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Extraction of a Comprehensive Steroid Panel from Human Serum Using Biotage® Mikro ABN SPE Microelution Plates Prior to LC/MS-MS Analysis (No DHEAS)

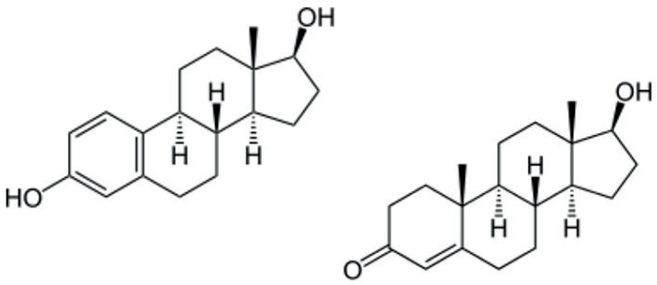

Figure 1. Structures of Estradiol and Testosterone.

Introduction

This application note describes the extraction of steroid hormones from human serum using Biotage® Mikro ABN microelution plates prior to LC/MS-MS analysis. The simple sample preparation procedure delivers clean extracts and analyte recoveries mostly greater than 90% with RSDs lower than 5% for most analytes. Linearity of greater than 0.99 is achieved for all analytes from 1–1000 pg/mL.

Mikro plate extraction allows for very low elution volumes and enhanced workflow efficiency.

Analytes

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

The polar metabolite DHEAS is not included in this application note. If DHEAS is to be included in the panel, see application note AN939.

Internal Standards

Dihydrotestosterone- D_3 (DHT-D_3) and Aldosterone-D_4.

Sample Preparation Procedure

Format

Biotage® Mikro ABN Plate, 2 mg, part number 600-0002-LVP

Sample Pre-Treatment

Spike serum (200 µL) with internal standard solution and allow to equilibrate for 1 hour. Dilute with 1% formic acid (1:1, v/v). Mix.

Internal standard solution consisted of 10 pg/µL methanolic solution. 20 µL was added to 200 µL serum to give a 1 ng/mL spike concentration.

Conditioning

Condition wells with methanol (100 µL)

Equilibration

Equilibrate wells with 0.1% formic acid (100 µL)

Sample Loading

Load 400 µL of the pre-treated serum sample

Wash 1

Elute interferences with water (100 µL)

Wash 2

Elute interferences with H_2O:MeOH (60:40, v/v, 100 µL)

Dry

Dry plate for 2 minutes

Elution

Elute analytes with Ethyl Acetate (30 µL)

Collection Vessels

Collect the eluent in a 1 mL square well collection plate (p/n 121-5202).

Post Elution

Evaporate extracts to dryness at 40 °C, for approximately 5 mins. at a flow rate of 20–40 L/min. using the Biotage® SPE Dry-96.

Reconstitute

Reconstitute in MeOH:H_2O (50:50, v/v, 30 µL).

Vortex mix and cover plate with a sealing mat prior to injection.

Processing Conditions

Biotage® Mikro plates were processed using a Biotage® Pressure+ Positive Pressure Manifold.

Settings:

- Condition, equilibrate, load, wash and elute steps: 7–9 psi (fine control setting)
- Plate dry step: 40 psi coarse setting for 2 minutes

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UHPLC Conditions

Instrument
Shimadzu Nexera x2 UHPLC

Column
ACE C18 (100 mm x 2.1 mm, 1.7 µm)
with a Restek EXP holder and ARC-18 guard

Mobile Phase
A: 0.2 mM Ammonium Fluoride (aq)

B: Methanol

Flow Rate
0.4 mL/min

Column Temperature
40 °C

Injection Volume
5 µL

MS 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 1. HPLC Gradient.

