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Article

Automated Supported Liquid Extraction for the analysis of a panel of 12 endogenous steroids in human plasma by LC-MS/MS

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Abstract: Steroid analysis is important in the clinical assessment of endocrine function in health and disease. Although tandem mass spectrometry methods coupled with chromatographic separation are considered the gold standard analytical technique in this setting, enabling profiling of multiple steroids in a single sample, sample processing can be labour-intensive. Here we present a simple, efficient automated 96-well Supported Liquid Extraction method with dichloromethane/isopropanol as organic solvent, carried out on an automated sample handler which completes sample preparation of 80 plasma samples (200µL) in 90 minutes. Compounds were separated on a Kinetex C18 column (150x3mm; 2.6µm) using a mobile phase of methanol and water (0.1% formic acid). The run time was 16 minutes on a uHPLC tandem mass spectrometry instrument. Precision ranged 8.1 to 18.1% RSD, bias -10.1-5.8%, and extraction recoveries 73.5-111.9%. Limits of Quantitation ranged between 0.025–0.500 ng/mL.

The method was used to profile 12 endogenous glucocorticoids, androgens and progestogens in 54 female human plasma samples, at baseline and following a low dose Synacthen® test. Increases in steroid intermediates such as 11-deoxycorticosterone and 11-deoxycortisol were detected following Synacthen treatment. This simple automated sample preparation of 200 µL human plasma followed by LC-MS/MS analysis, is sensitive and specific, and suitable for profiling steroids for endocrine studies.

Keywords: steroids; steroid panel; clinical mass spectrometry; plasma; sample automation; endocrine; Synacthen

1. Introduction

Steroid hormones such as corticosteroids and gonadal steroids are critical in numerous physiological and pathophysiological processes. Corticosteroids produced in the adrenal cortex play

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critical roles in many physiological processes. Glucocorticoids, mineralocorticoids and adrenal androgens are produced via the steroidogenesis pathway from cholesterol through a series of enzyme-controlled steps (Figure 1). Over the past two decades it has become increasingly clear that, as well as steroid hormones themselves, intermediates in the biosynthetic pathway and steroid metabolites have bioactivity. Dysfunction of the adrenal glands has broad spectrum systemic effects and can be fatal if untreated. Assessing the steroidogenic cascade is used in diagnosis and monitoring of a number of endocrine disorders e.g. congenital adrenal hyperplasia (CAH) [1, 2, 3, 4, 5], Cushing's [6,7] syndrome, Addison's disease [8, 9], adrenal cancers [10,11,12,13] and polycystic ovary syndrome (PCOS) [14, 15, 16]. Many of these conditions display elevations in androgens such as testosterone and androstenedione while 21-hydroxylase deficiency can be determined through elevation of 17hydroxyprogesterone. While cortisol is the main glucocorticoid in humans, measurement of corticosterone is becoming more relevant, showing increases in 17-hydroxylase deficiencies with evidence of potential benefit as a treatment in glucocorticoid deficiency [17].

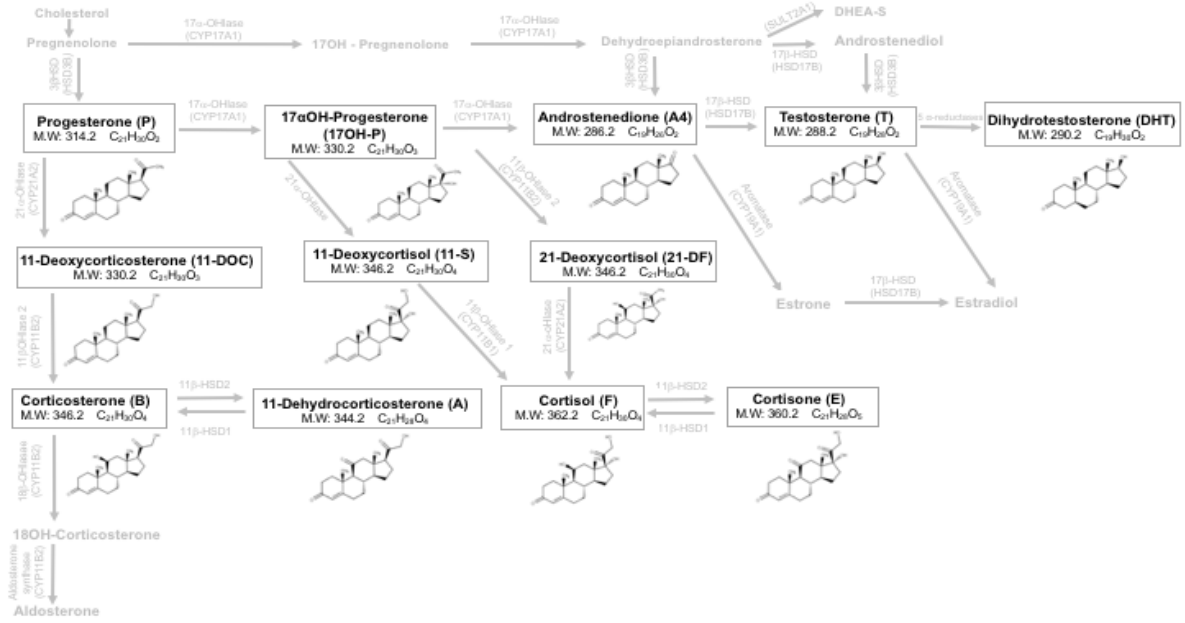

Figure 1 – Steroid Biosynthetic Pathway. Steroids included in this LC-MS/MS method panel are indicated with the chemical structure and molecular weight (MW) as the monoisotopic mass. Enzymes are above arrows and genes are in brackets

In the clinical setting, approaches used to measure steroid concentrations in human biomatrices include immunoassays, and more recently chromatographic methods coupled to tandem mass spectrometry (LC-MS/MS) [18,19,20,21]. LC-MS/MS has a number of advantages over immunoassays, including its increased specificity and sensitivity [21, 22, 23, 18]. Immunoassays have a number of limiting factors, such as cross reactivity and numbers of concomitant analytes, leading to reduced assay specificity, inadequate standardisation and limited dynamic range, all of which may produce inaccurate results rendering them unreliable for clinical applications [24].

