Fast and Reliable Method for the Analysis of Testosterone, Androstenedione, and 17-hydroxy Progesterone from Human Plasma

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Application Note 21173

Key Words
Steroid hormones, steroids, SOLA, SOLAμ HRP, reversed phase SPE, LC-MS/MS, Syncronis C18, micro-scale SPE, testosterone, androstenedione, 17-hydroxy progesterone

Goal

To describe an accurate and precise high-throughput analytical technique for the analysis of testosterone, androstenedione, and 17-hydroxy progesterone utilizing micro-scale solid phase extraction (SPE), followed by liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS).

Introduction

Steroid hormone levels can vary from elevated to depressed states, so a fast, sensitive, and reliable analysis method is required. Measuring steroid hormones presents issues for the bioanalyst due to the high endogenous levels; obtaining blank matrices for matched standards can present a problem. Phosphate buffer saline (PBS) is regularly used as a surrogate matrix because of its low cost and availability; however, it is often not a close match for the sample requiring analysis. Treated or stripped plasma can be used as a surrogate matrix, but in order to remove trace levels of any particular compound, the level of processing required can be so high that the surrogate is no longer a close matrix match.

Solid phase extraction (SPE) can be employed as a technique to achieve high levels of recovery and low levels of matrix effects. Provided monitoring of matrix to surrogate is carried out on a batch-by-batch basis; samples can be extracted alongside calibration standards prepared in a surrogate matrix with confidence in the accuracy of the data.

Thermo Scientific™ SOLAμ™ products provide reproducibility, robustness, and ease-of-use at low elution volumes by utilizing the revolutionary SOLA solid phase extraction technology. This removes the need for frits, delivering a robust, reproducible format that ensures highly consistent results at low elution volumes.

SOLAμ products deliver:

- Lower sample failures due to high reproducibility at low elution volumes
- Increased sensitivity due to lower elution volumes
- The ability to process samples that are limited in volume
- Improved stability of biomolecules by reduction of adsorption and solvation issues

By utilizing SOLAμ HRP plates, a fast, sensitive, accurate, and precise method for the analysis of steroids from human plasma was developed. The use of micro-scale SPE ensures the fastest possible time from sample to extract, while maintaining a high level of matrix clean-up to ensure minimal discrepancy from matrix to surrogate matrix.

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In addition, the Thermo Scientific™ Syncronis™ column range has been engineered to provide exceptional reproducibility due to its highly pure, high-surface-area silica, dense bonding, and double endcapping, all controlled and characterized through the use of rigorous testing. The Syncronis C18 1.7 µm, 100 x 2.1 mm column was chosen as the small particle size allowed for a higher backpressure to be maintained for separation. This was achieved at a lower flow rate than with larger particle size columns, which presented a higher level of sensitivity when using atmospheric pressure chemical ionization (APCI).

Here, a fast and reliable method for the analysis of testosterone, androstenedione, and 17-hydroxy progesterone (Figures 1–3) is presented.

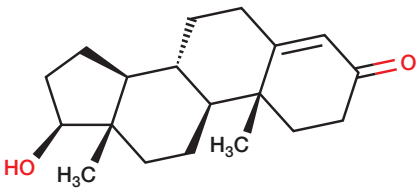

Figure 1. Testosterone.

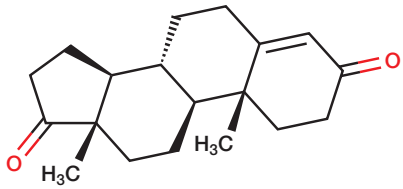

Figure 2. Androstenedione.

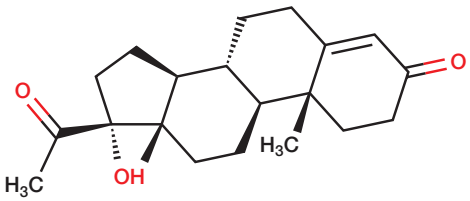

Figure 3. 17-hydroxy progesterone.

