Extraction of a Comprehensive Steroid Panel from Human Serum Using Biotage® Mikro ABN SPE Microelution Plates Prior to LC/MS-MS Analysis

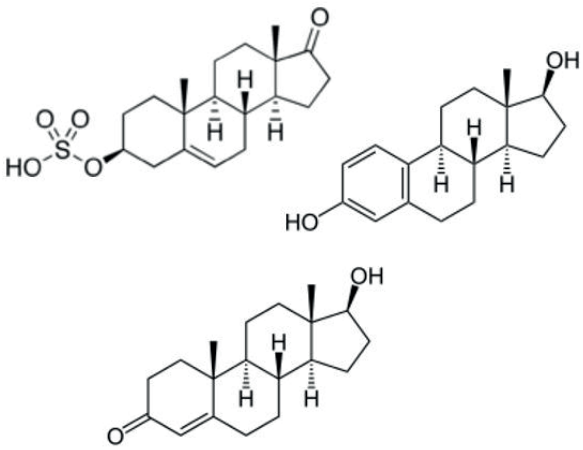

Figure 1. Structures of DHEAS, Estradiol and Testosterone.

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 70% with RSDs lower than 5% for most analytes. Linearity of greater than 0.99 is achieved for all analytes from 1–1000 pg/mL. An elution volume of 30 µL is used, which is simply diluted prior to analysis, avoiding a time consuming evaporation step.

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, Dehydroepiandrosterone sulfate (DHEA-S), Testosterone, Dihydrotestosterone (DHT), Pregnenolone, Androstenedione, 11-deoxycorticosterone, Progesterone

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 methanol (30 µL)

Collection Vessels

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

Post Elution: Dilute

Add a aliquot of water (30 µL) to each well. 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

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
17L/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
DHEAS	367.1 > 97.05 (367.1 > 191.05)	33	-
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

Recovery data for the steroid panel is shown in figure 2. Typical analyte recoveries above 70%, with corresponding RSDs below 10% were achieved.

Note that for recovery of the polar DHEAS metabolite, methanol is used the elution solvent. This has the added advantage that it can be diluted with water and injected directly into the LC-MS/MS system, eliminating the need for time consuming evaporation steps. Direct injection of the elution solvent is also possible, but some peak broadening of early eluting analytes was observed, reducing the sensitivity for these 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 in the range 1–1000 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. Example calibration curves are shown in Figure 4. (See page 4).

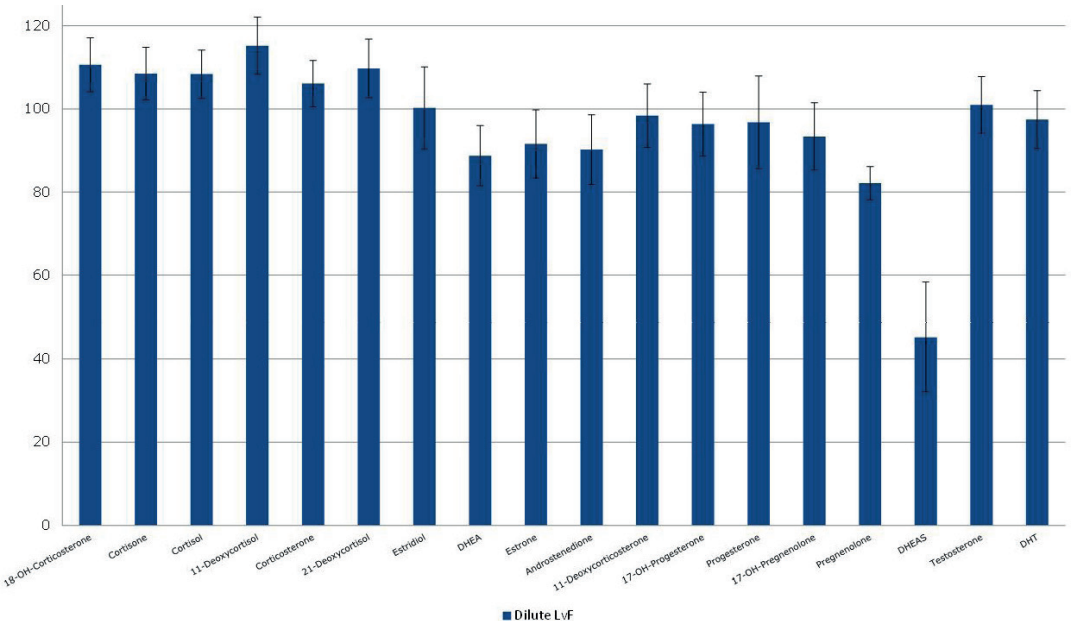

Figure 2. Typical analyte % extraction recoveries (n=7) using the extraction procedure described in this application note.