Due to the complexities of the steroid biosynthetic pathway, mass spectrometric techniques have become an invaluable tool, not only for their applications in steroid quantitation, but also for their ability to analyse many compounds simultaneously, enabling the mapping of novel biosynthetic pathways [22]. Developing multi-analyte methods comes with analytical challenges, including identifying a reliable method of sample preparation that results in a repeatable recovery of all analytes. This is confounded by the wide and often challengingly low circulating concentrations of some steroids [23].

In this study we describe automated sample preparation of plasma (200 µL) by SLE for analysis of a panel of steroids followed by ESI-LC-MS/MS analysis. The method has been validated for the quantitation of 12 major, diagnostically important adrenocortical steroids from the glucocorticoid,

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mineralocorticoid, androgen and progestogen classes. These are testosterone (T), dihydrotestosterone (DHT), androstenedione (A4), progesterone (P4), 17-hydroxyprogesterone (17-OHP), 11-deoxycortisol (S), 21-deoxycortisol (21-DF), 11-deoxycorticosterone (11-DOC), corticosterone (B), 11-dehydrocorticosterone (A), cortisol (F), cortisone (E). Steroid intermediates such as S and 21-DF are usually at very low circulating levels, unless there has been a disruption to the steroidogenesis pathway. Measuring steroid levels following treatment of healthy subjects with a synthetic analogue of ACTH - Synacthen® - which stimulates steroidogenesis for a short period - is an ideal model to assess reliable detection of these intermediates in human clinical samples.

2. Results

2.1 Method development

2.1.1 LC-MS/MS method

There are analytical challenges in developing a method for the global steroid biosynthetic pathway. The first is the occurrence of isobars in the pathway, as shown in Figure 1, such as Dehydroandrosterone (DHA), Dehydroepiandrosterone (DHEA) and testosterone (T); 21-deoxycortisol (21DF), corticosterone (B) and 11-deoxycortisol (S); Aldosterone (Aldo) and cortisone (E), 17-hydroxyprogesterone (17-OHP) and 11-deoxycorticosterone (11-DOC). Another important consideration is the isotopologues contribution of steroids that differ only by 2 Da due to the natural abundance of ^{2}H and ^{13}C. To address these concerns, reliable chromatographic separation of steroids by time is necessary. The superior selectivity and specificity of LC-MS/MS bioanalytical methods derives from directly measuring the analyte of interest as a mass transition of precursor to product ion. The isolated precursor ion is collisionally dissociated into diagnostic product ions in a tandem mass spectrometer. However, many steroids undergo collisional activated dissociation to typical steroid product ions and this can reduce specificity of the method. To address this, in addition to ensuring complete separation of the steroids of interest chromatographically from that of known isobars and isotopologues, the ratio of the quantifier and qualifier ions in standards and biological samples was monitored.

2.1.1.2 Mass Spectrometric parameters

We compared ionisation modes: APCI with ESI and selected ESI as the response was more consistent across the panel of steroids. Optimised positive molecular ion transitions in ESI were identified following infusion of solutions of each steroid. Each individual steroid was tuned to maximise the precursor ion and, product ions were selected following product ion scans for Selected Reaction Monitoring (SRM) transitions. Two transitions were selected for each steroid – the quantitative and qualitative ion - their optimised declustering potential, collision exit potential and collision energy are listed in Table 1.

Table 1 – Selected Reaction Monitoring parameters for each steroid. Following electrospray ionisation at 5.5 kV, 600°C. DP= Declustering Potential; CE = Collision Energy; CXP = Collision Cell Exit Potential. Quantifier (1) and qualifier (2), indicated accordingly

Steroid	Q1 Mass (*m*/*z*)	Q3 Mass (*m*/*z*)	DP (V)	CE (V)	CXP (V)
Cortisol (F) 1	363.1	121.2	76	31	8
Cortisol (F) 2	363.1	91.1	76	83	10
Cortisone (E) 1	361.1	163.1	81	31	26
Cortisone (E) 2	361.1	77.1	81	107	10
Corticosterone (B) 1	347.1	121.1	76	29	8

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Corticosterone (B) 2	347.1	90.9	76	75	12
11-Dehydrocorticosterone (A) 1	345.1	121.2	66	31	12
11-Dehydrocorticosterone (A) 2	345.1	91.2	66	83	40
11-Deoxycortisol (11-S) 1	346.9	109	76	27	14
11-Deoxycortisol (11-S) 2	346.9	97.0	76	25	11
21-Deoxycortisol (21-S) 1	346.9	120.8	76	41	11
21-Deoxycortisol (21-S) 2	346.9	105.0	76	27	11
11-Deoxycorticosterone (11-DOC) 1	331.2	97.0	46	26	11
11-Deoxycorticosterone (11-DOC) 2	331.2	109.1	66	31	12
Testosterone (T) 1	289.1	97.0	101	29	12
Testosterone (T) 2	289.1	109.2	101	31	6
Androstenedione (A4) 1	287.1	97.0	61	27	14
Androstenedione (A4) 2	287.1	78.9	61	67	10
Dihydrotestosterone (DHT) 1	291.3	255.2	116	21	30
Dihydrotestosterone (DHT) 2	291.3	91.0	116	55	10
Dehydroepiandrosterone (DHEA) 1	289.0	253.3	21	13	50
Dehydroepiandrosterone (DHEA) 2	289.0	213.1	21	11	14
Progesterone (P4) 1	315.0	97.1	96	25	10
Progesterone (P4) 2	315.0	109.1	96	27	10
17OH-Progesterone (17aOH-P) 1	331.0	109.1	66	31	12
17OH-Progesterone (17aOH-P) 2	331.0	96.9	66	29	12
d4-Cortisol (d4F)	367.2	121.1	80	29	16
d8-Cortisone (d8E)	369.2	169.0	96	33	20
d8-Corticosterone (d8B)	355.3	125.1	56	31	8
$^{13}C_3$-Testosterone ($^{13}C_3$-T)	292.2	100.2	101	27	4
$^{13}C_3$--Androstenedione ($^{13}C_3$-A4)	290.2	100.1	31	27	12
$^{13}C_3$-DHT ($^{13}C_3$-DHT)	294.1	258.3	61	21	12
d9-Progesterone (d9P4)	324.1	100.0	151	31	15

