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Analysis of Corticosteroids and Androgens in Serum for Clinical Research

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APPLICATION BENEFITS

- Analytical selectivity of the chromatographic method provides separation of isobaric species
- LC-MS/MS enables high sample-throughput using multi-well plate automation
- Excellent agreement to the EQA Mass spectrometry mean for testosterone, androstenedione, 17-OHP, DHEAS, and cortisol

WATERS SOLUTIONS

Oasis® PRiME HLB µElution Plate

ACQUITY UPLC® HSS T3 Column

ACQUITY UPLC HSS T3 VanGuard™ Pre-column

ACQUITY UPLC I-Class System (FTN)

Xevo® TQ-S micro

MassLynx® Software

TargetLynx™ Application Manager

MassLynx LIMS Interface v3.0

Tecan File Converter v2.0

KEYWORDS

Testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, 21-deoxycortisol SPE, LC-MS/MS, Xevo TQ-S micro, automation

INTRODUCTION

Steroid hormones encompass a large class of small molecules that play a central role in metabolic processes, such as regulation of sexual characteristics, blood pressure and inflammation. Enzymes that form part of the steroid biosynthetic pathway are pivotal in these metabolic processes and their dysfunction can be examined through measurement of steroid hormones in the pathway. Measurement of these steroids by immunoassay can be prone to analytical interference as a result of cross reactivity of reagent antibodies with structurally related steroid hormones and synthetic derivatives. Liquid chromatography – tandem mass spectrometry (LC-MS/MS) can provide analytically sensitive, accurate, and precise measurement of these steroid hormones.

Here we describe a clinical research method utilizing Oasis PRiME HLB µElution Plate technology for the extraction of testosterone, androstenedione, 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone sulfate (DHEAS), cortisol, 11-deoxycortisol and 21-deoxycortisol from serum, which has been automated using the Tecan Freedom EVO 100/4 liquid handler. Chromatographic separation was performed on an ACQUITY UPLC I-Class System using an ACQUITY UPLC HSS T3 VanGuard Pre-column and ACQUITY UPLC HSS T3 Column, followed by detection on a Xevo TQ-S micro Mass Spectrometer (Figure 1). In addition, we have examined External Quality Assessment (EQA) samples for testosterone, androstenedione, 17-OHP, DHEAS and cortisol to evaluate the bias and therefore suitability of the method for analyzing these steroids for clinical research.

Figure 1. The Waters® ACQUITY UPLC I-Class system and Xevo TQ-S micro.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC I-Class (FTN)
Needle:	30 µL
Column:	ACQUITY UPLC HSS T3 2.1 x 50 mm, 1.8 µm (P/N 186003538)
Pre-column:	ACQUITY UPLC HSS T3 VanGuard 2.1 x 5 mm, 1.8 µm (P/N 186003976)
Mobile phase A:	Water with 2 mM ammonium acetate + 0.1% formic acid
Mobile phase B:	Methanol with 2 mM ammonium acetate + 0.1% formic acid
Needle wash solvent:	Methanol
Purge solvent:	40% methanol$_{(aq)}$
Column temp:	50 °C
Injection volume:	20 µL
Flow rate:	0.60 mL/min
Gradient:	See Table 1
Run time:	4.7 min

MS conditions

System:	Xevo TQ-S micro
Resolution:	MS1 (0.75 FWHM) MS2 (0.5 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (see Table 2 for details)
Polarity:	ESI +/-
Capillary:	1.0 kV
Source temp.:	150 °C
Desolvation temp.:	600 °C
Inter-scan delay:	0.01 s
Inter-channel delay:	0.02 s

Data management

MassLynx v4.1 Software with TargetLynx Application Manager

Sample preparation

Testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol and 21-deoxycortisol certified reference solution and stable labeled internal standards for testosterone, androstenedione, 17-OHP, DHEAS, cortisol, and 11-deoxycortisol were purchased from Sigma Aldrich (Poole, UK). 21-deoxycortisol stable labeled internal standard was purchased from Isosciences (King of Prussia, PA).

