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Analysis of Plasma 17-Hydroxyprogesterone, Androstenedione, and Cortisol Using a Novel Solid-Phase Extraction (SPE) Sorbent, Oasis PRiME HLB, for UPLC-MS/MS Analysis in Clinical Research

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APPLICATION BENEFITS

- Faster, simplified sample preparation workflow
- Elimination of at least 97% of phospholipids compared to protein precipitation
- No evaporation or reconstitution necessary
- Linear, accurate, and precise results for all analytes

WATERS SOLUTIONS

Oasis PRiME HLB µElution Plate (p/n 186008052)

ACQUITY® 96-well Sample Collection Plate, 700 µL Round well (p/n 186005837)

ACQUITY UPLC® HSS T3 Column 100Å, 1.8 µm, 2.1 x 50 mm (p/n 186003538)

ACQUITY UPLC® I-Class

Xevo® TQ-S Mass Spectrometer

KEY WORDS

Androstenedione, cortisol, 17-alpha-OH progesterone, uElution, sample preparation, SPE, steroids, matrix effects, phospholipid removal, LC-MS

INTRODUCTION

Sample preparation is an important consideration for any bioanalytical LC-MS/MS method for clinical research. Waters has developed a novel sample preparation sorbent, Oasis® PRiME HLB, which is designed to have some key advantages over traditional SPE sorbents. These include the ability to eliminate sorbent preconditioning and equilibration, allowing a more rapid workflow compared to traditional SPE products, and the ability to remove greater than 95% of phospholipids, resulting in a cleaner extracts and reducing the risk of short column lifetimes or MS source fouling.

This application note details the extraction and UPLC-MS/MS analysis of 17α-hydroxyprogesterone (17-OHP), androstenedione (Adione), and cortisol using Oasis PRiME HLB. Measurement of these compounds by immunoassay can be prone to cross reactivity with antibodies of chemically similar compounds. Immunoassays must also be done individually, requiring separate samples and analyses for each compound. LC-MS/MS offers greater discrimination and selectivity and the ability to multiplex methods, allowing the simultaneous determination of multiple compounds. The use of Oasis PRiME HLB resulted in consistent and highly reproducible recoveries of all compounds with minimal matrix effects. Phospholipids were almost completely eliminated compared to protein precipitation. Finally, the use of Waters® patented µElution format allowed for the concentration of the sample on the SPE column, eliminating the need to evaporate and reconstitute the sample. This resulted in a method that was linear, accurate and precise for all analytes, with limits of quantification of 50 pg/mL for androstendione and 17-OHP.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC HSS T3 Column, 100Å, 1.8 µm, 2.1 x 50 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Mobile phase A (MPA):	Water with 0.1% formic acid
Mobile phase B (MPB):	ACN with 0.1% formic acid
Purge solution:	25:25:50 ACN:MeOH:Water
Wash solution:	10% ACN

The gradient ramp is shown in Table 1.

Time (min.)	Flow (mL/min.)	%A	%B
0	0.6	70	30
1.0	0.6	50	50
2.0	0.6	45	55
2.5	0.6	5	95
3.5	0.6	5	95
3.6	0.6	70	30
4.5	0.6	70	30

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the Methods section.

Mass spectrometry

MS System:	Xevo TQ-S Mass Spectrometer
Ionization mode:	ESI Positive
Capillary Voltage:	1.0 kV
Cone voltage:	Optimized for each analyte
Desolvation Gas:	1000 L/hr
Cone Gas:	150 L/hr
Desolvation Temp:	500 °C
Source Temp:	150 °C

Data were acquired and analyzed using MassLynx® Software (V4.1; SCN 876). Quantification was performed using TargetLynx.™

METHODS

All standards and stable isotope labelled internal standards were purchased from Cerilliant (Round Rock, TX). A combined stock standard (20 µg/mL cortisol; 1 µg/mL androstenedione and 17-OHP) was prepared in 25% methanol. A stock solution of 10 µg/mL cortisol-d4 and 0.5 µg/mL androstenedione-^{13}C3 and 17-OHP-d8 was prepared in methanol. A working internal standard solution of 750 ng/mL cortisol-d4 and 37.5 ng/mL androstenedione-^{13}C3 and 17-OHP-d8 was prepared in 25% methanol. Individual calibrators and quality control standards were prepared daily in 25% methanol. 25 µL of each working calibrator or QC standard was added to 475 µL of double charcoal stripped human plasma (Golden West Biological, Temecula, CA) to make calibration curves and QC samples.