All steroidal analytes and internal standards generated singly charged positive ions and underwent transitions in SRM to product ions typical of steroids. The common product ions of *m/z* 121, *m/z* 97 and *m/z* 109 are from the A-ring of the steroid structure, first described by Williams et al, [40] using stable isotope labelled testosterone to identify fragments from collision induced dissociation. This was subsequently confirmed by Ronquist-Nii et al in corticosteroids [41]. The two most abundant product ions were selected for each compound and assessed as the quantifier and qualifier ions.

2.1.3 Chromatographic conditions

The best separation was seen using a Kinetex C18 column and a methanol/water gradient system. 3 mm and 2.1 mm id versions of the 150 mm Kinetex C18 were compared, but once analysis of

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biological samples commenced, it was found that the 3 mm id column gave more consistent retention times and did not suffer from pressure build up, which was seen with the 2.1 mm column. Formic Acid (0.1%) was used as modifier of the mobile phase system of water and methanol. FA suppresses negative ion formation and as such only a minimal signal was achieved for Aldo, but separation from its isobar E was confirmed (2.5 mins vs 3.3 mins). Methanol was needed to encourage retention of the more polar steroids on the stationary phase of the column, being a weaker organic solvent than acetonitrile. Optimal results were achieved with flow rates of 0.5 mL/min and temperatures of 40°C. Starting at 55% B, held for 4 minutes, rising to 100% B to 10 minutes, held for 2 minutes, followed by a return to 55%B and re-equilibration after 16 minutes (Figure 2). At early and late time points the flow was diverted to waste (0-1.5 min; 10-16 mins) and a high organic gradient was employed to minimise build-up on the ESI source and on the column - required for robustness during 96-well plate analysis. Separation of 17OHPreg, Preg, Aldo and DHA are indicated in Figure 2, but their validation was not pursued in this steroid panel method as the limits of quantitation were below the clinical range expected. Separation of 21DF, B and S is shown in the *m/z* 347→121 transition as is 11-DOC and 17OHProg in *m/z* 331→109. Although DHEA, T and DHA are isobaric, DHEA can only be detected in the *m/z* 289 → 253 appearing between T and DHA, and not seen in the mass transition *m/z* 289→97, used as quantifier for T, illustrating that certain transitions are specific to certain steroids

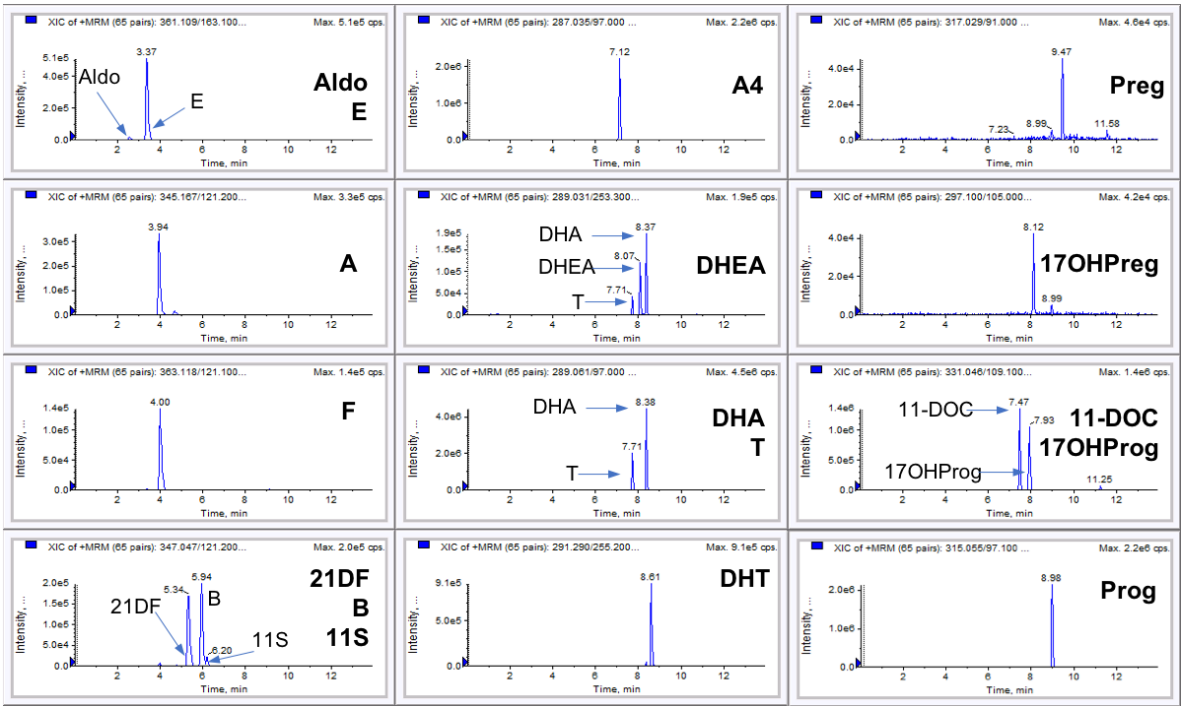

Figure 2 – Extracted Ion Chromatograms showing isobar separation and resolution of all possible isotopologues using a Kinetex C18 (150 x 2.1 mm; 2.6 um) with gradient mobile phase of methanol and water with formic acid (0.1%, v/v). Retention times of the peaks are indicated on chromatograms. A – 11-dehydrocorticosterone, Aldo – aldosterone, A4 – androstenedione, B – corticosterone, DHA – dehydroandrosterone, DHEA- dehydroepiandrosterone, DHT- dihydrotestosterone, 21DF – 21-deoxycortisol, 11-DOC – 11-deoxycorticosterone, E – cortisone, F- cortisol, Preg- pregnenolone, 17-OHPreg – 17-hydroxypregnenolone, P4 –progesterone, 17OHP- 17-hydroxyprogesterone, S – 11-deoxycortisol, T- testosterone

