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Extraction of a Comprehensive Steroid Panel from Human Serum Using ISOLUTE® SLE+ Prior to LC/MS-MS Analysis

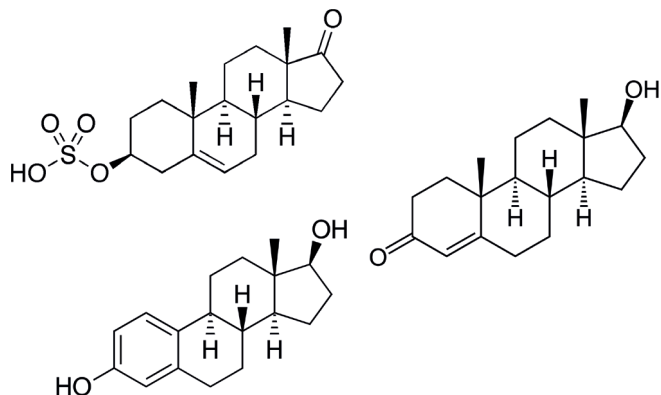


Figure 1. Structures of DHEAS, Estradiol and Testosterone.

Introduction

This application note describes the extraction of a panel of steroid hormones from human serum using ISOLUTE® SLE+ Supported Liquid Extraction plates prior to LC/MS-MS analysis. The simple sample preparation procedure delivers clean extracts and high, reproducible recoveries (>75%, RSD <10%) for all analytes in human serum, with linearity >0.99 in the range 5–5000 pg/mL.

ISOLUTE SLE+ Supported Liquid Extraction plates offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation.

Analytes

Cortisol, 18-OH-Corticosterone, 21-Deoxycortisol, Cortisone, Estradiol, 17-OH-Pregnenolone, Aldosterone, 11-Deoxycortisol, Corticosterone, Estrone, DHEA, 17-OH-Progesterone, DHEAS, Testosterone, Dihydrotestosterone (DHT), Pregnenolone, Androstenedione, 11-deoxycorticosterone, Progesterone

Internal standards

DHT-d³ and Aldosterone-d⁴

Sample Preparation Procedure

Format

ISOLUTE® SLE+ 400 µL Supported Liquid Extraction plate, part number 820-0400-P01.

Sample Pre-treatment

Add 25 µL of methanolic ISTD to human serum (total conc. 250 pg/mL). Mix.

Sample Loading

Apply 300 µL of sample into each well of the ISOLUTE SLE+ plate. Using a Biotage® PRESSURE+96 Positive Pressure Manifold, apply 2–5 psi of pressure to load samples onto the sorbent. Wait 5 minutes for the sample to equilibrate on the sorbent.

Analyte Extraction

Apply ethyl acetate (500 µL) and allow to flow under gravity for 5 minutes. Apply a further aliquot of ethyl acetate (500 µL) and allow to flow for 5 minutes under gravity. Apply pressure (5–10 seconds) to remove any remaining extraction solvent.

*this elution procedure should be used when the polar metabolite DHEAS is included in the steroid panel. If DHEAS is not required, and alternative elution solvent system (ethyl acetate: hexane, 75:25, v/v, 2 x 500 µL) should be used. See results section.

Post Elution and Reconstitution

Dry the extract in a stream of air or nitrogen using a Biotage® SPE Dry at 40 °C, 20 to 40 L/min, for 20 minutes. Reconstitute evaporated samples with 200 µL of 50:50 (v/v) mobile phase A: mobile phase B, and mix thoroughly.

UHPLC Conditions

Instrument

Shimadzu Nexera UHPLC

Column

ACE C18 (100 mm x 2.1 mm, 1.7 µm)

Mobile Phase

A: 0.2 mM Ammonium Fluoride (aq)

B: Methanol

Flow Rate

0.4 mL/min

Column Temperature

40 °C

Injection Volume

10 µL

Table 1. UHPLC Gradient

Time (min)	%A	%B
0	50	50
3	40	60
8	10	90
9	5	95
9.1	5	95
9.5	50	50



Mass Spectrometry Conditions

Instrument

Shimadzu 8060 Triple Quadrupole MS using ES interface

Nebulizing Gas Flow

3 L/min

Drying Gas Flow

3 L/min

Heating Gas Flow

17 L/min

Interface Temperature

400 °C

DL Temperature

250 °C

Heat Block Temperature

400 °C

CID Gas Flow

270 kPa

Table 2. MS conditions and retention times for target analytes in positive and negative mode.

