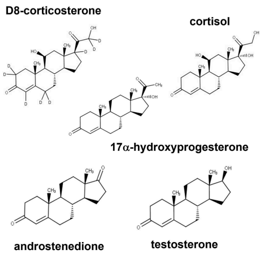
Feb 28, 2024 Version 2

LC-MS/MS analysis of 5 steroids in plasma in a clinical study of Congenital Adrenal Hyperplasia V.2



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Catriona Kyle[1,2], George Just[3], Scott G Denham[3], Natalie ZM Homer[1,3]

[1]University of Edinburgh; [2]NHS Lothian;
[3]Mass Spectrometry Core, Edinburgh Clinical Research Facility, University of Edinburgh

Clinical Mass Spectrom...

Natalie ZM Homer
University of Edinburgh

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We use this protocol and it's working

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Abstract

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders that affects adrenal steroidogenesis, resulting in deficiency of the glucocorticoid cortisol and in many cases the mineralocorticoid aldosterone[1]. This resulting lack of glucocorticoid (and mineralocorticoid) activates the hypothalamic-pituitary-adrenal (HPA) axis causing excessive release of adrenocorticotrophic hormone (ACTH) and excess adrenal androgen synthesis.

Diagnosing and treating CAH requires reliable methods for steroid analysis. Tandem mass spectrometry methods coupled with chromatographic separation are considered the gold standard analytical technique for steroid analysis[2] with the added benefit of enabling simultaneous analysis of multiple steroids. There are a range of methods that have been developed to measure multiple steroids in CAH[3]. Here we have developed a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for application to a clinical study that specifically explores the administration of d8-corticosterone as an alternative to hydrocortisone for CAH treatment.

Plasma samples (200 µL) were enriched with isotopically labelled steroids, diluted with water (0.1% formic acid v/v) and extracted alongside a (0.0025 - 400 ng) calibration curve, by automated 96-well supported liquid extraction (SLE), using dichloromethane and isopropanol as an organic solvent, on a Biotage Extrahera automated sample handler. Extracted steroids were separated on a Shimadzu Nexera uHPLC with gradient elution on a Kinetex C18 column (150 × 3 mm; 2.6 µm) and a mobile phase of methanol and water (0.1% formic acid in water and methanol). The run time was 16 minutes, followed by mass spectral analysis on an AB Sciex 6500+ tandem quadrupole mass spectrometer operated in multiple reaction mode, positive ionisation. The method measures five steroids - hydrocortisone (cortisol) and d8-corticosterone - combined with markers of CAH - androgens and the intermediate 17α-hydroxyprogesterone, alongside the internal standards - in plasma. Validation demonstrated that this method is sensitive, specific, and reliable.

Attachments

XLSX

ROCC STUDY PLATE MA...

20KB

Guidelines

Ensure all training is up-to-date for operating the necessary laboratory instrumentation and equipment.

Materials

Consumables Table

	A	B	C	D
	Item	**Supplier**	**Part no.**	**Quantity**
	1.75 mL glass vials with lids	Scientific Laboratory Supplies Ltd	TUB1200	10
	7 mL glass vials with lids	Scientific Laboratory Supplies Ltd	TUB1220	5
	Isolute SLE+ 400 96 well plate	Biotage	820-0400-P01	1
	96-well plate sealing film	VWR	391-1250	1
	Adhesive Plate Seal	Waters	186006336	1
	Kinetex C18 (150 × 3 mm; 2.6 um)	Phenomenex	00F-4462-Y0	1
	Kinetex KrudKatcher, 0.5 um	Phenomenex	AFO-8497	1
	Deep well 96 well collection plate	Biotage	121-5203	1
	Deep well (2 mL) 96 well collection plate	Waters	186002482	1

Consumables for homogenisation of bone marrow and steroid extraction by supported liquid extraction (SLE)

Chemicals and Analytical Standards Table

	A	B	C
	Item	**Supplier**	**Article no.**
	Water (HPLC grade)	Fisher Scientific	C-10449380-X
	Acetonitrile (LC-MS grade)	VWR	83640.320
	Methanol (LC-MS grade)	VWR	83638.320
	Water (LC-MS grade)	VWR	83645.320

