

Analysis of 17-Hydroxyprogesterone in Serum for Clinical Research

Dominic Foley, Michelle Wills, and Lisa Calton
Waters Corporation, Wilmslow, UK

APPLICATION BENEFITS

- Analytical sensitivity enables the quantification of low physiological levels of 17-OHP
- Analytical selectivity improves method performance in terms of reproducibility through removal of interferences
- LC-MS/MS enables high sample throughput using multi-well plate automation

WATERS SOLUTIONS

[Oasis® PRiME HLB \$\mu\$ Elution Plate](#)

[ACQUITY UPLC® HSS T3 Column](#)

[ACQUITY UPLC HSS T3 VanGuard™
Pre-column](#)

[ACQUITY UPLC I-Class System \(FTN\)](#)

[Xevo® TQD](#)

[MassLynx® Software](#)

[TargetLynx™ Application Manager](#)

KEYWORDS

17-OHP, SPE, LC-MS/MS

INTRODUCTION

17-hydroxyprogesterone (17-OHP) is a glucocorticoid steroid hormone derived from both progesterone via 17-hydroxylase and 17-hydroxypregnenolone via 3 β -hydroxysteroid dehydrogenase. 17-OHP is converted to 11-deoxycortisol via 21-hydroxylase, followed by conversion to cortisol via 11 β -hydroxylase. Measurement of 17-OHP by immunoassay can be prone to analytical interference as a result of cross reactivity of reagent antibodies with structurally-related steroid hormones. Analysis of these structurally-similar steroid hormones by liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides three levels of selectivity: sample preparation, liquid chromatography, and detection by Multiple Reaction Monitoring (MRM) mass spectrometry.

It has previously been demonstrated that Oasis PRiME HLB Solid Phase Extraction (SPE) has been suitable for extracting steroid hormones from serum samples.¹ This application note describes a clinical research method utilizing Oasis PRiME HLB μ Elution Plate technology for the extraction of 17-OHP, which was automated using a liquid handler – the Tecan Freedom EVO® 100/4. Chromatographic separation was performed on an ACQUITY UPLC I-Class System using an ACQUITY UPLC HSS T3 VanGuard pre-column and an 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 17-OHP to evaluate the bias and therefore suitability of the method for analyzing 17-OHP 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, 1.8 µm, 2.1 mm x 50 mm, (P/N 186003538)
Pre-column:	ACQUITY UPLC HSS T3 VanGuard, 1.8 µm, 2.1 mm x 5 mm (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+
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

17-OHP certified reference solution and its stable labeled internal standard 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). 17-OHP calibrators were prepared over the range of 0.76–303 nmol/L, with quality controls (QCs) at 1.5 nmol/L, 30 nmol/L, and 227 nmol/L.

To convert SI units to conventional mass units divide by 3.028 (nmol/L to ng/mL).

Sample extraction

Sample extraction was performed using a liquid handler. 25 µL of 75 nmol/L 17-OHP-¹³C₃, 200 µL methanol, and 550 µL water were added to 100 µL of sample. 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 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 to reduce potential ionic interference. 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	0.600	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 17-OHP. Operating backpressure at the initial conditions was approximately 8500 psi.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Dwell time (s)	Cone voltage (kv)	Collision energy (eV)
17-OHP (quan)	331.2	97.0	0.034	40	24
17-OHP (qual)	331.2	109.0	0.034	40	24
17-OHP- ¹³ C ₃	331.2	100.0	0.034	40	24

Table 2. MRM parameters for 17-OHP and its stable isotope labeled internal standard. The scan window for 17-OHP was 2.61–3.8 minutes. Mobile phase was directed to waste at all other times.

RESULTS

No interferences were observed at the retention time of 17-OHP when eleven structurally-related compounds were examined (11-deoxycortisol, 21-deoxycortisol, 21-hydroxyprogesterone, testosterone, androstenedione, corticosterone, cortisol, DHEA, DHEAS, epitestosterone, and dihydrotestosterone). The chromatographic selectivity of the column is demonstrated through the baseline resolution of 17-OHP and its isobar: 21-hydroxyprogesterone (11-deoxycorticosterone) (Figure 2).