Analytes	MRM Transition	Collision Energy	Ion Mode
DHEAS	367.1 > 97.05	33	-
Cortisol	363.4 > 121.25	-24	+
18-OH-Corticosterone	363.3 > 269.2	-16	+
Cortisone	361.3 > 163.15	-22	+
21-Deoxycortisol	347.1 > 311.2	-16	+
Estradiol	271.1 > 145.2	39	-
Aldosterone d ⁴	363.1 > 190.3	19	-
Aldosterone	359.1 > 189.25	18	-
17-OH-Pregnenolone	315.3 > 297.2	-13	+
11-Deoxycortisol	347.3 > 109.25	-27	+
Corticosterone	347.3 > 329.25	-16	+
Estrone	269.2 > 145.2	37	-
11-Deoxycorticosterone	331.3 > 109.05	-25	+
DHEA	289.3 > 253.2	-13	+
Testosterone	289.3 > 97.05	-23	+
DHT-d ³	294.4 > 258.25	-16	+
DHT	291.3 > 255.25	-15	+
Androstenedione	287.3 > 97.2	-21	+
Pregnenolone	299.3 > 159.25	-20	+
17-OH-Progesterone	331.3 > 97.1	-22	+
Progesterone	315.2 > 97.2	-22	+

Results

If the polar metabolite DHEAS is to be included in the steroid suite, ethyl acetate should be used as the elution solvent. If DHEAS is not required, an alternative elution solvent, ethyl acetate: hexane (75:25, v/v) can be used. Recovery data for both elution solvent systems is shown below.

The optimized ISOLUTE® SLE+ protocol using ethyl acetate demonstrated analyte recoveries greater than 75% as shown in Figure 2. The optimized ISOLUTE SLE+ protocol using ethyl acetate:hexane (75:25, v/v) demonstrated analyte recoveries greater than 80% (except DHEAS) as shown in Figure 3. RSDs were below 10% for all analytes using both protocols.

Removal of phospholipids was demonstrated by monitoring MRM transitions using the common product ion at m/z 184. Figure 3. Shows the serum phospholipid profile comparing lysophospholipids (bottom trace) and larger molecular weight phospholipids (top trace) extracted using the optimized ISOLUTE SLE+ protocols and 100 μ L of serum protein precipitated with 400 μ L of acetonitrile. Both elution solvents (EtOAc and EtOAc/Hexane(75/25, v/v) eliminated phospholipids compared to a protein precipitated sample.

Recoveries

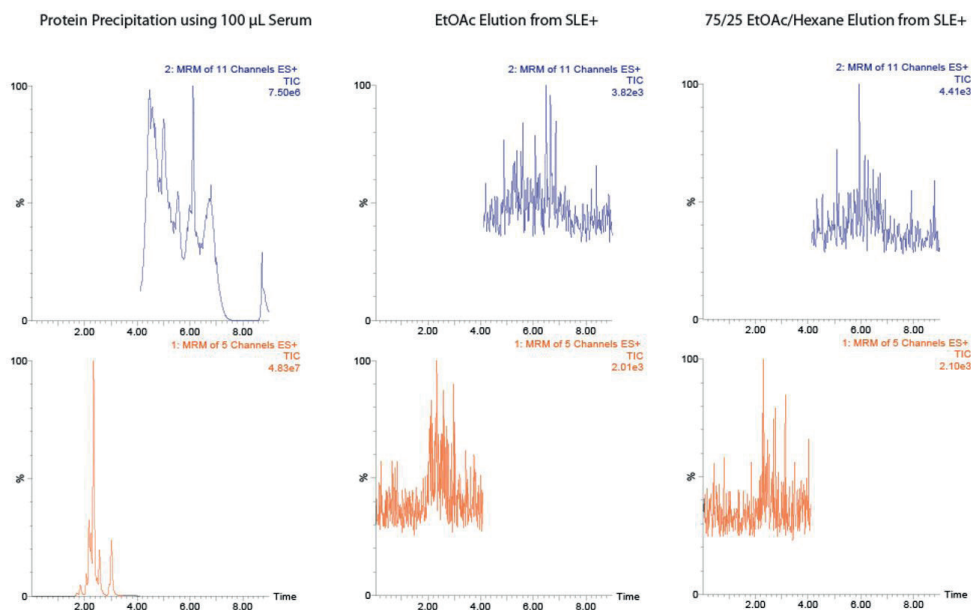
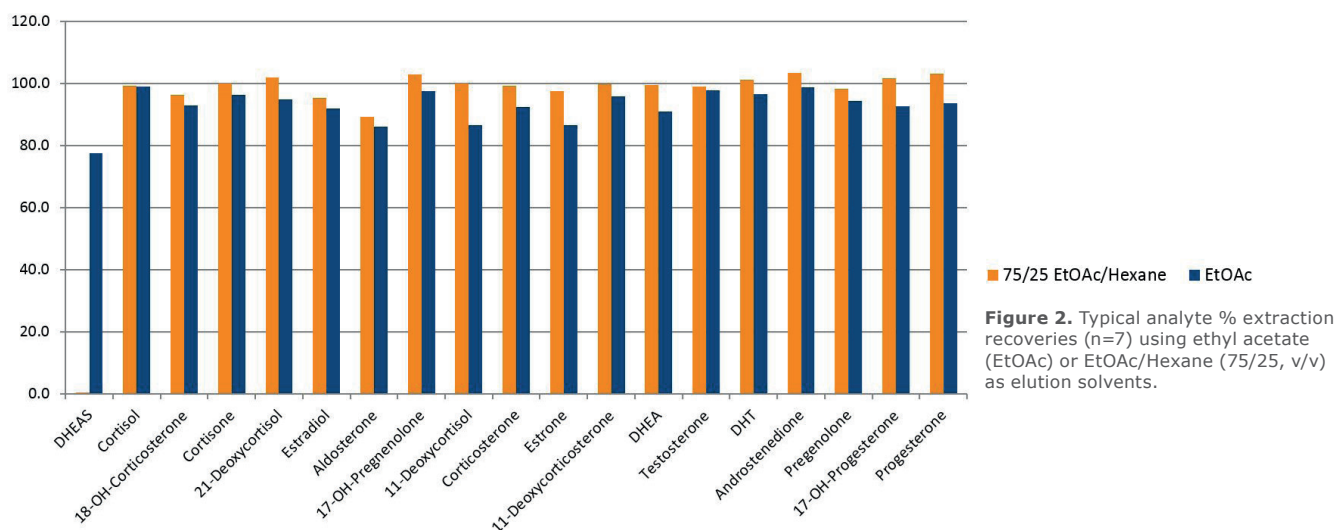


