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应用纪要

Analysis of Testosterone, Androstenedione, and Dehydroepiandrosterone Sulfate in Serum for Clinical Research

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Abstract

This application note describes a clinical research method utilizing Oasis PRiME HLB µElution Plate

technology for the extraction of testosterone, androstenedione, and DHEAS from serum.

Benefits

- Analytical sensitivity enables the quantification of low physiological levels of the steroids
- Analytical selectivity improves reproducibility through removal of interferences
- LC-MS/MS enables high sample-throughput when utilizing multi-well plate automation

Introduction

Testosterone, androstenedione, and dehydroepiandrosterone sulfate (DHEAS) are androgenic steroid hormones that are involved in the regulation of sexual characteristics. Analysis of these structurally similar steroid hormones by LC-MS/MS provides three levels of selectivity; sample preparation, liquid chromatography, and detection by multiple reaction monitoring (MRM) mass spectrometry. A selective sample preparation method has previously been developed which demonstrates excellent analytical sensitivity for testosterone and androstenedione using the Oasis MAX µElution SPE Plates.[1] However, with the inclusion of DHEAS into the analysis, this SPE is unsuitable, as it irreversibly binds DHEAS through an anion exchange mechanism. Therefore, a more suitable sample preparation protocol for this panel of steroids is required.

Here we describe a clinical research method utilizing Oasis PRiME HLB µElution Plate technology for the extraction of testosterone, androstenedione, and DHEAS from serum, which has been automated on a 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 TQD Mass Spectrometer (Figure 1). In addition, we have evaluated External Quality Assessment (EQA) samples for testosterone, androstenedione, and DHEAS to evaluate the bias and therefore suitability of the method for analyzing testosterone, androstenedione, and DHEAS for clinical research.

Figure 1. The Waters ACQUITY UPLC I-Class and Xevo TQD.

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 (Waters P/N 186003538)
Pre-column:	ACQUITY UPLC HSS T3 VanGuard 2.1 x 5 mm 1.8 µm (Waters 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:	80% methanol(aq)
Purge solvent:	40% methanol(aq)
Column temp.:	50 °C
Injection volume:	15 µL

Flow rate:	0.60 mL/min
Gradient:	See Table 1
Run time:	4.7 minutes

MS conditions

System:	Xevo TQD
Resolution:	MS1 (0.75 FWHM) MS2 (0.75 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (see Table 2 for details)
Polarity:	ESI positive/negative
Capillary:	0.4 kV
Source temp.:	150 °C
Desolvation temp.:	450 °C
Inter-scan delay:	0.01 seconds
Inter-channel delay:	0.02 seconds

Data management

MassLynx v4.1 Software with TargetLynx Application Manager

Sample preparation

Testosterone, androstenedione, and DHEAS certified reference solutions and their stable labeled internal standards were purchased from Sigma Aldrich (Poole, UK). Calibrators were prepared in a surrogate matrix of MSG4000 stripped human serum purchased from Golden West Biologicals (Temecula, CA). Testosterone and androstenedione calibrators were prepared over the range of 0.17–69 nmol/L, with quality controls (QCs) at 0.52 nmol/L, 5.2 nmol/L and 35 nmol/L. DHEAS calibrators were prepared over the range of 0.14–54 µmol/L with QCs at 0.41 µmol/L, 4.1 µmol/L, and 27 µmol/L.

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) and 2.716 for DHEAS (µmol/L to µg/mL).

Sample extraction

Extraction was performed using a Tecan Freedom Evo 100/4 Liquid Handler. To 100 µL of sample; 25 µL of 28 nmol/L testosterone-$^{13}C_3$ /androstenedione-$^{13}C_3$, and 1.4 µmol/L DHEAS-2H5 , 200 µL methanol and 550 µL water were added. The samples were mixed after each reagent addition. Samples were centrifuged for 5 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 (Waters P/N 186008052) and slowly pulled through at low vacuum (100 mbar). Consecutive washes with 200 µL of 0.1% (v/v) formic acid in 35%

(v/v) methanol(aq) and 200 µL 0.1% (v/v) ammonia in 35% (v/v) methanol(aq) were performed. Analytes were eluted using 45 µL of methanol, followed by 55 µL water.

Method conditions

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.6	55	45	Initial
1	0.6	55	45	6
3.5	0.6	35	65	6
3.51	0.6	2	98	11
4	0.6	55	45	11

Table 1. Gradient table for the separation of testosterone, androstenedione, and DHEAS. Operating backpressure at the initial conditions was approximately 8500 psi.