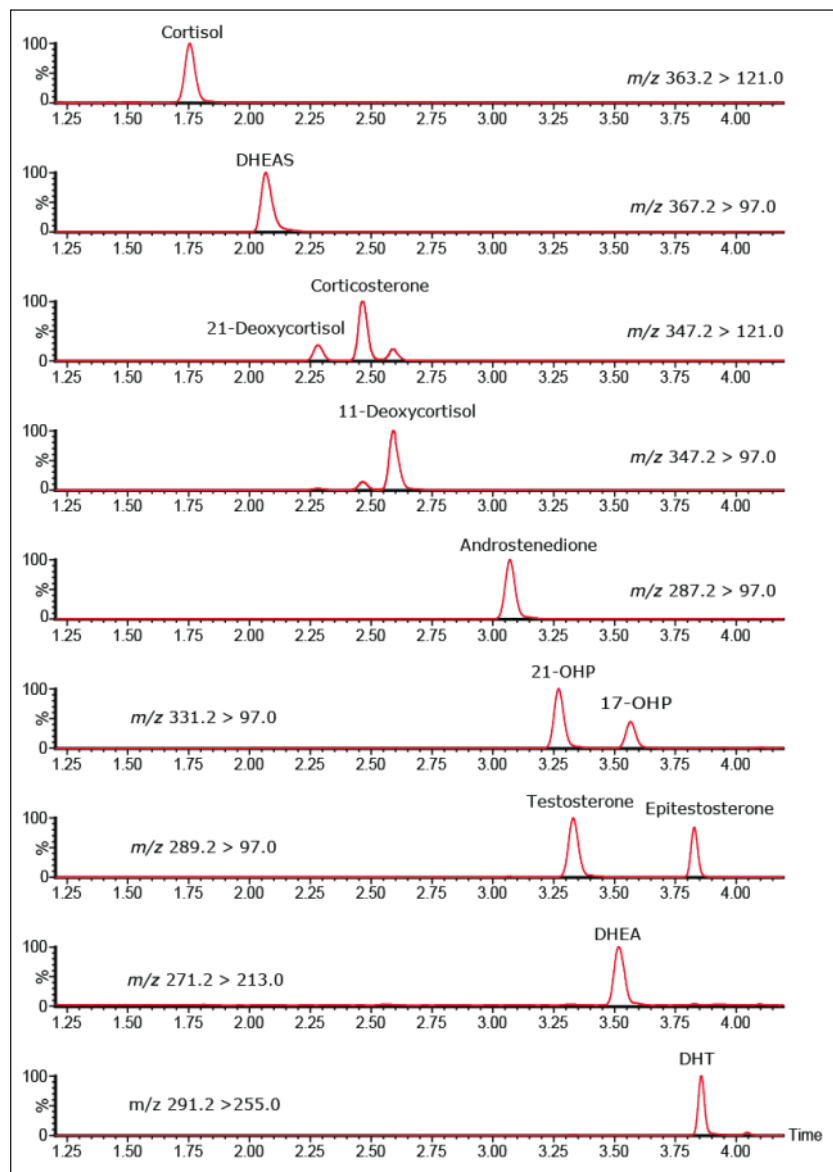


Figure 2. Chromatographic selectivity on the ACQUITY UPLC HSS T3 Column for a selection of steroid hormone solvent standards, including 17-OHP.

It was determined that in terms of analytical sensitivity, the method would allow for precise quantification (<20% RSD) at 0.76 nmol/L. Signal-to-noise ratio of the lowest calibration standard was >10:1 on ten separate occasions.

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 1.5, 30, and 227 nmol/L, respectively.

Total QC precision (CV %)			QC repeatability (CV %)		
Low	Mid	High	Low	Mid	High
8.2%	4.5%	5.7%	8.2%	3.8%	5.0%

Table 3. Total precision and repeatability for the analysis of 17-OHP.

The method was shown to be linear over the range of 0.68–333 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.

Matrix effect investigations 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-to-internal standard response ratio, produced similar values to peak area matrix factor – both demonstrating minimal ion suppression.

Mean matrix factor (range) analyte peak area	% RSD	Mean matrix factor (range) response Ratio	% RSD
0.92 (0.87–1.00)	5.5%	0.96 (0.90–1.02)	4.4%

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

EQA samples were analyzed for 17-OHP (n=50). The data obtained was compared to the mass spectrometry mean for the samples, and Deming regression was performed. The correlation can be seen in Table 5 and shows excellent agreement with the EQA scheme MS mean. Altman-Bland agreement demonstrated a mean bias of -5.0%.

Deming equation	Proportional bias?	Constant bias?	Linear Fit (r)
$y = 0.93x + 0.14$	Y	N	0.998

Table 5. Deming regression comparing the Waters LC-MS/MS method to the EQA scheme MS method for 17-OHP.

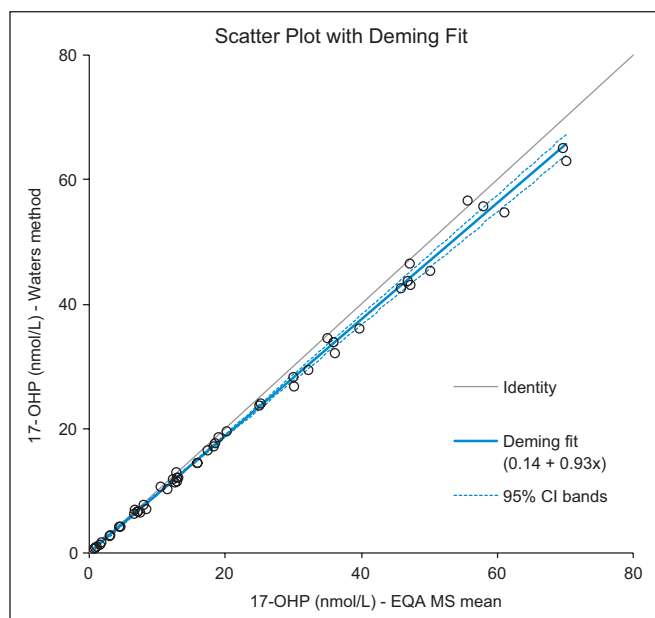


Figure 3. Deming regression comparing the Waters LC-MS/MS method to the EQA scheme MS mean for 17-OHP.

CONCLUSIONS

An analytically sensitive and selective clinical research method has been developed for the analysis of 17-OHP in serum.

Using only 100 μ L sample volume, this method provides sufficient analytical sensitivity to analyze low physiological levels of 17-OHP. Automation of the analytical method in combination with sample tracking capabilities of the liquid handler improves laboratory workflow and reduces sample handling, minimizing the potential for operator error.

References

1. Foley D., Wills M., and Calton L. Analysis of Testosterone, Androstenedione, and Dehydroepiandrosterone Sulfate for Clinical Research. Waters Application Note. 2016. [720005554EN](#).

For Research Use Only. Not for use in diagnostic procedures.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters, The Science of What's Possible, Oasis, ACQUITY UPLC, Xevo, and MassLynx are registered trademarks of Waters Corporation. VanGuard and TargetLynx are trademarks of Waters Corporation. All other trademarks are the property of their respective owners.

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com