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2.2 *Assay Validation*

2.2.1 *Extraction of steroids*

The recovery of steroids from plasma by automated SLE and the matrix effects, were assessed (Table 2). Isotopically labelled steroids were used to assess recovery due to the presence of endogenous steroids in plasma. The results show between 81.4 -111.6% recovery for the isotopically labelled steroids from plasma using automated SLE400. A slightly lower recovery is seen for the less polar d9-P4 of only 73.5%. This suggests that it is not completely eluting from the SLE material using the solvent system of dichloromethane/propan-2-ol (98:2) and that a small proportion may be remaining on the SLE material. The effect of polar and non-polar solvents on the recovery of progesterone from serum using SLE has previously been discussed [41]. Although other solvents were assessed in preliminary experiments such as MTBE, the elution solvent used in this method is dichloromethane which has a polarity index of 3.1 and propan-2-ol of 3.9. These were selected as recovery was 70% or more across the panel, within international acceptance criteria.

Table 2 – Recovery and matrix effects in plasma (200 µL; n=6) following automated SLE

	$^{13}C_3$T	$^{13}C_3$DHT	$^{13}C_3$A4	d9P4	d8B	d4F
Recovery (%)	81.4	86.2	97.4	73.5	111.6	111.9
Matrix Effect (%)	45.8	46.2	58.1	31.3	64.3	61.4

Matrix effects assessment show the signal for the glucocorticoids is ~60%, for the androgens it is approximately ~50% but for the progesterone it is ~30% in this study. Further optimization of the elution solvent from SLE may reduce the matrix effects, but a balance must be struck between breadth of the method and analytical performance when extending the number of analytes in a panel. It will be important in the future to assess matrix effects in a wider population in larger studies to fully determine the robustness of the sample preparation method.

2.2.2 *Specificity of steroid analysis through chromatography and MS/MS settings*

The ratio of peak areas of quantifier to qualifier mass transitions for steroids was monitored in plasma and standards to further ensure specificity. The ratios in standards were comparable to those in plasma (Table 3), showing differences less than 20%, apart from 21-DF which could not be compared to endogenous 21-DF as it was not detectable in the pool of QC plasma tested.

Table 3 – Quantifier/qualifier ratio of steroids in standards (n=6) and plasma extracts (n=6); A – 11-dehydrocorticosterone, A4 – androstenedione, B – corticosterone, DHT- dihydrotestosterone, 21DF – 21-deoxycortisol, 11-DOC – 11-deoxycorticosterone, E – cortisone, F- cortisol, P4 – progesterone, 17OHP- 17-hydroxyprogesterone, S – 11-deoxycortisol, T- testosterone, Av = mean; n.d. = no data.

	T	**DHT**	**A4**	**P4**	**17OHP**	**S**	**21DF**	**11DOC**	**B**	**A**	**F**	**E**
Standards	1.2	6.6	5.3	1.1	1.5	1.2	1.4	2.1	1.4	2.7	2.0	4.3
Plasma	1.1	5.9	5.5	1.2	1.6	1.3	-	2.3	1.3	2.5	1.9	4.3

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2.2.3 Limits of Quantitation, precision and accuracy of the SLE-LC-MS/MS analysis compared to clinical expected ranges

The full validation results for this SLE-LC-MS/MS steroid panel method are summarised in Table 4. Standard curves were prepared over a broad range 0.025-500 ng/mL with acceptable linearity over clinically expected ranges [43] for each steroid and a mean r value of 0.999. Best fit was achieved using weighting of 1/x for all steroids.

Table 4 – Bioanalytical Method Validation results. Inter-assay precision and accuracy (Acceptance%RSD <20% for low and <15% for med and high; %RME (accuracy) is from -20 to 20%) RSD - relative standard deviation; RME - relative mean error. The precision of the QC plasma batch (n=6) is reported. Clinical expected ranges in women are shown, where available [42].

	T	**DHT**	**A4**	**DHEA**	**P4**	**17OHP**	**S**	**21DF**	**11DOC**	**B**	**A**	**F**	**E**
Female Clinical Expected Range (ng/mL)	0.07-10.8	0.1-0.95	0.2-2.9	2.3-13	0.2-24	0.5-6.3	0.1-3.44	<0.05	0.1-0.3	0.53-15.6	//	20 - 250	6-35
Precision %RSD													
LLOQ (ng/mL)	0.05	0.025	0.05	2.5	0.05	0.25	0.125	0.25	0.05	0.05	0.5	0.5	0.25
Med (ng/mL)	5	0.5	0.5	50	5	5	5	1	1	5	5	10	5
ULOQ (ng/mL)	25	50	50	50	25	125	12.5	12.5	50	25	12.5	500	250
low	18.1	13.4	18.1	11.7	8.1	10.7	9.8	17.1	8.8	19.2	6.0	12	12.2
med	6.5	9.1	13.3	4.7	3.7	8.6	8.5	9.8	6.0	6.7	8.5	11	5.3
high	7.8	11.2	6.4	12.1	8.0	5.0	9.6	5.8	10.3	4.0	7.2	6.0	7.3
QC	2.2	8.6	0.9	2.0	2.1	19.4	3.2	n.d	7.9	8.5	1.3	5.9	4.3
Accuracy %RME	**T**	**DHT**	**A4**	**DHEA**	**P4**	**17OHP**	**S**	**21DF**	**11DOC**	**B**	**A**	**F**	**E**
low	2.1	5.8	4.7	-2.1	-1.8	2.2	1.8	4.9	-10.1	-7.0	-2	0.5	-12
med	6.0	-2.0	-3.0	3.0	4.0	-1.0	1.0	-6.0	-7.0	7.0	3.0	10.0	0.0
high	9.0	3.0	5.0	2.0	-9.0	0.0	2.0	2.0	3.0	-11	-11	8.0	-11