Figure 4. shows representative chromatography obtained from stripped serum spiked at 5 ng/mL. Satisfactory resolution of the various isobars was obtained using the ACE C18 UPLC column. In order to achieve low level detection of analytes in positive and negative ion modes a combination of 0.2 mM NH₄F (aq) and MeOH was utilized.

Linearity was investigated using stripped serum spiked between 5–5000 pg/mL. Good linearity was observed for all analytes typically delivering r^2 values greater than 0.99. Table 3. details linearity performance and associated LOQ for each analyte using EtOAc and EtOAc/Hexane (75/25, v/v) as elution solvents. Selected calibration curves using EtOAc as elution solvent are shown in Figure 5.

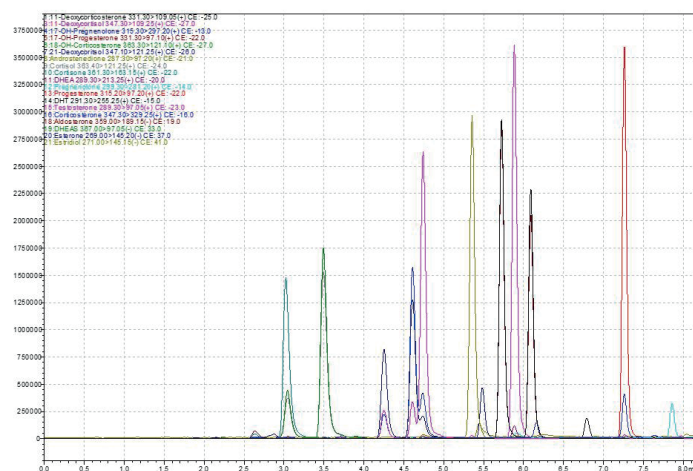


Figure 4. Representative chromatography for analytes spiked at 5 ng/mL in stripped serum.

Table 3. Analyte calibration curve r^2 and LOQ performance.