Calibrators and Quality Controls (QCs) were prepared in a surrogate matrix of MSG4000 stripped human serum purchased from Golden West Biologicals (Temecula, CA). Testosterone calibrators were prepared over the range of 0.10–69 nmol/L, with QCs at 0.35 nmol/L, 3.5 nmol/L, and 49 nmol/L. Androstenedione calibrators were prepared over the range of 0.09–349 nmol/L, with QCs at 0.52, 17.5, and 245 nmol/L. 17-OHP calibrators were prepared over the range of 0.19–757 nmol/L, with QCs at 0.76 nmol/L, 38 nmol/L, and 530 nmol/L. DHEAS calibrators were prepared over the range of 0.065–43 µmol/L with QCs at 0.41 µmol/L, 2.2 µmol/L, and 30 µmol/L. Cortisol calibrators were prepared over the range of 0.69–1380 nmol/L, with QCs at 0.83 nmol/L, 69 nmol/L, and 966 nmol/L. 11-deoxycortisol and 21-deoxycortisol calibrators were prepared over the range of 0.72–144 nmol/L. QC concentrations were 1.44, 7.2, and 101 nmol/L for 11-deoxycortisol and 2.2, 7.2, and 101 nmol/L for 21-deoxycortisol.

To convert SI units to conventional mass units divide by 3.470 for testosterone (nmol/L to ng/mL), 3.494 for androstenedione (nmol/L to ng/mL), 3.028 for 17-OHP (nmol/L to ng/mL), 2.716 for DHEAS (nmol/L to ng/mL), 2.761 for cortisol (nmol/L to ng/mL), and 2.889 for 11-deoxycortisol and 21-deoxycortisol (nmol/L to ng/mL).

Sample extraction

Extraction was performed using a liquid handler. To 100 µL of sample; 25 µL of 28 nmol/L testosterone-$^{13}C_3$ and androstenedione-$^{13}C_3$, 75 nmol/L 17-OHP-$^{13}C_3$, 1.4 µmol/L DHEAS-$^{2}H_5$, 137 nmol/L Cortisol-$^{13}C_3$, 71 nmol/L 11-deoxycortisol-$^{13}C_3$, and 21-deoxycortisol-$^{2}H_4$, 200 µL methanol, and 550 µL water were added. The samples were mixed after each reagent addition. Samples were centrifuged for five minutes at 4000 g.

An aliquot of each of the pre-treated samples (600 µL) was loaded into individual wells of the Oasis PRiME HLB µElution Plate and slowly pulled through at low vacuum (100 mbar). Consecutive washes with 150 µL of 0.1% (v/v) ammonia in 35% (v/v) methanol$_{(aq)}$ and 150 µL 0.1% (v/v) formic acid in 35% (v/v) methanol$_{(aq)}$ were performed to reduce potential ionic interference. Analytes were eluted using 30 µL of 85/15 (v/v) acetonitrile/methanol, followed by addition of 70 µL water.

Method conditions

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.600	55	45	Initial
1.0	0.600	55	45	6
3.5	0.600	35	65	6
3.51	0.600	2	98	11
4.0	0.600	55	45	11

Table 1. Gradient table for the separation of the steroid hormones. Operating backpressure at the initial conditions was approximately 8500 psi.