Experimental

Consumables

- SOLAµ HRP plate (P/N 60209-001)
- Syncronis C18 1.7 µm, 100 × 2.1 mm (P/N 97102-102130)
- Thermo Scientific™ Hypersil GOLD™ 5 µm, 10 × 4 mm drop-in guard (P/N 25005-014001)
- 96-well square well microplate (P/N 60180-P212)
- Thermo Scientific™ Webseal™ mat (P/N 60180-M120)
- Fisher Scientific™ LC-MS grade water (P/N 10095164)
- Fisher Scientific LC-MS grade methanol (P/N 10636545)
- Fisher Scientific LC-MS grade acetonitrile (P/N 10055454)
- Fisher Scientific analytical grade formic acid (P/N 10063427)
- Phosphate buffer saline (PBS) (P/N 10151570)

Sample Handling Equipment

SPE hardware

- Thermo Scientific™ HyperSep™ 96 vacuum manifold (P/N 60103-351)
- Vacuum pump, European version (P/N 60104-241)
- Vacuum pump, North American version (P/N 60104-243)

Compounds and Matrix

Compounds

- Testosterone, androstenedione, and 17-hydroxy progesterone

Internal standards

- Corticosteroid-d_8

Matrix

- Human plasma and phosphate buffer saline

Calibration and Quality Control (QC) Preparation

First, 5 µL of each working solution were prepared in methanol and added to 95 µL of PBS to give concentrations in the range 0.5 to 500 ng/mL. QC samples were then prepared in PBS and control human plasma with lithium heparin anti-coagulant at 1.5 and 400 ng/mL levels.

Endogenous Level Investigation Samples

Blank plasma was spiked with internal standard to measure endogenous levels of steroids.

Sample Pretreatment

First, 20 µL of 500 ng/mL methanol stock solution of internal standard (corticosteroid-d8) was added to each PBS or plasma calibration and QC sample. Then, 20 µL of methanol was added to each blank sample. Finally, 400 µL of 0.1% formic acid in water was added to each sample and the samples were vortex mixed.

Sample Preparation

SPE plate type	SOLAμ HRP
Condition	Apply 200 µL of methanol
Equilibrate	Apply 200 µL of 0.1% formic acid in water
Load	Load the entire pre-treated sample
Wash 1	Apply 500 µL of 0.1% formic acid in water
Wash 2	Apply 200 µL of 40% methanol in water
Elution	2 × 25 µL of methanol
Post extraction	Dilute extract with 50 µL of 0.1% formic acid was added to each extract prior to injection onto the HPLC system

Separation Conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system
Column	Syncronis C18 1.7 µm, 100 × 2.1 mm
Guard column	Hypersil GOLD 5 µm, 10 × 4 mm drop-in guard
Mobile phase A	0.1% formic acid in water
Mobile phase B	Acetonitrile
Gradient	See Table 1
Flow rate	0.300 mL/min
Column temperature	45 °C
Injection details	15 µL
Injection wash solvent 1	Mobile phase A
Injection wash solvent 2	30:30:30:10 methanol/propan-2-ol (IPA)/water/acetone

Table 1. LC gradient conditions.

Time (min)	A	B
0.0	50	50
3.5	0	100
4.0	0	100
4.01	50	50
5.0	50	50

MS Conditions

Instrumentation	Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer
Ionization source	APCI
Polarity	Positive
Scan time	0.02 s
Q1 (FWHM)	0.75
Q3 (FWHM)	0.75
Compound transition details	See Table 2

Table 2. Compound transition details.

Compound	Testosterone	Androstenedione	17-hydroxy progesterone	Corticosteroid-d8
Precursor (*m/z*)	289.2	287.2	331.2	355.3
Products (*m/z*)	97.1	109.1	97.2	125.1
Collision energy (V)	26	24	28	31

Results and Discussion

Due to the high endogenous levels of steroids present in human plasma, it is common practice to use a surrogate matrix of phosphate buffer saline in preparation of calibration and quality control samples. To enable the measurement of endogenous levels of each steroid, control human plasma was used to spike additional QC samples. An evaluation was made of the endogenous steroid level, and the accuracy, precision, and matrix effects were assessed in both PBS and plasma. This was carried out to determine the validity of the surrogate matrix measurements.

Chromatography

Full analyte separation was achieved using a Syncronis C18 analytical column in less than 4 minutes as shown in Figures 4, 5, 6, and 7.