Analyte	ESI mode	Precursor ion (m/z)	Product ion (m/z)	Dwell time (s)	Cone voltage (kv)	Collision energy (eV)
Testosterone (Quan)	+	289.2	97.0	0.034	40	24
Testosterone (Qual)	+	289.2	109.0	0.034	40	24
Testosterone-$^{13}C_3$	+	292.2	100.0	0.034	40	24
Androstenedione (Quan)	+	287.2	97.0	0.034	40	24
Androstenedione (Qual)	+	287.2	109.0	0.034	40	24
Androstenedione-$^{13}C_3$	+	290.2	100.0	0.034	40	24
DHEAS	–	367.2	97.0	0.250	40	30
DHEAS-$^{2}H_5$	–	372.2	98.0	0.250	40	30

Table 2. MRM parameters for testosterone, androstenedione, DHEAS, and their internal standards. The scan window for DHEAS was 1.5–2.6 minutes. The scan window for testosterone and androstenedione was 2.61–3.8 minutes. Mobile phase was directed to waste at all other times.

Results and Discussion

No interferences were observed at the retention time of testosterone, androstenedione, and DHEAS when the analytes themselves and eight structurally related compounds were examined (11-deoxycortisol, 21-deoxycortisol, 21-hydroxyprogesterone, 17-hydroxyprogesterone, corticosterone, cortisol, DHEA, and dihydrotestosterone). The chromatographic selectivity of the column is demonstrated through the baseline resolution of testosterone and its epimer; epitestosterone (Figure 2). In addition, separation of androstenedione, 17-hydroxyprogesterone, and DHEA from testosterone is necessary due to the detection of these analytes or their isotopes in the testosterone MRM trace at concentrations of >1 µmol/L.

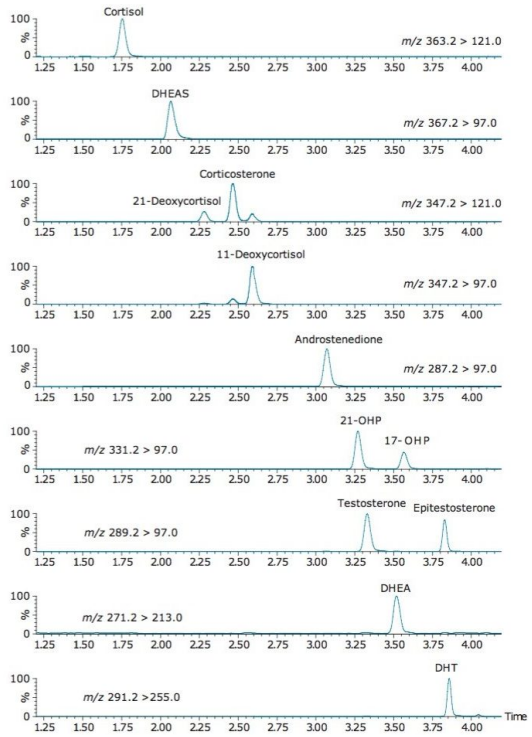

Figure 2. Chromatographic selectivity on the ACQUITY UPLC HSS T3 columns for a selection of steroid hormones, including testosterone, androstenedione, and DHEAS.

No system carryover was observed from high concentration samples into subsequent blank injections. High concentration samples were at 175 nmol/L for testosterone and androstenedione, and 135 µmol/L for DHEAS. A 1:5 dilution was successfully performed on the high concentration carryover sample, providing a mean accuracy of 97%, 101%, and 96% for testosterone, androstenedione, and DHEAS respectively.

Analytical sensitivity investigations reveal that the

analytical sensitivity of this method would allow precise quantification (<20% RSD) at 0.17 nmol/L for testosterone and androstenedione, and at 0.14 µmol/L for DHEAS. Signal:noise (S/N) of the lowest calibration standard was >10:1 on 10 separate occasions for all analytes.

Total precision was determined by extracting and quantifying three replicates of tri-level QC material on 2 occasions per day over 5 separate days (n=30). Repeatability was assessed by analyzing three replicates at each QC level. Low, mid, and high QC concentrations were 0.52, 5.2, and 35 nmol/L for both testosterone and androstenedione. Low, mid, and high QC concentrations were 0.41, 4.1, and 27 µmol/L for DHEAS. Total precision and repeatability using the Tecan Freedom Evo 100 Liquid Handler was ⩽6.3% for all analytes (Table 3).

Compound	Total QC Precision			QC Repeatability		
	Low	Mid	High	Low	Mid	High
Testosterone	4.7%	3.3%	3.8%	3.6%	2.0%	3.4%
Androstenedione	6.3%	2.6%	4.6%	5.2%	2.3%	3.9%
DHEAS	3.3%	3.0%	3.9%	2.1%	1.8%	2.7%

Table 3. Total precision and repeatability for the analysis of testosterone, androstenedione, and DHEAS.