Analyte	ESI mode	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Testosterone (Quan)	+	289.2	97.0	45	20
Testosterone (Qual)	+	289.2	109.0	45	20
Testosterone-$^{13}C_3$	+	292.2	100.0	45	20
Androstenedione (Quan)	+	287.2	97.0	45	20
Androstenedione (Qual)	+	287.2	109.0	45	20
Androstenedione$^{13}C_3$	+	290.2	100.0	45	20
17-OHP (Quan)	+	331.2	97.0	55	24
17-OHP (Qual)	+	331.2	109.0	55	24
17-OHP-$^{13}C_3$	+	331.2	100.0	55	24
DHEAS	-	367.2	97.0	45	30
DHEAS-$^{2}H_5$	-	372.2	98.0	45	30
Cortisol (Quan)	+	363.2	97.0	45	24
Cortisol (Qual)	+	363.2	121.0	45	24
Cortisol-$^{13}C_3$	+	366.2	124.0	45	24
11-deoxycortisol (Quan)	+	347.2	97.0	45	24
11-deoxycortisol (Qual)*	+	347.2	121.0	45	24
11-deoxycortisol-$^{13}C_3$	+	350.2	100.0	45	24
21-deoxycortisol	+	347.2	175.1	45	30
21-deoxycortisol-$^{2}H_4$	+	351.2	177.1	45	30

*Table 2. MRM parameters for testosterone, androstenedone, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, 21-deoxycortisol, and their stable isotope labelled internal standards. The scan window for the analytes were 1.4–2.8 minutes for DHEAS, cortisol, 11-deoxycortisol and 21-deoxycortisol (+/- switching), and 2.61–3.8 minutes for testosterone, androstenedione, and 17-OHP. Mobile phase was directed to waste at all other times. Dwell times were set to automatic with 15 points across the peak over six seconds. *Also used for the 21-deoxycortisol qualifier transition.*

RESULTS

The chromatographic selectivity of the column is demonstrated through the baseline resolution of isobaric steroid species; 11-deoxycortisol, corticosterone, and 21-deoxycortisol; 17-OHP and 21-OHP; testosterone and epitestosterone (Figure 2). No interferences were observed at the retention time of testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol when each of these steroids and ten other structurally related compounds were individually examined (21-hydroxyprogesterone, corticosterone, DHEA, epitestosterone, dihydrotestosterone, aldosterone, cortisone, 18-hydroxycorticosterone, 17-hydroxypregnenolone, and prednisone). It has been noted, at high prednisolone concentrations (>0.5 µmol/L), >20% bias is observed at low concentrations (20 nmol/L) of cortisol. This is related to the isotopic contribution of prednisolone to the cortisol MRM trace. This interference can be detected through a change in the ion ratio of the quantifier and qualifier ions. No significant interference (≤10% bias) for testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol were observed when other endogenous compounds were examined (albumin, bilirubin, uric acid, intralipid, triglycerides, and cholesterol).

No system carryover was observed from high concentration samples into subsequent blank injections. A 1:5 dilution was successfully employed on high concentration samples, providing a mean accuracy of 102%, 98%, 99%, 96%, 94%, 107%, and 97% for testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol, respectively, with RSDs <7%.

The method would allow for precise quantification (<20% RSD) at 0.10 nmol/L for testosterone, 0.09 nmol/L for androstenedione, 0.19 nmol/L for 17-OHP, 0.65 µmol/L for DHEAS, 0.69 nmol/L for cortisol, and 0.72 nmol/L for 11-deoxycortisol and 21-deoxycortisol. Signal:noise at these concentrations in stripped serum were >10:1 on 10 separate occasions.

