS method was successfully developed and validated for the simultaneous quantification of 18 steroids using human serum. The method, implemented on the Vanquish Horizon UHPLC coupled to the TSQ Certis MS equipped with the OptaMax Plus ion source, demonstrated excellent linearity, precision, and reproducibility across a wide dynamic range. All calibration curves achieved R^2 values greater than 0.99, and quantitative accuracy was maintained within 30% of expected concentrations for the commercial QC samples.

Optimization of MS parameters, particularly vaporizer temperature, significantly enhanced analyte response and sensitivity. The method also exhibited exceptional robustness, with %RSD values below 17% for both peak area and response ratio over 311 consecutive injections.

These results confirm that the TSQ Certis MS platform provides the sensitivity, selectivity, and long-term stability required for high-throughput steroid analysis in complex biological matrices. The workflow offers a reliable and efficient solution for clinical research laboratories seeking to improve the measurement of steroid biomarkers.

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