Time (min.)	%A	%B
0	50	50
2	50	50
5	40	60
8	10	90
9	5	95
9.1	5	95
9.2	50	50

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

Analytes	MRM Transition	Collision Energy	Ion Mode
Cortisol	363.4 > 121.25 (363.40>327.15)	-24	+
18-OH-Corticosterone	363.3 > 269.2 (363.30 > 121.10)	-16	+
Cortisone	361.3 > 163.15 (361.30 > 329.15)	-22	+
21-Deoxycortisol	347.1 > 311.2 (347.10 > 269.20)	-16	+
Estradiol	271.1 > 145.2 (271.10 > 183.25)	39	-
Aldosterone-D_4	363.1 > 190.3	19	-
Aldosterone	359.1 > 189.25 (359.00 > 297.15)	18	-
17-OH-Pregnenolone	315.3 > 297.2 (315.30 > 251.00)	-13	+
11-Deoxycortisol	347.3 > 109.25 (347.30 > 283.15)	-27	+
Corticosterone	347.3 > 329.25 (347.30 > 283.15)	-16	+
Estrone	269.2 > 145.2 (269.20 > 143.20)	37	-
11-Deoxycorticosterone	331.3 > 109.05 (331.30 > 97.25)	-25	+
DHEA	271.10 > 253.20 (271.10 > 213.20)	-13	+
Testosterone	289.3 > 97.05 (289.3 > 109.2)	-23 -25	+
DHT-D_3	294.4 > 258.25	-16	+
DHT	291.3 > 255.25 (291.3 > 199.05)	-15 -15	+
Androstenedione	287.3 > 97.2 (287.30 >109.20)	-21	+
Pregnenolone	299.3 > 159.25 (299.30 > 281.20)	-20	+
17-OH-Progesterone	331.3 > 97.1	-22	+
Progesterone	315.2 > 97.2 (331.30 > 109.15)	-22	+

Results

In this application note, ethyl acetate is used as the elution solvent. Compared to methanol, improved analyte recoveries are achieved. However, if the polar metabolite dehydroepiandrosterone sulfate (DHEAS) is to be extracted, methanol should be used as the elution solvent (see application note AN939).

Using Biotage® Mikro ABN plates, very low elution volume (30µL) of ethyl acetate is possible. This extract is evaporated and reconstituted prior to analysis in a solvent compatible with the LC-MS/MS mobile phase.

Recovery data for ethyl acetate elution is shown below in Figure 2. The optimized SPE protocol delivers typical analyte recoveries above 90%, with corresponding RSDs below 10%, for most analytes.

Figure 3. demonstrates 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 NH_4F (aq) and MeOH was utilized.

Calibration curve performance was investigated from stripped serum spiked between 1–1000 pg/mL. Good linearity was observed for all analytes typically delivering r2 values greater than 0.99. Table 3. details linearity performance and associated LOQ for each analyte. Selected calibration curves are shown in Figure 4. (See page 4).