SAMPLE PREPARATION

Samples were prepared as follows: 20 µL of the working internal standard solution was added to 150 µL of each calibrator or QC sample. All samples were precipitated with 300 µL of a solution of 4:1 MeOH:89 g/L $ZnSO_4$. The samples were aspirated several times to ensure full precipitation and then centrifuged at 3220 rcf for 10 minutes. 300 µL of the resulting supernatant was then added to 900 µL of 4% H_3PO_4 and aspirated to fully mix the sample. The resulting pretreated sample was then directly applied to the Oasis PRiME HLB µElution Plate in 2 aliquots. All wells of the SPE plate were subsequently washed with 2 x 200 µL aliquots of 25% methanol. The samples were then eluted with 2 x 25 µL aliquots of 90:10 ACN:MeOH and diluted with 25 µL of water. 7.5 µL was injected onto the UPLC-MS/MS system. The sample extraction procedure is summarized in Figure 1.

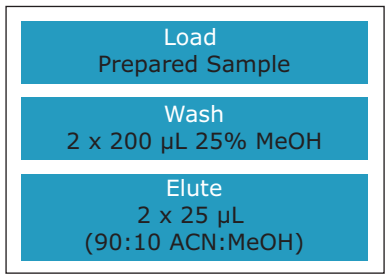

Figure 1. Oasis PRiME HLB extraction methodology for plasma corticosteroids. With no conditioning and equilibration, sample extraction is simplified to just three steps.

RESULTS AND DISCUSSION

Chromatography

Figure 2 shows the chromatography of the three steroids from an extracted calibrator. All compounds eluted within 2 minutes. The HSS T3 column offered a distinct advantage over other C_{18} columns. Even though these compounds are not polar, the enhanced retentivity of the T3 column eliminated solvent effects observed on other columns when samples were injected in high proportions of organic solvent.

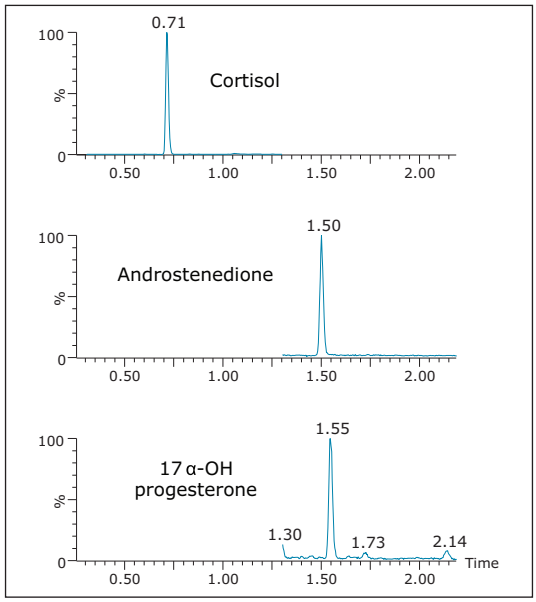

Figure 2. Chromatography of cortisol, androstendione, and 17-OHP from an extracted plasma sample on the ACQUITY UPLC HSS T3 Column, 100Å, 1.8 µm; 2.1 x 50 mm. The concentration of cortisol was 20 ng/mL. The concentration of the other compounds was 1 ng/mL.

Table 2 lists the retention time and individualized MS parameters of the steroids and their stable isotope labelled internal standards, including MRM transitions, cone voltage, and collision energy. Two MRM transitions were used for each compound, a primary (listed first) and a confirmatory transition (listed second).

Recovery and matrix effects

Extraction recovery was very consistent.
As Figure 3 shows, recovery for all compounds was 72–73% with %RSDs under 5%, demonstrating the reproducibility of Oasis PRiME.

