Comparison of Sample Preparation Options for the Extraction of a Panel of Endogenous Steroids from Serum Prior to UHPLC-MS/MS Analysis

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Introduction

This poster compares sample preparation options for the extraction of a panel of endogenous steroids from serum. LC-MS/MS parameters were investigated for increased sensitivity: MRM transitions, chromatography and mobile phase additives for use with positive and negative ionisation modes. Particular emphasis was placed on the sample preparation to provide high reproducible recoveries whilst minimizing matrix effects and co-extracted materials such as proteins and phospholipids. Solid phase extraction was compared to supported liquid extraction in terms of recoveries, ion suppression, phospholipid content, calibration curve performance and overall sensitivity.

Experimental

Reagents

Standards, ammonium acetate and ammonium fluoride were purchased from Sigma-Aldrich Company ltd. (Gillingham, UK). LC/MS grade solvents were from Honeywell Research Chemicals (Bucharest, Romania). Water (18.2 MΩ.cm) was drawn fresh daily from a Direct-Q 5 water purifier (Merck Millipore, Watford, UK). Pooled human plasma was from The Welsh Blood Service (Pontyclun, UK) or Golden West Biologicals, Inc. (Temecula CA).

Sample Preparation

Extractions were developed using supported liquid extraction or polymer-based SPE in 96 fixed well plate format. ISOLUTE® SLE+ was used in the 400 µL capacity 96-well plate format (P/N 820-0400-P01) following a load-wait-elute procedure (***Figure 2***).

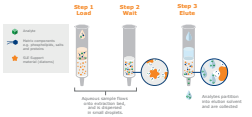

Figure 2. *Schematic of ISOLUTE® SLE+ Supported Liquid Extraction Procedure.*

EVOLUTE® EXPRESS ABN and CX were used in 10 and 30 mg formats (P/N 600-0010-PX01 and 601-0010-PX01) following a typical SPE procedure incorporating additional wash steps (***Figure 3***).

Figure 3. *Schematic of a Typical SPE Procedure.*

Full method optimization was performed for each sample preparation technique with final extraction protocols for each shown in ***Table 1***.

Post extraction: Extracts were evaporated at 40 °C and reconstituted in 200 µL of 50:50 mobile phase A:B prior to injection.

Table 1. *Optimized Extraction Protocols.*

Step	ISOLUTE® SLE+ 400 µL	EXPRESS ABN 10 mg	EXPRESS CX 10 mg
Condition	-	MeOH 500 µL	MeOH 500 µL
Equilibration	-	0.1% Formic Acid (aq) 500 µL	0.1% Formic Acid (aq) 500 µL
Sample load	Serum 300 µL	1:1 1% HCOOH (aq) 400 µL	1:1 1% HCOOH (aq) 400 µL
Wash 1	-	H_2O 500 µL	H_2O 500 µL
Wash 2	-	60:40 H_2O:MeOH 500 µL	70:30 H_2O:MeOH 500 µL
*** Elution**	2x 500 µL of EtOAC **OR** 75:25 EtOAc:Hexane	150 µL of MeOH **OR** EtOAc	150 µL of MeOH **OR** EtOAc
* Elution is dictated by the presence/absence of DHEAS in the panel			

UHPLC Conditions

Instrument: Shimadzu Nexera UHPLC (Shimadzu Europa GmbH, Duisburg, Germany)
Column: ACE C18 1.7 µm 100 x 2.1 mm + guard (ACT, UK)
Mobile phase: A, 0.2 mM NH_4F (aq); B, MeOH **Flow rate:** 0.4 mL/min
Gradient: Initial 50/50; linear to 60% B, 3 min; linear to 90% B, 8 min; linear to 95% B, 9 min; hold, 0.1 min; resume initial conditions, 9.5 min.
Column temp: 40 °C **Injection volume:** 10 µL

Mass Spectrometry

Instrument: Shimadzu 8060 Triple Quadrupole mass spectrometer equipped with and ES interface for mass analysis (Shimadzu Europa GmbH, Duisburg, Germany). Positive or negative ions were acquired in the MRM mode (***Table 2***).
Heat Block Temp: 400° C **Interface Temp:** 400° C **DL Temp:** 250° C
Nebulizing Gas: 3 L/min **Drying Gas:** 3 L/min **Heating Gas:** 17 L/min
CID Gas: 270 kPa

Table 2. **MRM Parameters**

Analyte	Transition	Ion Mode	Collision Energy, V
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 > 258.2	+	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

Evaporation Optimization

Steroids are well known to exhibit non-specific binding to plastic collection plates resulting in signal losses. On evaluation, these evaporation losses using Biotage 2 mL collection plates were minimal when using reconstitution solvents incorporating 50% MeOH. No advantage was observed when incorporating glycol to avoid complete evaporation as demonstrated in ***Figure 3***.