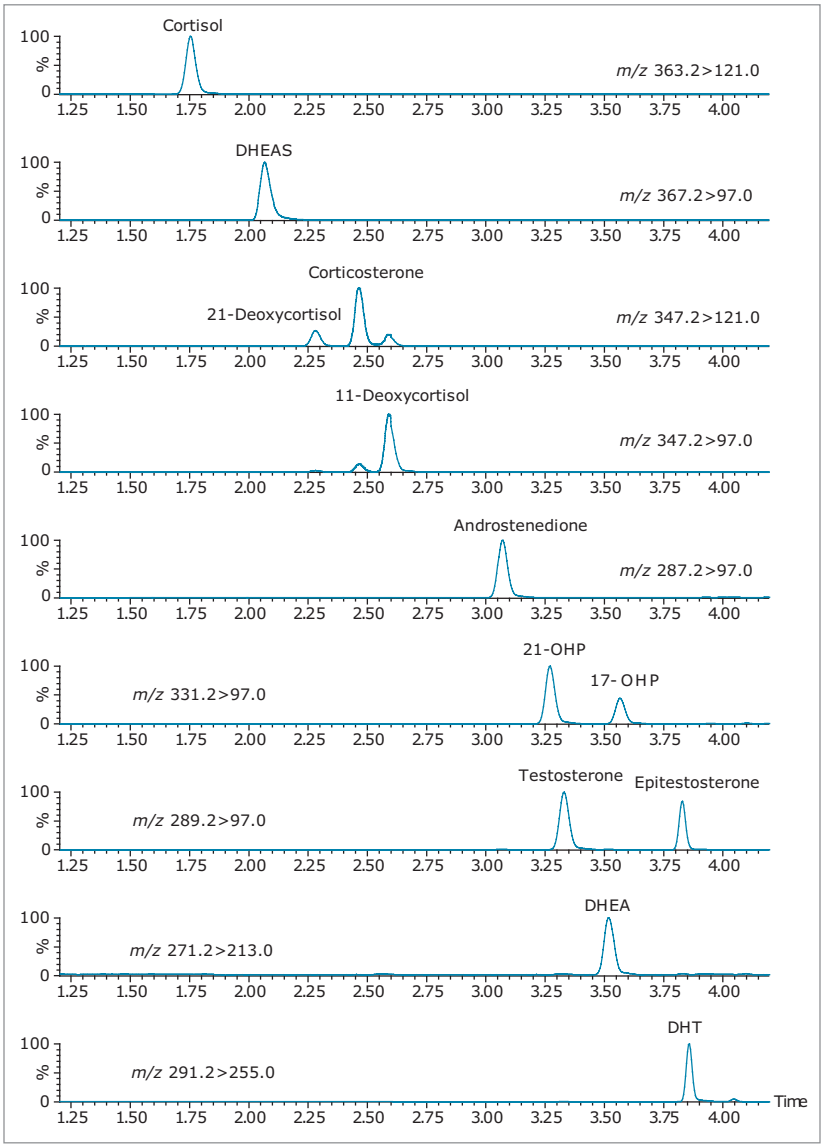

Figure 2. Chromatographic selectivity on the ACQUITY UPLC HSS T3 Column for a selection of steroid hormones.

Total precision was determined by extracting and quantifying three replicates of tri-level QC material on two occasions per day over five separate days (n=30). Repeatability was assessed by analyzing three replicates at each QC level. Low, mid, and high concentrations were 0.35 nmol/L, 3.5 nmol/L, and 49 nmol/L for testosterone; 0.52, 17.5, and 245 nmol/L for androstenedione; 0.76 nmol/L, 38 nmol/L, and 530 nmol/L for 17-OHP; 0.41 µmol/L, 2.2 µmol/L, and 30 µmol/L for DHEAS; 0.83 nmol/L, 69 nmol/L and 966 nmol/L for cortisol; 1.44, 7.2 and 101 nmol/L for 11-deoxycortisol and 2.2, 7.2 and 101 nmol/L for 21-deoxycortisol. As seen in Table 3, total QC precision and repeatability for the steroid hormones was ≤ 7.6%.

	Total QC precision			QC repeatability		
Compound	Low	Mid	High	Low	Mid	High
Testosterone	5.6%	6.0%	6.3%	3.2%	2.7%	3.2%
Androstenedione	6.0%	5.2%	4.8%	4.0%	2.7%	3.5%
17-OHP	5.1%	5.3%	5.1%	3.4%	2.7%	3.4%
DHEAS	5.5%	7.6%	6.0%	3.9%	2.1%	3.4%
Cortisol	7.3%	5.4%	5.1%	7.3%	2.7%	3.5%
11-deoxycortisol	5.3%	5.7%	5.5%	3.5%	3.3%	3.5%
21-deoxycortisol	6.9%	6.2%	6.6%	4.9%	4.2%	5.2%

Table 3. Total precision and repeatability for the analysis of testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol.