Analyte	RT (min)	MRM transitions (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
Cotisol	0.72	336.17>121.06	42	22
		336.17>91.03	42	52
d4 Cortisol (IS)	0.71	367.17>121.12	42	22
Androstenedione	1.50	287.17>97.08	58	20
		287.17>109.04	58	26
^{13}C3 A-dione (IS)	1.50	290.17>100.07	58	20
17-OHP	1.55	331.17>97.08	58	26
		331.17>295.20	58	16
d8-17-OHP (IS)	1.53	339.23>100.07	58	26

Table 2. Mass spectral parameters for all analytes and internal standards.

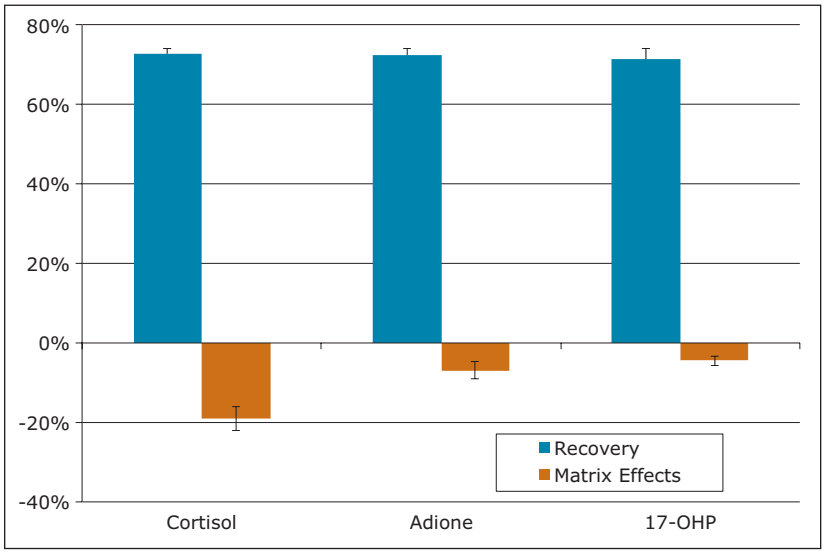

Figure 3. Recovery and matrix effects of cortisol, androstendione, and 17-OHP after extraction using the Oasis PRiME HLB µElution Plate. %RSDs for extraction recovery were less than 5% for all compounds. Matrix effects were -19% for cortisol and less than 10% for the other two steroids.

Matrix effects were low for all compounds. The matrix effect for cortisol was -19%, indicating minor ion suppression and was minimal for the other compounds. The average matrix effect for all compounds was -10.1%. Once again, the low standard deviations (3.1% or less) demonstrate the consistency of extraction and cleanup seen with Oasis PRiME. All recovery and matrix effect data are summarized in Table 3.

	Recovery			Matrix effects	
	Mean	S.D.	%RSD	Mean	S.D.
Cortisol	72.7%	3.1%	4.2%	-19.0%	3.1%
Adione	72.5%	1.9%	2.7%	-6.9%	2.2%
17-OHP	71.5%	1.9%	2.6%	-4.5%	1.3%
			Mean	**-10.1%**	

Table 3. Recovery and Matrix effects (N=4 for all tests).

Quantitative results

Calibration and quality control samples ranged prepared as previously described in the materials and method section. Calibration ranges were from 1–500 ng/mL for cortisol and from 0.05–25 ng/mL for the remaining compounds, mirroring the expected concentrations of these compounds in plasma. Quality control samples were prepared at low, medium, and high concentrations as appropriate for the calibration ranges.

All compounds had linear responses over the entire calibration range with R^2 values of 0.99 or greater with 1/x weighting. Table 4 summarizes the data from the calibration curves. Lower limits of quantification (LLOQ) were 1.0 ng/mL for cortisol and 0.05 ng/mL for androstenedione and 17-OHP. In each case, all FDA recommendations for accuracy, precision and analytical sensitivity were met for validated methods.[1]

	R^2	Mean % dev.
Cortisol	0.996	8.39
Adione	0.989	8.03
17-OHP	0.993	9.72

Table 4. Calibration Curve Summary. Linear fits with 1/x weighting were used for all compounds.