The method was shown to be linear over the range of 0.15–76 nmol/L for testosterone, 0.15–74 nmol/L for androstenedione, and 0.13–58 µmol/L for DHEAS, 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) >0.998 on 10 separate occasions for all analytes.

Matrix effect investigations were performed using individual donor serum samples. The endogenous peak areas were separately quantified. Post-spiked samples were adjusted using the mean endogenous peak area to enable comparison to solvent spiked samples. The matrix factor calculated is shown in Table 4. Normalized matrix factor calculations, based on the analyte:internal standard response ratio produced similar values to peak area matrix factor.

Compound	Mean matrix factor (range) based on analyte peak area	RSD
Testosterone	1.02 (0.93–1.09)	5.9%
Androstenedione	1.02 (0.94–1.07)	4.6%
DHEAS	0.93 (0.86–1.02)	6.8%

Table 4. Mean (range) matrix factor and %RSD based on peak area of testosterone, androstenedione and DHEAS.

Samples were selected (n=50) for comparison against an independently developed LC-MS/MS method for testosterone, androstenedione, and DHEAS. Comparison data was processed using Analyse-it v2.3. Altman-Bland agreement demonstrated a mean bias of 5.0%, -3.3%, and -6.3% for testosterone, androstenedione, and DHEAS, respectively.

EQA samples were analyzed for testosterone (n=30), androstenedione and DHEAS (n=50). The data obtained was compared to the mass spectrometry mean for the samples and Deming regression was performed. The

correlation for testosterone, androstenedione, and DHEAS can be seen in Table 5 showing the excellent agreement with the EQA scheme MS mean. Proportional and constant bias was observed for DHEAS when all samples were analysed (0–40 µmol/L), however, the bias over the range 10.2–14.9 µmol/L was ⩽6.9%. Altman-Bland agreement demonstrated a mean bias of -0.50%, 0.4%, and 5.8% for testosterone, androstenedione, and DHEAS, respectively.

Analyte	Deming equation	Proportional bias?	Constant bias?	Linear Fit (r)
Testosterone	y =1.01x–0.02	N	N	0.999
Androstenedione	y =1.03x–0.10	Y	N	0.999
DHEAS (0–40 µmol/L)	y =1.10x–0.48	Y	Y	0.996
DHEAS (0–15 µmol/L)	y =1.05x–0.09	Y	N	0.996

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

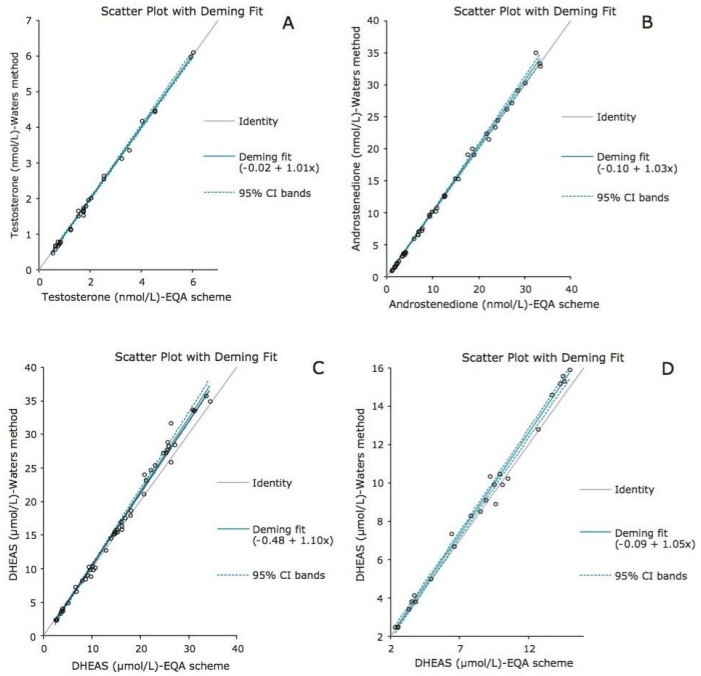

Figure 3. Deming regression comparing the Waters LC-MS/MS method to the EQA scheme MS mean for A) Testosterone, B) Androstenedione, C) DHEAS 0–40 µmol/L, and D) DHEAS 0–15 µmol/L.

Conclusion

An analytically sensitive and selective clinical research method has been developed for the analysis of testosterone, androstenedione, and DHEAS in serum.

Using only 100 µL sample volume, this method provides sufficient analytical sensitivity to analyze low physiological levels of testosterone, androstenedione, and DHEAS. Automation of the analytical method in combination with sample tracking capabilities

improves laboratory workflow and reduces sample handling, which alleviates the potential for operator error.

Acknowledgement

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References

1. Foley D. and Calton L. A Clinical Research Method for the Analysis of Serum Testosterone and Androstenedione. Waters application note. 2015. p/n 720005274EN.

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