The method was shown to be linear for testosterone (0.10–69 nmol/L), androstenedione (0.09–349 nmol/L), 17-OHP (0.19–757 nmol/L), DHEAS (0.065–43 µmol/L), cortisol (0.69–1380 nmol/L), and 11-deoxycortisol and 21-deoxycortisol (0.72–144 nmol/L) when different ratios of high and low concentration pools of the analytes were combined and analyzed. In addition, calibration lines in spiked serum were linear with coefficient of determinations (r^2) >0.994 for all analyses.

Matrix effect investigations for testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol were performed using individual donor serum samples (n=6). The matrix factor calculated is shown in Table 4. Normalized matrix factor calculations, based on the analyte:internal standard response ratio demonstrated that the internal standards compensated for any ion suppression observed.

Compound	Mean matrix factor (range) peak area	%RSD	Mean matrix factor (range) response ratio	%RSD
Testosterone	0.70 (0.63–0.75)	6.2%	1.00 (0.97–1.02)	1.8%
Androstenedione	0.79 (0.74–0.84)	4.0%	1.00 (0.97–1.02)	1.8%
17-OHP	0.79 (0.76–0.82)	3.3%	0.99 (0.96–1.01)	1.7%
DHEAS	0.94 (0.92–0.96)	1.6%	0.99 (0.96–1.01)	1.7%
Cortisol	0.83 (0.76–0.91)	6.7%	0.99 (0.87–1.06)	6.6%
11-deoxycortisol	0.80 (0.75–0.84)	4.1%	0.93 (0.91–0.95)	1.5%
21-deoxycortisol	0.79 (0.72–0.85)	7.3%	1.00 (0.98–1.02)	1.6%

Table 4. Mean (range) matrix factor and %RSD based on analyte peak area and response ratio.

Accuracy was assessed for testosterone, androstenedione, 17-OHP, DHEAS, and cortisol through the analysis of EQA samples. The data obtained was compared to the mass spectrometry method mean for the samples and Deming regression, Altman-bland agreement and Linear regression was performed (Table 5). No statistically significant proportional or constant bias was observed for testosterone and cortisol. Statistically significant proportional bias was observed for androstenedione and 17-OHP and statistically significant constant bias was observed for DHEAS. Altman-Bland agreement for testosterone, androstenedione, 17-OHP, DHEAS, and cortisol demonstrated a mean method bias within ±5.8%, demonstrating excellent agreement with the EQA mass spectrometry method mean for the steroid hormones (Figures 3A–E).

Compound	Samples	Deming equation	Mean bias	Linear fit (r)
Testosterone	33	Y = 1.00x – 0.00	-0.1%	0.998
Androstenedione	55	Y = 0.94x + 0.11	-5.1%	0.998
17-OHP	54	Y = 1.07x – 0.25	5.2%	0.997
DHEAS	45	Y = 0.98x – 0.45	-5.8%	0.991
Cortisol	55	Y = 0.99x + 0.25	-1.0%	0.996

Table 5. Deming regression comparing the Waters LC-MS/MS method to the EQA scheme MS method for testosterone, androstenedione, 17-OHP, DHEAS, and cortisol analysis.