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

Analyte	r^2 Dilute	LLOQ (pg/mL) Dilute
DHEAS	0.9990	50
Cortisol	0.9991	50
18-OH-Corticosterone	0.9991	50
Cortisone	0.9997	25
21-Deoxycortisol	0.9998	100
Estradiol	0.9995	50
Aldosterone	0.9995	50
11-Deoxycortisol	0.9991	25
Corticosterone	0.9995	250
Estrone	0.9991	25
11-Deoxycorticosterone	0.9992	50
DHEA	0.9994	250
Testosterone	0.9992	5
DHT	0.9991	100
Androstenedione	0.9995	10
Pregnenolone	0.9997	500
17-OH-Progesterone	0.9997	100
Progesterone	0.9997	1

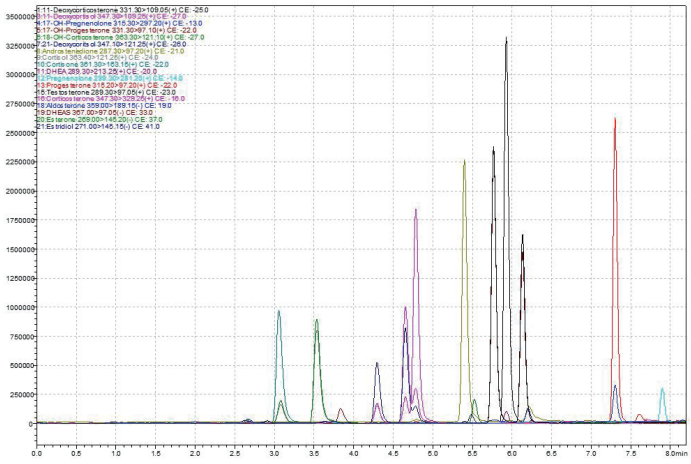

Figure 3. Representative chromatography for stripped serum spiked at 5 ng/mL.

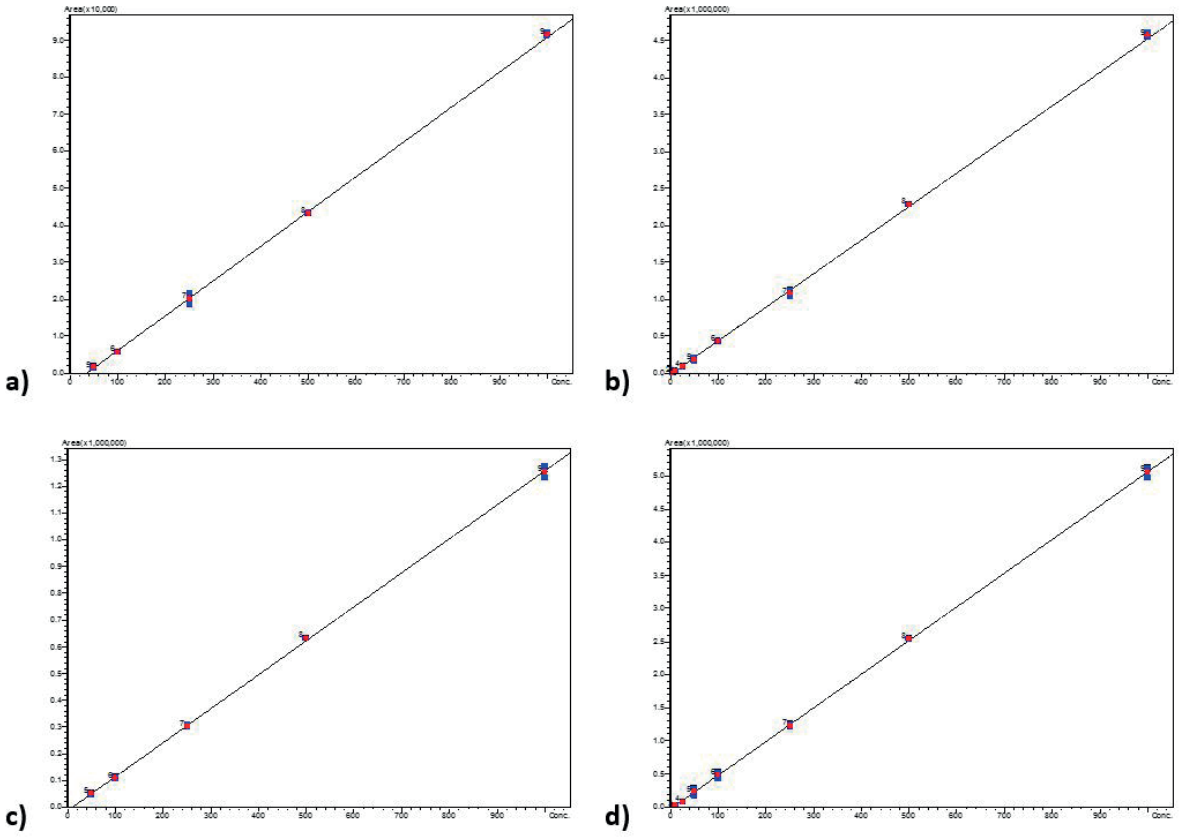

Figure 4. Calibration curves for Estradiol (a), Testosterone (b), 17-OH-Progesterone (c) and Androstenedione (d) for extracts diluted 1:1 (v/v) prior to analysis.

Chemicals and Reagents

- Methanol (LC-MS grade), Ultra-Pure Methanol (Gradient MS) 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 950 µL of MeOH. 20 µL of this solution was then added to each calibration sample.
- The pre-treatment 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 solution 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.

Additional Information

- All data shown in this application note was generated using serum both stripped and unstripped purchased from Golden West.
- The use of ammonium fluoride in the mobile phase increased sensitivity in both positive and negative ion modes.
- Increasing sensitivity:
  - For later eluting analytes direct injection of the methanol elution solvent, without dilution is possible.
- 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 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
121-5202	Collection Plate, 1 mL Square	50
121-5204	Pierceable Sealing Mat	50

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