A – 11-dehydrocorticosterone, A4 – androstenedione, B – corticosterone, DHEA-dehydroepiandrosterone, DHT- dihydrotestosterone, 21DF – 21-deoxycortisol, 11-DOC – 11-deoxycorticosterone, E – cortisone, F- cortisol, P4 –progesterone, 17OHP- 17-hydroxyprogesterone, S – 11-deoxycortisol, T- testosterone, Av = mean; n.d. = no data.

2.3 Method application

The volume of sample (200 μL) required for this method and the LOQs obtained compared favourably with previous literature for steroid analysis by LC-MS/MS. The complexity of the separation and the analytical challenges encountered whilst establishing a broad panel quantitative method for steroids have been addressed to ensure precision and accuracy in biological samples. The method was applied to a cohort of plasma samples. Levels of steroids (ng/mL) detected at baseline, prior to a low dose Synacthen® test, are shown in Table 5. All concentrations reported are within the quantitation limits (LLOQ-ULOQ) range of the validated method for each steroid.

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Table 5 –Steroid hormone profile of female plasma (n=54; Age 20-30 y) in ng/mL. The average in nmol/L (nM) is calculated and compared with the median values reported using LC-MS/MS [25].

Analyte	T	DHT	A4	P4	17OHP	S	21DF	11DOC	B	A	F	E
lowest	0.10	0.01	0.45	0.10	0.70	0.12	0.25	0.02	0.50	0.60	23.90	7.40
highest	0.66	0.33	3.03	13.30	5.10	1.73	0.47	0.78	20.25	6.45	350.50	40.1
Av (ng/mL)	0.27	0.12	1.21	1.27	0.89	0.25	0.11	0.12	2.38	1.92	119.43	20.6
Av (nM)	0.94	0.41	4.20	4.04	2.70	1.06	0.32	0.36	6.87	5.58	329.00	57.2
Median (nM)[25]	0.93	//	3.04	0.38	0.79	0.37	0.03	0.10	5.2	n.d.	247.00	51.8

A – 11-dehydrocorticosterone, A4 – androstenedione, B – corticosterone, DHEA-dehydroepiandrosterone, DHT- dihydrotestosterone, 21DF – 21-deoxycortisol, 11-DOC – 11-deoxycorticosterone, E – cortisone, F- cortisol, P4 –progesterone, 17OHP- 17-hydroxyprogesterone, S – 11-deoxycortisol, T- testosterone, Av = mean; n.d. = no data.

In the samples measured, the concentrations of glucocorticoids and progestogens were within the clinical ranges expected in adult females and within the validation ranges of the method. T and A4 are also within the clinically expected ranges, while DHT is slightly below. The LLOQ for DHEA in this method is not sensitive enough to reach lower expected clinical ranges (Table 5) and so the results are not included. Sulphated DHEA (DHEAS) is a more common measure of that steroid; and it is found at higher circulating levels, however conjugates were not included in the panel. The average values across the steroid panel correlate well with the median values of female plasma steroids, measured using a different LC-MS/MS method and reported by Eisenhofer *et al* (Table 5). For the androgens our method reports T and A4 with an average of 0.94 and 4.2 nM while Eisenhofer *et al* had median values of 0.93 and 3.04 nM, respectively. For glucocorticoids (this method reports F, E and B with an average of 329, 57 and 6.87 nM, while Eisenhofer et al had median values of 247, 51.8 and 5.2 nM) indicating that these steroid results analysed using this SLE-LC-MS/MS method are comparable. Eisenhofer et al. did not report values for DHT or A.

Monitoring the steroid profile following treatment with low-dose Synacthen ® reveals the predicted increase in the concentrations of the intermediate steroids 17OHP and S (Figure 3), followed by return of the intermediate to low levels at 60 minutes. F is elevated and remains high at 60 minutes (Figure 3). Monitoring along the steroid pathway towards B production, sees detectable levels of the intermediate 11-DOC. The level of B falls faster than that of F (Figure 3), in keeping with its faster clearance.

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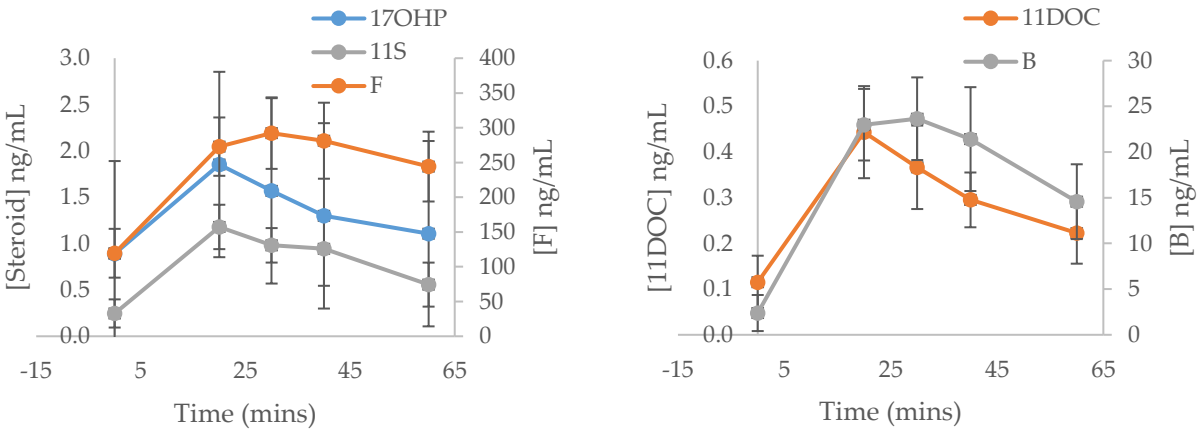