Quality control samples were accurate and precise, with all results within 10% of expected values and %CVs no greater than 7% (N=6). This data can be seen in Table 5. The excellent accuracy and precision demonstrate the consistency and robustness of this sorbent.

	Accuracy					
	Androstenedione			17α-OH progesterone		
QC Level (ng/mL)	Mean	S.D.	%CV	Mean	S.D.	%CV
0.15	94.3%	5.4%	5.7%	93.7%	6.1%	6.5%
1.5	95.0%	3.4%	3.6%	92.3%	4.7%	5.6%
15	95.4%	5.3%	**5.5%**	**93.7%**	6.1%	6.5%
Mean	**94.9%**			**92.6%**		

	Accuracy		
	Cortisol		
QC Level (ng/mL)	Mean	S.D.	%CV
3	92.3%	4.9%	5.4%
30	94.8%	2.9%	3.0%
300	94.9%	5.7%	6.0%
Mean	94.0%		

Table 5. Quality control results from extracted plasma samples (N=6 for each compound at all three levels).

Phospholipid removal

One of the key features of Oasis PRiME HLB over other reversed-phase SPE sorbents or liquid-liquid extraction is its enhanced ability to remove phospholipids, which can be major contributors to matrix effects,[2,3] accumulate on analytical columns, and may contribute to fouling of MS sources, necessitating frequent cleaning. To assess the removal of phospholipids, we analyzed the extracts for several of the most common phospholipids and compared that data to samples that had been subject to protein precipitation only. Figure 4 compares chromatograms of identical plasma samples that were ether extracted as described above, or subject only to the protein precipitation step detailed. The total area of phospholipids in the Oasis PRiME HLB extracted samples was 3% that of precipitated samples. The true removal is likely even greater than 97% as there was no sample concentration for the precipitated samples. Taking the concentration factor into account (approximately 6X) would result in a removal of greater than 99%.

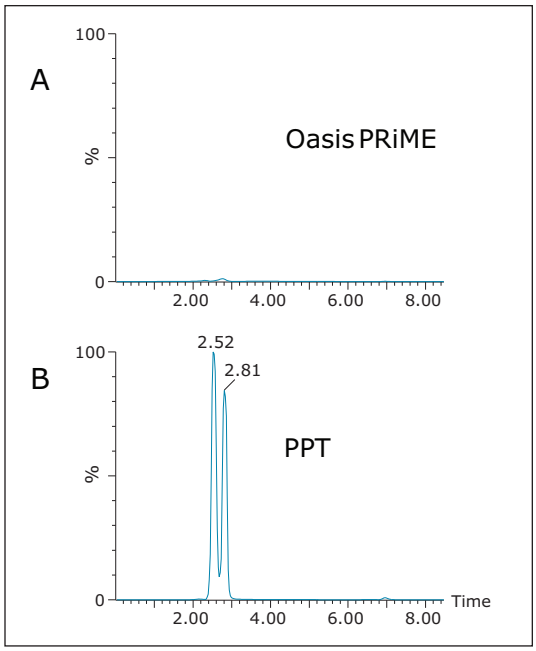

Figure 4. Removal of phospholipids compared to protein precipitaion. A. Summed chromatogram of phospholipids from a plasma sample prepared using Oasis PRiME HLB. B. Summed chromatogram of phospholipids from an identical plasma sample prepared by protein precipitation. Chromatographic scales are linked.

CONCLUSIONS

This application note details the extraction of cortisol, androstendione and 17-OHP from plasma samples using a novel SPE sorbent, Oasis PRiME HLB, in a µElution format for clinical research. The unique nature of this sorbent enabled the elimination of conditioning and equilibration steps, simplifying the extraction procedure and speeding up the sample preparation workflow. In addition, residual phospholipids were nearly eliminated compared to protein precipitation. The µElution format enabled the direct injection of extracts without evaporation or reconstitution.

Recoveries were very consistent for all compounds, and matrix effects were less than -20% for cortisol and under -10% for the other compounds. Linearity, accuracy, precision and analytical sensitivities were excellent for all compounds. All accuracies were within 8% of target concentrations and all %CVs were less than 7% for QC samples demonstrating the high reproducibility of this sorbet.

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