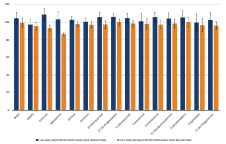

Figure 3. *Non-specific Binding Investigation (with and without glycol).*

ISOLUTE® SLE+ Optimization

Initial experiments focused on pre-treatment for potential disruption of protein binding. Due to nature of extraction process protein binding was not seen to be a problem as demonstrated in ***Figure 4***. In order to maximize matrix loading, future experiments focused on serum modified with ISTD addition only.

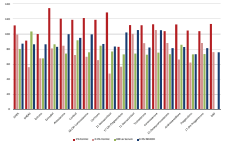

Figure 4. *Pre-treatment buffer Investigation.*

Elution investigation demonstrated the ability of various solvent combinations to provide effective results, as show in ***Figure 5***. If DHEAS is present in the panel then EtOAc should only be considered for the elution solvent.

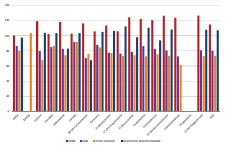

Figure 5. *Elution Solvent Investigation.*

SPE Optimization

Initial development was performed using the 30 mg ABN sorbent plate with extraction optimization focussed on wash and elution solvent combinations. ***Figure 6***. demonstrates the effect on recoveries of increasing the MeOH content in the wash solvent. For ABN it is possible to wash with 40% MeOH whereas the CX sorbent optimized at around 30% (data not shown).

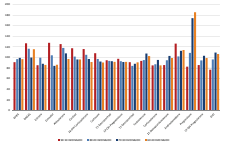

Figure 6. *ABN H_2O:MeOH Wash Solvent Investigation.*

Figure 7. demonstrates the elution solvent screen. Multiple elution solvents may be used for steroid analysis. However, if DHEAS is present then MeOH should only be considered for the elution solvent. EtOAc was selected as the best performing solvent if DHEAS was not present due to extract cleanliness and phospholipid content.

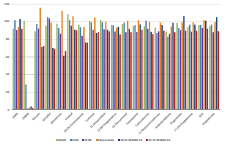

Figure 7. *ABN Elution Solvent Investigation.*

Final methods were scaled to the 10 mg plate format to minimize elution volumes. ***Figure 8***. demonstrates final optimized extraction recovery performance for SPE and SLE+ protocols. CX typically didn't perform as well as ABN for the entire suite so further work was discontinued.

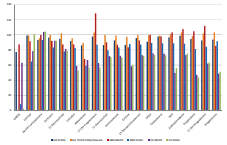

Figure 8. *Recovery Profiles for Optimized protocols.*

Phospholipid Removal

Figure 9. compares the efficiency of phospholipid removal using the optimized protocols for ISOLUTE® SLE+ and EVOLUTE® EXPRESS ABN.

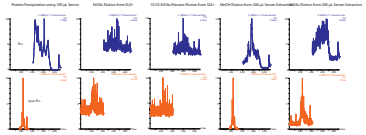

Figure 9. *Phospholipid Removal using Optimized Extraction Protocols.*

Calibration curves were constructed in stripped human serum from 5-5000 pg/mL. ***Table 3.*** summarizes the LOQ and coefficients of determination for ISOLUTE® SLE+ using 72/25 EtOAc/hexane and ABN using MeOH as elution solvents.

Table 3. *Summary of method performance.*

Analyte	ISOLUTE® SLE+ 75/25	ISOLUTE® SLE+ LOQ pg/mL	ABN MeOH	ABN LOQ pg/mL
DHEAS	-	-	0.991	25
Cortisol	0.998	<10	0.992	<50
18-OH-Corticosterone	0.997	<100	0.996	<250
Cortisone	0.994	<5	0.996	<5
21-Deoxycortisol	0.998	25	0.995	50
Estradiol	0.997	<25	0.997	<50
Aldosterone	0.999	25	0.976	25
17-OH-Pregnenolone	0.999	<500	0.996	<500
11-Deoxycortisol	0.998	5	0.993	25
Corticosterone	0.999	<50	0.992	<100
Estrone	0.999	10	0.996	<25
11-Deoxycorticosterone	0.999	<10	0.999	<10
DHEA	0.994	<1000	0.994	<1000
Testosterone	0.998	<5	0.997	<5
DHT	0.999	<250	0.998	<250
Androstenedione	0.998	10	0.997	25
Pregnenolone	0.998	<250	0.994	<250
17-OH-Progesterone	0.998	<10	0.995	<25
Progesterone	0.999	<5	0.995	10

Conclusions

- This poster presents optimized supported liquid extraction and solid phase extraction approaches to low level analysis of steroid hormones.
- Good recoveries, LOQs and linearity were obtained while demonstrating excellent removal of matrix interferences such as phospholipids.

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