Analytes	r^2 EtOAc	LLOQ (pg/mL) EtOAc	r^2 EtOAc/Hexane 75/25 (v/v)	LLOQ (pg/mL) EtOAc/ Hexane 75/25 (v/v)
DHEAS	0.991	<1000	-	-
Cortisol	0.998	< 10	0.998	< 10
18-OH-Corticosterone	0.996	< 100	0.997	< 100
Cortisone	0.992	< 5	0.994	< 5
21-Deoxycortisol	0.997	25	0.998	25
Estradiol	0.996	< 25	0.997	< 25
Aldosterone	0.998	25	0.999	25
17-OH-Pregnenolone	0.999	< 500	0.999	< 500
11-Deoxycortisol	0.999	5	0.998	5
Corticosterone	0.997	< 50	0.999	< 50
Estrone	0.997	10	0.999	10
11-Deoxycorticosterone	0.998	< 10	0.999	< 10
DHEA	0.994	< 1000	0.994	< 1000
Testosterone	0.994	< 5	0.998	< 5
DHT	0.994	< 250	0.999	< 250
Androstenedione	0.990	10	0.998	< 10
Pregnenolone	0.991	< 250	0.998	< 250
17-OH-Progesterone	0.994	< 10	0.998	< 10
Progesterone	0.993	< 5	0.999	< 5

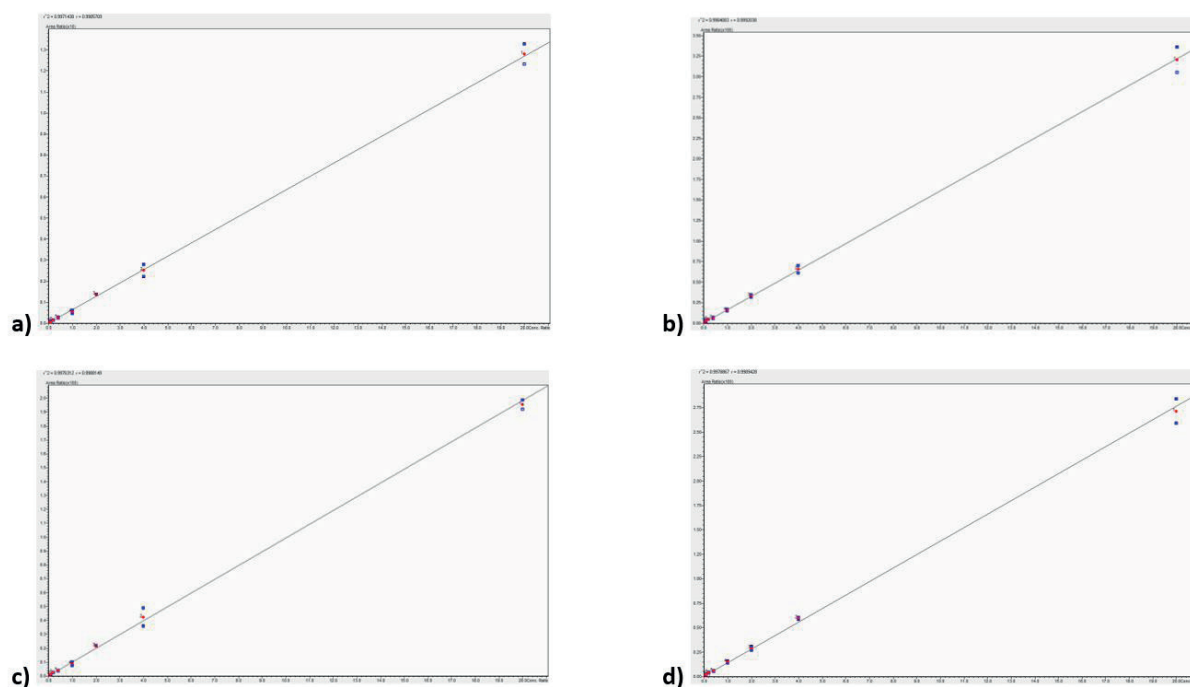


Figure 5. Calibration curves for Estradiol (a), Testosterone (b), 17-OH-Progesterone (c) and Androstenedione (d).

Additional Information

- » Ammonium fluoride increased sensitivity in both positive and negative ion modes.
- » For increased sensitivity:
 - » Increase matrix loading volumes to 350 μ L
 - » Decrease reconstitution solvent volume below 200 μ L
 - » Increase injection volumes above 10 μ L.
- » Steroids can exhibit non-specific binding to plastic collection plates. Different plastics exhibit different binding characteristics. Addition of 2 μ L of ethylene glycol to the collection plate prior to evaporation can mitigate this issue. Note: No ethylene glycol was used in generation of the data shown in this application note, utilizing collection plate p/n 121-5203.

Ordering Information

Part Number	Description	Quantity
820-0400-P01	ISOLUTE SLE+ 400 μ L Supported Liquid Extraction Plate	1
120-5203	Collection plate, 2 mL square	50
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage® SPE Dry 96 Sample Evaporator 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry 96 Sample Evaporator 100/120 V	1

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