(a) **(b)**

Figure 3 – (a) Time dependent change in concentrations of intermediates 17OHP and 11S and end point F in female subjects (n=54) over 60 minutes following low dose Synacthen ® treatment; (b) Time dependent change in concentrations of steroid intermediate 11DOC and end point B in female subjects (n=54) over 60 minutes following low dose Synacthen ® treatment. Data points show average with standard error of mean. 11-DOC – 11-deoxycorticosterone, 17OHP- 17-hydroxyprogesterone, B-corticosterone, F- cortisol, S – 11-deoxycortisol

3. Discussion

When analysing compounds from complex biomatrices such as plasma, ion suppression is a common problem in LC-MS/MS [24]. This can be reduced by maximising the efficiency of chromatographic separation of the analytes, and by optimising the clean-up steps prior to mass spectrometry analysis. There are a number of options for sample clean-up which include protein precipitation, liquid-liquid extraction (LLE) and solid phase extraction (SPE). Protein precipitation is the simplest approach but the resultant sample is less clean than that obtained from other approaches and also the method dilutes the sample meaning that detection of low concentrations are even more challenging. LLE requires manual intervention, while SPE generates cleaner extracts, but the wash and elution steps add time to the process and can favour specific compound groups. As the steroidogenesis pathway contains a number of families of steroids with subtly different chemical characteristics; glucocorticoids, androgens, progestogens and mineralocorticoids, they can be differentially retained or lost during wash-steps of SPE.

An alternative extraction method to SPE and LLE is supported liquid extraction (SLE). SLE immobilises the aqueous sample on an inert solid support matrix and the analytes of interest are extracted with organic solvents. In recent times the ability to automate SLE using a liquid handing robot has improved its scope, although it is also possible to automate LLE and SPE. An advantage of automated SLE over traditional LLE is a reduction in the volume of solvent necessary and less reliance on the expertise of the analyst. There is a growing body of evidence recommending automation of sample handling for the generation of reliable and robust data [25] and a further economic consideration in increasing sustainability of human clinical laboratories [26,27]. Not only does automation minimise sample handling and error, it also increases the throughput of analysis, with the ability to prepare multiple 96 well plates per day [28]. SLE has been adopted as a method of sample clean up in toxicology such as the screening of drugs of abuse in urine [29] and blood [30]. It has been used for sample preparation of serum (100 µL) prior to derivatisation and GC-MS steroid profiling in mouse [31]. It has also been applied to the extraction of single steroids, showing

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improved recovery rates over SPE, such as for aldosterone [32,33]. SLE has recently been reported as a method of sample preparation prior to LC-MS/MS analysis of saliva and plasma for adrenal-specific androgens in 21-hydroxylase deficiency in the clinical setting [34].

Volumes of serum or plasma in previous LC-MS/MS steroid profiling methods range between 80 and 500 µL and the number and type of steroids included varies. For example, Methlie et al. used human serum (80 µL) to detect 7 endogenous and 3 synthetic steroids in Addison's disease [9]. In diagnosis of adrenocortical carcinoma Taylor et al. used human serum (200 µL) for quantification of 13 endogenous steroids, including pregnenolone and DHEA-S, but this method does not include A or DHT [10]. Schweitzer et al. recently reported use of a commercially available kit for the analysis of 13 steroids in plasma (500 µL) in adrenal tumour patients [34]. Foster et al., measured cortisol, cortisone, DHEA, androstenedione and testosterone in the serum of patients with major trauma from injury, both within 24 hours of the accident and 6 months post-accident [35].

In developing and validating this bioanalytical method we have carefully considered and balanced a number of analytical factors identified as important in the validation, recently discussed by Yu and Bashaw [44]. These include the source of the reference standards, where we have using certified reference materials where ever possible, optimised chromatographic separation of isobars and isotopologues, by use of a relatively long chromatographic run, while selecting and assessing quantitative and qualitative ions, for maximum selectivity. Comprehensive and systematic tuning of compound specific parameters for each steroid ensured the most sensitive and reliable quantitation possible. Matching isotopically labelled internal standards to track the steroids and ensure a sufficiently wide dynamic range for each steroid was also important.

The automated SLE method is fast and efficient allowing for batches of 80 biological samples to be processed within 90 minutes, with minimal intervention once aliquoted, eliminating contamination, handling error and freeing up technical time. SLE is more affordable and faster than SPE, as it has only a diluent and eluent stage, without the need for equilibration and washing. As a result, method development and automation of SLE offers economic and practical advantages, generally doubling the speed of sample preparation compared to manual and other extraction techniques such as SPE.

The 12 endogenous steroids have been profiled in female plasma, at baseline and following Synacthen® treatment, which enabled detection of intermediates in the steroidogenic pathway as well as elevation in steroid end points.

This method has scope for further improvement as a steroid profiling tool. Exploring chlorobutane as an alternative elution solvent may lead to an improvement in progesterone recovery [42]. Including more compounds in the steroid biosynthetic pathway is also desirable, such as estrogens, aldosterone and DHEAS, by modifying the mobile phase of the LC system with ammonium fluoride, which is reported to enhance negative ion formation, post separation [45]. Successfully enhancing negative ion formation would address ion suppression issues in low abundance steroids and result in a broader steroidogenic pathway method. This would enable steroid profiling and allow detailed investigations into disease conditions in populations.