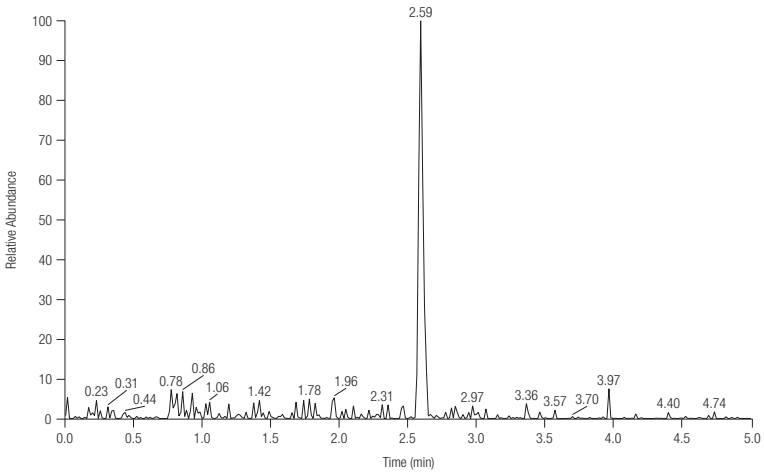

Figure 4. 0.5 ng/mL standard of testosterone.

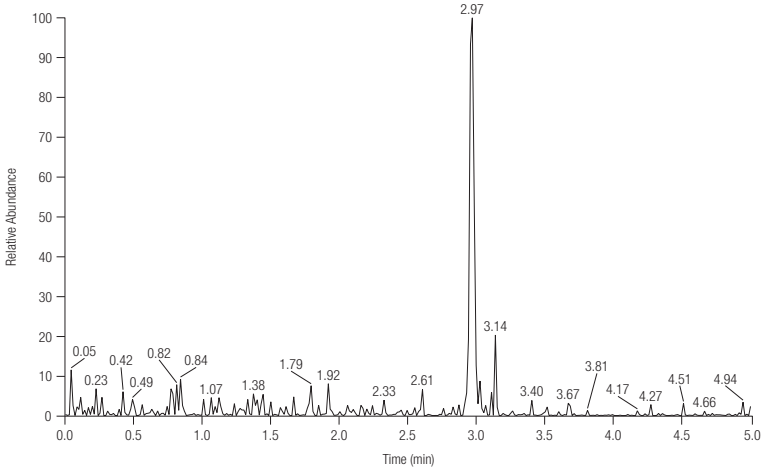

Figure 5. 0.5 ng/mL standard of androstenedione.

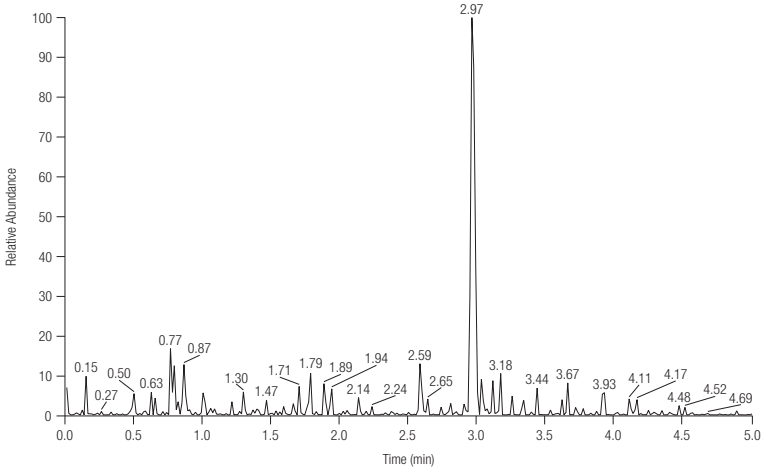

Figure 6. 0.5 ng/mL standard of 17-hydroxy progesterone.

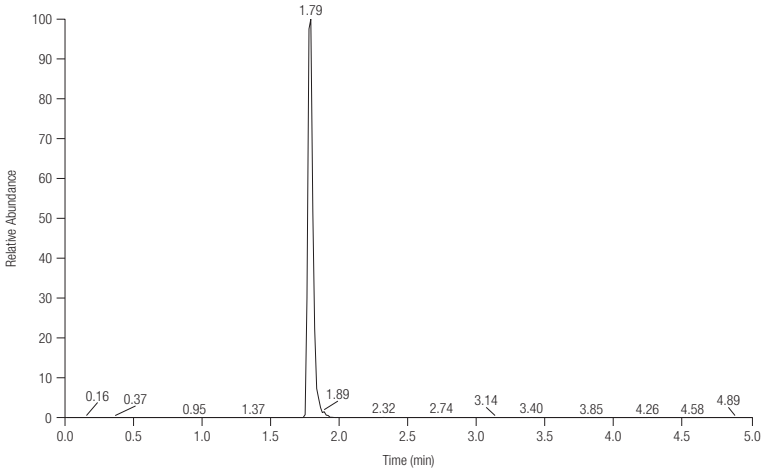

Figure 7. 100 ng/mL standard of corticosteroid d8 (internal standard).