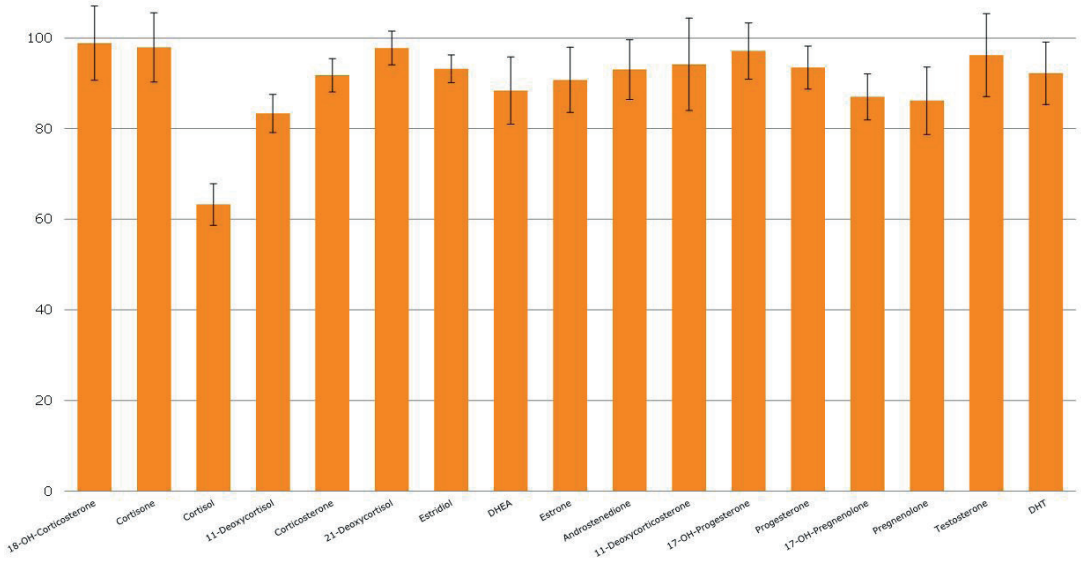

Figure 2. Typical analyte % extraction recoveries (n=7) using ethyl acetate as the elution solvent.

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

Analyte	r^2	LLOQ (pg/mL) Dilute
Cortisol	0.9996	100
18-OH-Corticosterone	0.9997	100
Cortisone	0.9992	25
21-Deoxycortisol	0.9994	100
Estradiol	0.9998	100
Aldosterone	0.9996	250
11-Deoxycortisol	0.9992	10
Corticosterone	0.9999	250
Estrone	0.9991	50
11-Deoxycorticosterone	0.9995	100
DHEA	0.9991	250
Testosterone	0.9994	10
DHT	0.9993	< 250
Androstenedione	0.9994	25
Pregnenolone	0.9990	500
17-OH-Progesterone	0.9994	250
Progesterone	0.9990	1

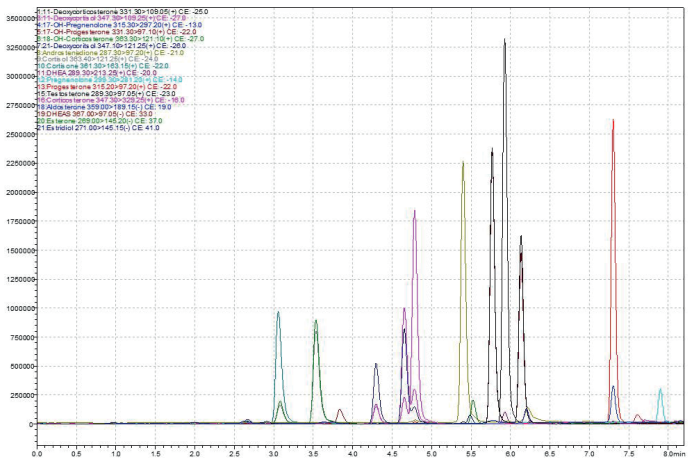

Figure 3. Representative chromatography for stripped serum spiked at 5 ng/mL.