This validated SLE-ESI-LC-MS/MS steroid profiling method illustrates the application of sample automation and supported liquid extraction of human plasma (200 µL) as a simple approach. The methodological approach has applicability to a number of clinically relevant conditions such as CAH and Addison's disease and owing to similarities in the steroidogenic pathway between species it can be adapted to measure steroids in rodent and avian samples. The method also includes the first reported measurement of the inactive glucocorticoid 11-dehydrocorticosterone in a steroid panel.

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4. Materials and Methods

4.1 Chemicals, reagents and consumables

Water (LC-MS grade), acetonitrile (LC-MS grade), methanol (LC-MS grade), dichloromethane (HPLC grade) and propan-2-ol (HPLC and LC-MS grade) were from VWR, Lutterworth, UK. Formic acid (LC-MS grade) and ammonium hydroxide (35%) were from Fisher Scientific, Loughborough, UK).

Certified reference materials (1 mg/mL in methanol or acetonitrile) for A4, T, DHT, P, 17-OHP, F, B were supplied by Cerilliant/Sigma-Aldrich, Dorset, UK, as were certified reference materials (100 µg/mL in methanol or acetonitrile) for isotopically labelled internal standards; 2,2,4,6,6,17α,21,21,21-[$^{2}H_{9}$]-progesterone (d9P4), 2,3,4-[$^{13}C_{3}$]-testosterone , 2,3,4-[$^{13}C_{3}$]-androstenedione ($^{13}C_{3}$-A4), 9,11,12,12-[$^{2}H_{4}$]-cortisol (d4F). 2,3,4-[$^{13}C_{3}$]-dihydrotestosterone ($^{13}C_{3}$-DHT) was from IsoSciences/QMX laboratories, Thaxted, Essex, UK. 2,2,4,6,6,17α,21,21-[$^{2}H_{8}$]-corticosterone (d8B) from CK Isotopes, Unthank, Leicestershire, UK. E and A were from Steraloids Inc, Newport, Rhode Island, USA. S, 11-DOC, 21-DF, pregnenolone (Preg), epi-testosterone (epi-T), 5α-dihydroandrostenedione (DHA), dehydroepiandrosterone (DHEA), 17-hydroxypregnenolone (17-OHPreg), Aldosterone (Aldo) were provided as powders from Sigma-Aldrich, UK. Reference standard solutions and powders were stored as directed by the manufacturers.

ISOLUTE®, SLE+ 400 µL Supported Liquid Extraction Plates were from Biotage (Uppsala, Sweden) and 2 mL collection plates from (Wilmslow, UK). Bulk pre-menopausal female EDTA plasma was supplied by Sera Laboratories International Ltd (West Sussex, UK).

4.2 Stock solutions and calibration standards

Unless bought as certified reference materials, standard solutions were prepared by dissolving a suitable amount of steroid powder in methanol (1 mg/mL). Further serial dilutions of the analytes were prepared in methanol. All solutions were stored in the dark, at -20ºC prior to use. Due to the presence of endogenous steroids in plasma, calibration standards were prepared by enriching water to achieve the required concentrations. Calibration standards were prepared over the range 0.0025 – 500 ng/mL. An endogenous human plasma quality control (QC) pool was prepared and analysed with each batch in duplicate. Nominal concentrations for the QC samples were assessed as 6 replicates prior to sample batch analysis.

4.3 Chromatographic and Mass Spectrometric conditions

Analysis was performed using a Shimadzu Nexera uHPLC (Shimadzu, Milton Keynes, UK) interfaced to a QTRAP 6500+ (Sciex, Warrington, UK) mass spectrometer. Instrument control and data acquisition were achieved using Sciex Analyst® 1.6.3 Software. Data were integrated using the Quantitate section of the Analyst software. The mass spectrometer was operated in positive ion electrospray ionisation (ESI) mode using a TurboIonSpray source and data were collected in unit resolution (0.7 *m/z* full width at half maximum). The TurboIonSpray source was operated at 600ºC with an IonSpray voltage of 5.5 kV, a Curtain Gas of 30 psi, nitrogen nebuliser ion source gas 1 (GS1) and heater ion source gas 2 (GS2) of 40 psi and 60 psi, respectively. Compound specific parameters were optimised for selected reaction monitoring (SRM) transitions by infusing 1 ng/mL or 10 ng/mL of each steroid standard solution into the source at 3 µL/min with a Collision Activation Dissociation (CAD) gas at the Medium setting of 2.6 x 10-5 Torr. The curtain, source, exhaust and CAD gas was delivered using an MS Table 1N Nitrogen and dry air generator Table (Peak Scientific, Scotland, UK).

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Chromatographic separation of steroidal isobars and isotopologues was assessed using a variety of column chemistries and different mobile phase systems, comparing acetonitrile with methanol and the effect of different modifiers such as ammonium acetate (AmAc 0.5 mM and 0.2 mM) and formic acid (0.1% v/v). Columns trialled included ACE Excel Super 2 C18; ACE Excel 2 C18-AR and C18-PFP (2.1 x 150 mm; 2 µm); Advanced Chromatography Technologies, Aberdeen, UK and Kinetex C18 column (3 x 100 mm; 2.6 µm particle size), (2.1 x 100 mm; 2.6 µm particle size) and (3 x 150 mm; 2.6 µm particle size), Phenomenex, UK, all fitted with a KrudKatcher Ultra In-Line Filter (0.5 µm porosity) Phenomenex, UK.

4.4 Sample collection and preparation

4.4.1 Plasma samples

Female human plasma samples were obtained from subjects participating in experimental medicine studies for which ethical approval had been obtained under the Ministry of Defence Research Ethics Committee, 790/MoDREC/16. All participants provided informed consent and was conducted in accordance with the Declaration of Helsinki. Non-pregnant females (n=54; 20-30 years) had blood sampled and plasma collected into EDTA tubes following a low dose Synacthen® test [35,36]. Samples were stored at -80C prior to analysis.