Linearity

Extracted standards from PBS gave a linear calibration curve over the dynamic range 0.5 to 500 ng/mL (Figures 8, 9, 10 and Table 3).

Linear $\frac{1}{X^2}$ weighting was used in each case.

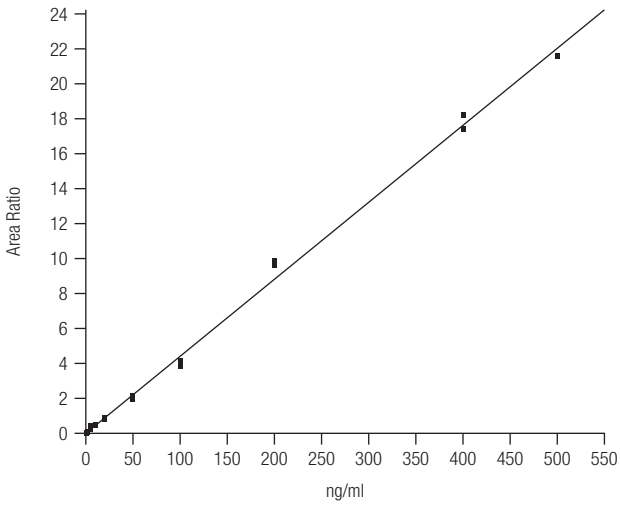

Figure 8. Testosterone linearity over a dynamic range of 0.5 to 500 ng/mL.

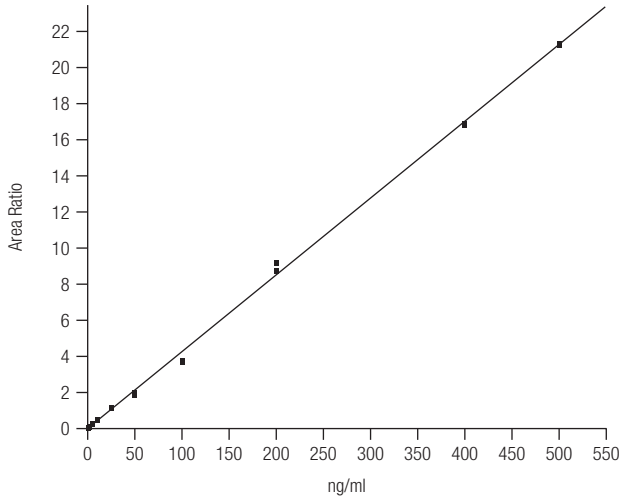

Figure 9. Androstenedione linearity over a dynamic range 0.5 to 500 ng/mL.

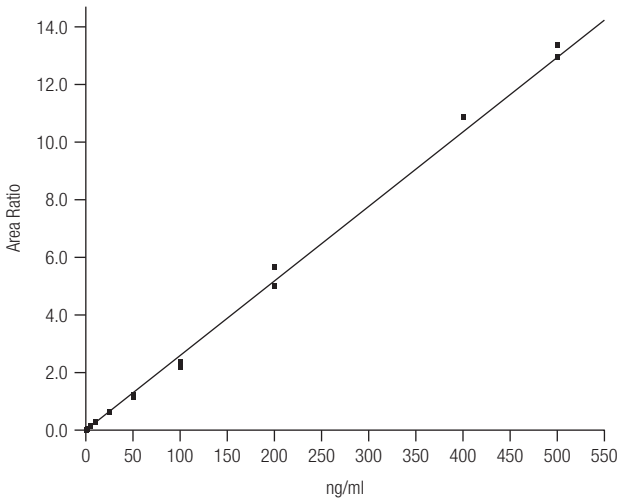

Figure 10. 17-hydroxy progesterone linearity over a dynamic range 0.5 to 500 ng/mL.

Table 3. Accuracy data for calibration standards PBS over a range of 0.5 to 500 ng/mL, n=2.