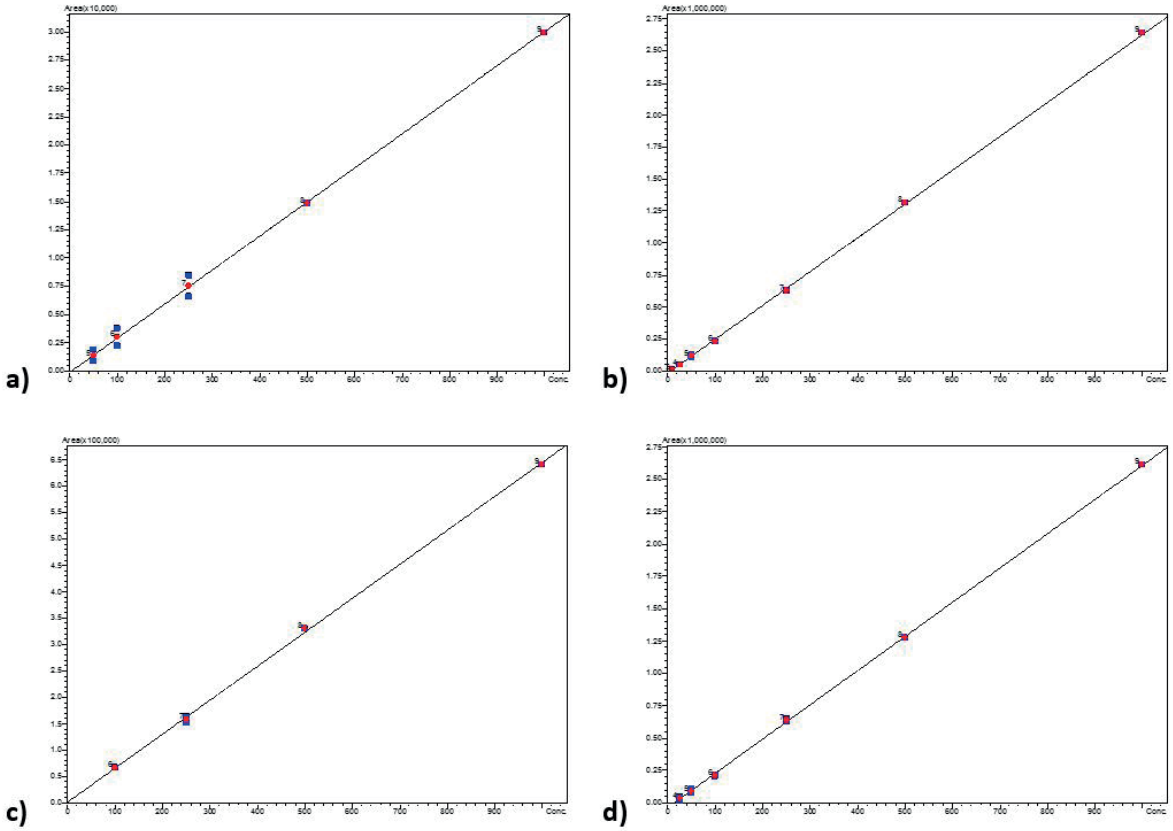

Figure 4. Calibration curves for Estradiol (a), Testosterone (b), 17-OH-Progesterone (c) and Androstenedione (d).

Chemicals and Reagents

- Methanol (LC-MS grade), Ultra-Pure Methanol (Gradient MS), ethyl acetate (99.7%) and formic acid (98%) were purchased from Honeywell Research Chemicals (Bucharest, Romania).
- All analyte standards and deuterated internal standards were purchased from Sigma- Aldrich Company Ltd. (Gillingham, UK).
- Water used was 18.2 MOhm-cm, drawn daily from a Direct-Q5 water purifier.
- Mobile phase A (0.2 mM ammonium fluoride (aq)) was prepared by adding 7.4 mg of ammonium fluoride to 1 L with purified water.
- Internal standards (100 pg/µL) were prepared from a 10 ng/µL stock solution by adding 10 µL of each of to 990 µL of MeOH. 20 µL of this solution was then added to each calibration sample.
- The pretreatment solvent 1% formic acid was made by adding 1 mL of formic acid to 99 mL of water (18.2 MOhm-cm).
- The equilibration solvent 0.1% formic acid was made by adding 100 µL of formic acid to 99.9mL of water (18.2 MOhm-cm).
- Wash 2: H_2O: MeOH (60:40, v/v) was made up by measuring out 60 mL of water (18.2 MOhm-cm) and 40 mL of methanol and adding both to a beaker.
- Reconstitution solvent was made by measuring out 50 mL of purified water (18.2 MOhm-cm) and 50 mL of mobile phase B and adding them to the same bottle.

Additional Information

- All data shown in this application note was generated using serum both stripped and unstripped purchased from Golden West.
- Ammonium fluoride in the mobile phase increased sensitivity in both positive and negative ion modes.
- Steroids exhibit non-specific binding to plastic collection plates. Different plastics exhibit different binding characteristics. The use of 2 µL of ethylene glycol can help to mitigate this issue. This application did not require the use of glycol.

Ordering Information

Part Number	Description	Quantity
600-0002-LVP	Biotage® Mikro ABN Plate, 2 mg	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS	Biotage® SPE Dry Sample Concentrator System	1
121-5202	Collection Plate, 1 mL Square	50
121-5204	Pierceable Sealing Mat	50

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