4.5 Sample Extraction

4.5.1 Automated Sample Extraction using a liquid handling robot

Plasma, calibration standards, quality control (QC) samples and blanks were dispensed manually as aliquots (200 µL) into individual wells of a 2mL 96 deep-well polypropylene plate (Waters, UK). An internal standard solution of all isotopically labelled standards ($^{13}C_3$-A4, $^{13}C_3$-T, $^{13}C_3$-DHT, d9P, d8B, d4F) in methanol was added (20µL; 10 ng) to each well except for the double blanks. The plate was agitated on a plate shaker (2 mins) and transferred to a Biotage® Extrahera™ automated sample processor (Biotage, Uppsala, Sweden) where ammonium hydroxide (200 µL, 0.5M) was added to each well. Samples were incubated at room temperature (18 22°C; 5 mins) and transferred to an SLE+ 400 plate by the robot, loaded onto the SLE material under positive pressure using compressed air. Following a wait (5 min) the analytes were eluted from the SLE material into a deep-well collection plate by positive pressure following the addition of dichloromethane/propan-2-ol (98:2; 4x 450 µL). The eluate was dried down under a stream of heated oxygen free nitrogen (OFN, 40°C) on an Argonaut SPE Dry™ Dual Sample Concentrator System (Biotage, Uppsala, Sweden). Once dry the extracts were reconstituted in water/methanol (70:30, 100 µL), the plate was sealed then shaken on a plate shaker (10 mins) before injecting directly from the 96-well plate for LC-MS/MS analysis.

4.6 Assay validation

The analytical validation of the method was assessed in terms of precision, accuracy, selectivity, sensitivity, and reproducibility of the measurement of each steroid, following recommendations of the Bioanalytical Method Validation (BMV) protocol of the Federal Drug Administration (FDA) (UCM070107)[38] and the European Medicines Agency[39] (EMA/CHMP/ICH/172948/2019, published 14/03/2019).

4.6.1 Extraction efficiency

The extraction efficiency of endogenous analytes was calculated from the recovery of stable isotope standards after enriching the plasma. A mixture of isotopically labelled internal standards (5 ng) was used to enrich plasma extracts, prior to extraction by SLE400 and the mean peak areas was compared to plasma that was extracted through the SLE400, and enriched after extraction (post-

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spiked, 20 ◎L; 1 ng/mL). Mean peak areas (n=6) of steroids following extraction were divided by those in post-spiked samples (n=6) and expressed as a percentage.

4.6.2 Assessment of matrix effects

Matrix effects of extracts of plasma on steroids was evaluated by comparing signal intensity of IS (5 ng) post-spiked into extracted plasma with that of aqueous IS solutions of the same concentration (n=6). Mean peak areas of steroids following extraction of steroids from post-spiked plasma were divided by mean peak areas of unextracted standards and expressed as a percentage.

4.6.3 Specificity for isobaric steroids

SRM chromatograms were inspected close to the retention times of steroids for possible interferences by other endogenous and common exogenous compounds in plasma. Known endogenous isobars were assessed for interference at anticipated retention times such as DHEA and DHA and epiT. The ratio of quantifier to qualifier ions of each steroid was measured in plasma and compared with those of standards and only accepted if within Relative Standard Deviation (RSD, (standard deviation/mean x 100)) 20%.

4.6.4 Limit of detection (LOD) and quantitation (LOQ)

LODs, LLOQs and ULOQs for all steroids were determined in water. The Signal/Noise (SNR) was calculated from peak areas of steroids and adjacent background noise, with matched time intervals. The LOD was assigned as a SNR ~3. The LLOQ and ULOQ were calculated as the concentration affording precision and accuracy of 15% or less following inter-day assessment of replicate aliquots (n=6).

4.6.5 Linearity of quantitation

Blank samples (IS only) and aliquots containing steroid analytes and internal standards were analysed by LC-MS/MS. Calibration curves were plotted as the peak area ratio (standard/IS) versus amount of steroid. Calibration lines of best fit were acceptable if the regression coefficient, r, was >0.99. Expected clinical ranges were consulted to ensure the reliable range of quantitation matched requirements. Weightings of 1, 1/x and 1/x2 were compared and the approach selected which offered the best data in relation to accuracy, with particular attention paid to the low range.

4.6.6 Accuracy and precision of the method

Aliquots of test plasma (200 µL) were extracted 6 times on the same day and 6 times on different days. The inter-assay precision and accuracy of the samples were assessed, prepared on different days, alongside a standard curve (n=6). The precision of these measurements was calculated as the RSD, and % accuracy was the bias ((measured value - theoretical value)/theoretical value x 100).

4.7 Method Application for steroid panel analysis following Synacthen® treatment

54 women (20-30 years), had blood sampled and plasma collected during a low-dose dynamic Synacthen® test [36]. A cannula was inserted into an antecubital or dorsal hand vein and a baseline blood sample (t=0) was obtained. After 10–15 min, 1 µg ACTH-(1-24) (tetracosactrin acetate as Synacthen®, Mallinckrodt, Dublin, Ireland) was injected followed by a 10 mL saline flush. Venous blood was sampled through the cannula 20, 30, 40 and 60 min after drug administration. The dose of ACTH-(1-24) was chosen to provide near-physiological levels of adrenal stimulation, as opposed to the 250 µg stimulation test, which is intended to induce maximal stimulation of the adrenal cortex to exclude adrenal insufficiency [37]. Eisenhofer *et al*. reported that plasma steroid levels measured by LC-MS/MS are consistently lower in women than men, with the exception of

progesterone [25]. As a result this group of subjects was deemed a suitable assessment of the sensitivity of the method. In addition, females tend to have more responsive adrenals, responding more briskly to ACTH stimulation, with a good likelihood of detecting intermediates in the steroid biosynthetic pathway during the time course.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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