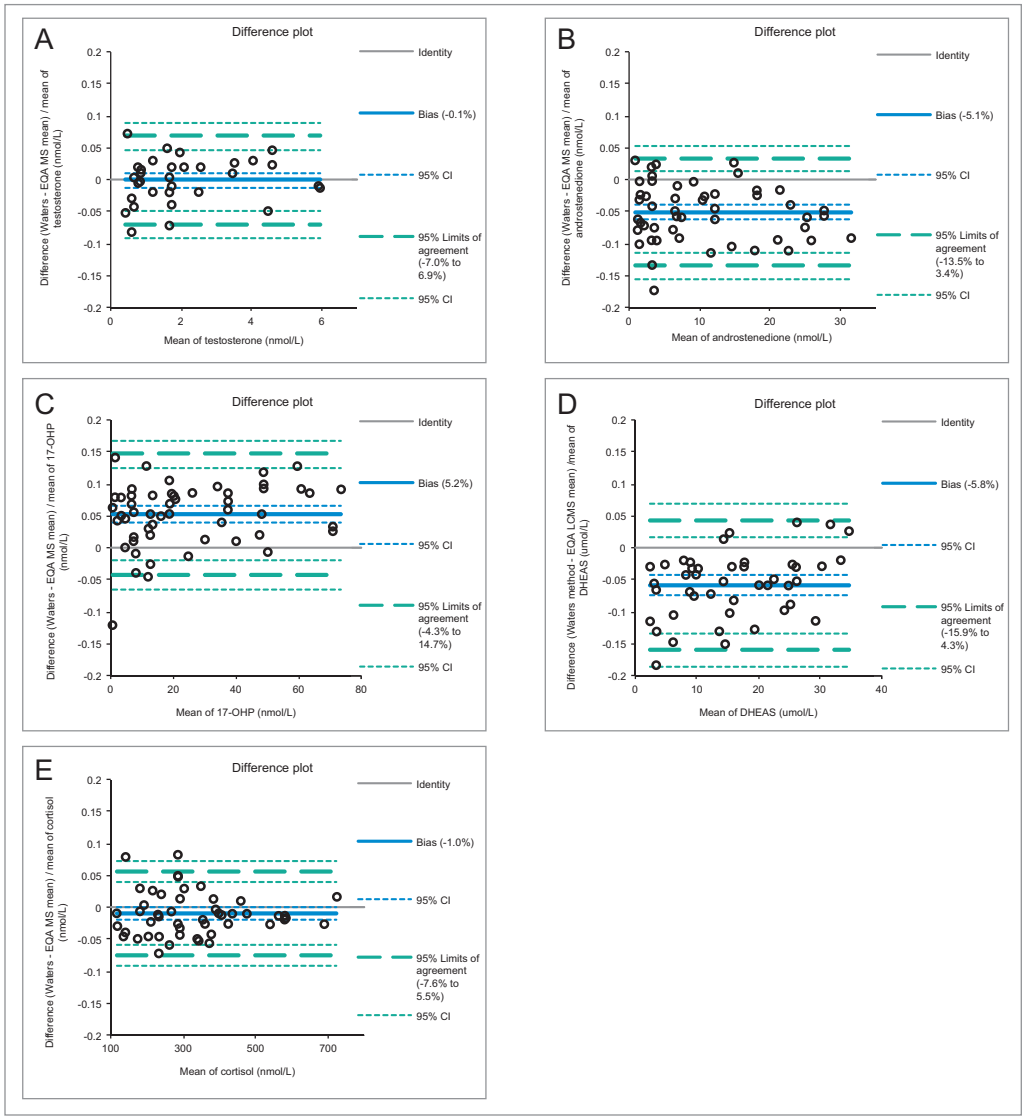

Figure 3. Altman-Bland agreement comparing the Waters LC-MS/MS method to the EQA scheme MS method mean for (a) testosterone (b) androstenedione (c) 17-OHP (d) DHEAS and (e) cortisol.

CONCLUSION

An analytically sensitive and selective clinical research method has been developed for the analysis of testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol in serum using the Xevo TQ-S micro.

The Xevo TQ-S micro enables this method to provide sufficient analytical sensitivity to analyze low levels of testosterone, androstenedione, 17-OHP, DHEAS, cortisol, 11-deoxycortisol, and 21-deoxycortisol by using only 100 µL sample volume. Excellent levels of imprecision across the calibration range have been demonstrated. Assessment of EQA samples has shown the method provides excellent agreement to the EQA LC-MS/MS mean for testosterone, androstenedione, 17-OHP, DHEAS, and cortisol. Automation of the analytical method in combination with sample tracking capabilities of the liquid handler, through the use of the Tecan File Converter and MassLynx LIMS Interface, improves laboratory workflow and reduces sample handling, which alleviates the potential for operator error.

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