	A	B	C
	Isopropanol (HPLC grade)	VWR	20880.320
	Dichloromethane (HPLC grade)	Fisher Scientific	C-23373320-X
	Cortisol	Sigma-Aldrich/Cerilliant	(C-106) 1 mg/mL in methanol (certified)
	Androstenedione	Steraloids	(A-075) 1 mg/mL in acetonitrile (certified)
	Testosterone	Sigma-Aldrich/Cerilliant	(T-037) 1 mg/mL in acetonitrile (certified)
	17a-hydroxyprogesterone	Sigma-Aldrich/Cerilliant	(P-069) 1 mg/mL in acetonitrile (certified)
	D8-Corticosterone	Cambridge Isotope Laboratories/CK Isotopes	(DLM-7347) Supplied as powder
	13C3-testosterone	Sigma-Aldrich/Cerilliant	(T-070) 100 ug/mL in acetonitrile (certified)
	13C3-androstenedione	Sigma-Aldrich/Cerilliant	(A-084) 100 ug/mL in acetonitrile (certified)
	d9-progesterone	Sigma-Aldrich/Cerilliant	P-070 100 ug/mL in acetonitrile
	Formic acid (LC-MS grade)	Fisher Scientific	10596814

Chemicals and analytical standards

Solutions Required

- 0.1% formic acid (aq) (200 mL) Make up to 200 mL with Water (HPLC grade). Mix thoroughly.
- 98:2 Dichloromethane:Isopropanol (1 L) - Add 20 mL Isopropanol (HPLC grade) to 980 mL Dichloromethane (HPLC grade). Mix thoroughly.
- Methanol (HPLC grade): for preparation of calibration standard/internal standard dilutions.
- Water (HPLC grade): for preparation of calibration standards.

- 70:30 Water:Methanol (100 mL) - Add 30 mL methanol (LC-MS grade) to 70 mL water (LC-MS grade). Mix thoroughly.

Equipment Table

	A	B	C
	Item	**Model**	**Supplier**
	2 x Liquid Chromatography Pumps	LC30AD	Shimadzu
	Autosampler	SIL-30ACMP	Shimadzu
	Column oven , Nexera X2	CTO-20AC	Shimadzu
	QTrap 6500+ mass spectrometer	5038125-J	AB Sciex
	Gilson Repetman	Gilson Repetman	Gilson
	Deepwell plate thermoshaker	TS-DW	Grant Scientific
	Liquid handling robot	Extrahera	Biotage, Sweden
	SPE Dry 96 dual evaporator	SPE Dry	Biotage, Sweden

Equipment required for homogenisation, extraction and steroid analysis

Troubleshooting

Safety warnings

Ensure risk assessments are up to date and that all local laboratory guidelines are followed for handling chemicals and biological samples

Ethics statement

All human studies were approved by the University of Edinburgh NHS Lothian ACCORD Ethical Review Board.

Before start

Ensure all consumables are in stock and all compounds and reagents are freshly prepared

Preparation of human plasma for extraction

1 Remove human plasma samples from the freezer and defrost on ice.

Preparation of calibration standard stock solutions

2 Prepare 1 mg/mL stock solution of d8-corticosterone (d8B) from powder then prepare separate 100 µg/mL stock solutions of each steroid - d8B, cortisol (F), 17a-hydroxyprogesterone (17OHP4), testosterone (T) and androstenedione (A4) in methanol.

2.1 Prepare a mixed stock of the 5 steroids - D8B, F, 17OHP4, T, A4 - by using 100 µg/mL stock solutions. Do this by adding 50 µL x 100 µg/mL D8B, 50 µL x 100 µg/mL F, 50 µL x 100 µg/mL 17OHP4, 50 µL x 100 µg/mL T and 50 µL x 100 µg/mL A4 + 750 µL methanol to give a **5 µg/mL stock**.

2.2 Dilute the **5 µg/mL stock** Mixed STOCK by 1:10 dilution (100 µL x 5 µg/mL + 900 µL methanol) to give **500 ng/mL stock**

2.3 Dilute the **500 ng/mL** mixed STOCK by 1:10 dilution (100 µL x 500 ng/mL + 900 µL methanol) to give **50 ng/mL stock**

2.4 Dilute the **50 ng/mL** mixed STOCK by 1:10 dilution (100 µL x 5 µg/mL + 900 µL methanol) to give **5 ng/mL stock**

2.5 Dilute the **5 ng/mL** Mixed STOCK by 1:10 dilution (100 µL x 5 µg/mL + 900 µL methanol) to give **500 pg/mL stock**

2.6 Dilute the **500 pg/mL** Mixed STOCK by 1:10 dilution (100 µL x 5 µg/mL + 900 µL methanol) to give **50 pg/mL stock** go to step #3

Preparation of internal standard solution

3 Prepare **100 µg/mL solutions** of each isotopically labelled internal standard (d4-cortisol, $^{13}C_3$-testosterone, $^{13}C_3$-androstenedione and d9-progesterone) in methanol.