Sample Name	Nominal Concentration (ng/mL)	Testosterone % Bias from Nominal	Androstenedione % Bias from Nominal	17-Hydroxy progesterone % Bias from Nominal
S1	0.5	-8.42	-1.68	-1.63
S2	1.5	-2.72	2.71	8.81
S3	5	9.51	10.6	2.42
S4	10	7.76	14.7	5.18
S5	25	9.23	-14.2	-7.27
S6	50	-6.44	-6.70	-9.03
S7	100	-12.4	-10.0	-11.4
S8	200	12.3	4.94	6.84
S9	400	3.37	-3.74	0.179
S10	500	-1.84	3.57	1.31
R^2 value	**-**	**0.9930**	**0.9918**	**0.9908**

Accuracy and Precision

QC samples were analyzed in replicates of six at concentrations of 1.5 and 400 ng/mL. The accuracy and precision of the QC extracted from PBS is presented in Table 4. The mean endogenous levels of each compound in human plasma are shown in Table 5. Accuracy and precision of the QC samples extracted from plasma is presented in Tables 6 and 7.

Table 4. Accuracy and precision data for QC samples from PBS, n=6.

Sample Name	Nominal Concentration (ng/mL)	Compound	Average Calculated Concentration (ng/mL)	% Bias from nominal	% RSD
Low QC	1.5	Testosterone	1.62	8.0	7.94
		Androstenedione	1.44	-4.0	10.5
		17-hydroxy progesterone	1.66	11	9.49
High QC	400	Testosterone	429	7.3	7.93
		Androstenedione	429	7.3	5.36
		17-hydroxy progesterone	419	4.8	7.49

Table 5. Mean endogenous levels of each compound in human plasma, n=6

Blank Plasma with Internal Standard	Compound	Mean Measured Endogenous Levels from Plasma (ng/mL)	% RSD
N=6	Testosterone	3.73	6.79
	Androstenedione	1.29	10.5
	17-hydroxy progesterone	2.63	2.63

Table 6. Calculation of accuracy data for QC samples from plasma, n=6.

Sample Name	Nominal Concentration (ng/mL)	Compound	Measured Endogenous Levels from Plasma (ng/mL)	Endogenous Plus Spiked Level (ng/mL)	Calculated Concentration	% Bias from Adjusted Nominal
Low QC	1.5	Testosterone	3.73	5.23	5.28	1.0
		Androstenedione	1.29	2.79	2.91	4.3
		17-hydroxy progesterone	2.63	4.13	4.31	4.4
High QC	400	Testosterone	3.73	404	401	-1.0
		Androstenedione	1.29	401	391	-2.5
		17-hydroxy progesterone	2.63	403	400	-1.0

Table 7. Accuracy and precision data for QC samples from plasma, n=6.

Sample Name	Nominal Concentration (ng/mL)	Compound	Calculated Concentration	% Bias from Adjusted Nominal	%RSD
Low QC	1.5	Testosterone	5.28	1.0	8.79
		Androstenedione	2.91	4.3	1.24
		17-hydroxy progesterone	4.31	4.4	3.36
High QC	400	Testosterone	401	-1.0	5.28
		Androstenedione	391	-2.5	9.70
		17-hydroxy progesterone	400	-1.0	10.6

Recovery and Matrix Effects

Recovery and matrix effects from both PBS and plasma are presented in Table 8.

Table 8. Recovery and matrix effects data for QC samples PBS and plasma, n=6.

Sample Name	Nominal Concentration (ng/mL)	Compound	% Recovery from PBS	% PBS Matrix Effects	% Recovery from Plasma	% Plasma Matrix Effects
Low QC	1.5	Testosterone	103	1.81	107	14.6
		Androstenedione	107	-0.344	105	-2.26
		17-hydroxy progesterone	111	4.54	107	5.12
High QC	400	Testosterone	92.4	-3.99	91.7	8.32
		Androstenedione	92.8	-6.80	105	-3.82
		17-hydroxy progesterone	93.9	-12.8	96.3	-8.22

Conclusion

- The use of SOLAμ HRP SPE allows for fast, reliable extraction of steroids from human plasma.
- The cleanliness of the final extraction allows the use of a surrogate matrix in preparation of calibration standards enabling analysis of normal and abnormal levels of steroids.
- Excellent levels of accuracy and precision were observed on all compounds using the SOLAμ HRP SPE plate. High levels of recovery were also seen as well as low levels of matrix effects on both PBS and plasma samples, giving a high degree of confidence in the analytical results.
- Separation was achieved on a Syncronis C18 1.7 µm column allowing a low flow through the column to aid with ionization but maintaining a backpressure suitable for separation from interferences.

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