3.1 Prepare a **mixed 5 µg/mL** Internal Standard mix stock solution of the three isotopically labelled steroids by adding 25 µL x 100 µg/mL d4-cortisol, 25 µL x 100 µg/mL

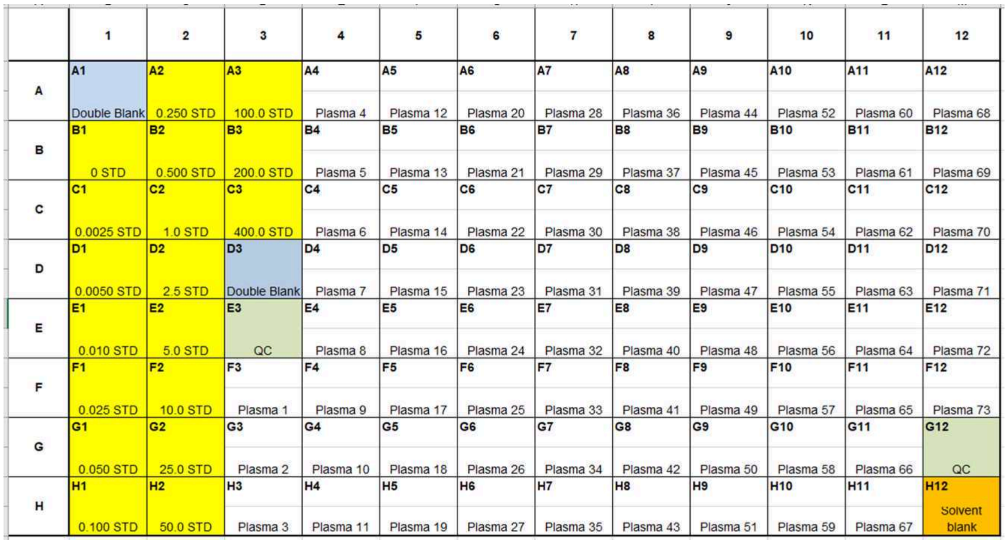
$^{13}C_3$-testosterone and 25 µL x 100 µg/mL $^{13}C_3$-androstenedione and 25 µL x 100 µg/mL d9-progesterone to 425 µL methanol.

3.2 Prepare a **5 ng/mL Working Internal Standard** solution by taking 10 µL x 5 µg/mL Int Std Mix + 1990 µL methanol.

Set up of supported liquid extraction of steroids from calibration standards and samples

10m

4 Label a 2 mL deep well 96-well collection plate (Biotage). Label a Supported Liquid Extraction SLE400 plate with batch details. Label a 2 mL deep well 96-well collection plate (Waters).
Design and prepare batch of standards and samples in Microsoft Excel template (see Files), following a column-wise plate map design as below (Table S1).



	1	2	3	4	5	6	7	8	9	10	11	12
A	A1 Double Blank	A2 0.250 STD	A3 100.0 STD	A4 Plasma 4	A5 Plasma 12	A6 Plasma 20	A7 Plasma 28	A8 Plasma 36	A9 Plasma 44	A10 Plasma 52	A11 Plasma 60	A12 Plasma 68
B	B1 0 STD	B2 0.500 STD	B3 200.0 STD	B4 Plasma 5	B5 Plasma 13	B6 Plasma 21	B7 Plasma 29	B8 Plasma 37	B9 Plasma 45	B10 Plasma 53	B11 Plasma 61	B12 Plasma 69
C	C1 0.0025 STD	C2 1.0 STD	C3 400.0 STD	C4 Plasma 6	C5 Plasma 14	C6 Plasma 22	C7 Plasma 30	C8 Plasma 38	C9 Plasma 46	C10 Plasma 54	C11 Plasma 62	C12 Plasma 70
D	D1 0.0050 STD	D2 2.5 STD	D3 Double Blank	D4 Plasma 7	D5 Plasma 15	D6 Plasma 23	D7 Plasma 31	D8 Plasma 39	D9 Plasma 47	D10 Plasma 55	D11 Plasma 63	D12 Plasma 71
E	E1 0.010 STD	E2 5.0 STD	E3 QC	E4 Plasma 8	E5 Plasma 16	E6 Plasma 24	E7 Plasma 32	E8 Plasma 40	E9 Plasma 48	E10 Plasma 56	E11 Plasma 64	E12 Plasma 72
F	F1 0.025 STD	F2 10.0 STD	F3 Plasma 1	F4 Plasma 9	F5 Plasma 17	F6 Plasma 25	F7 Plasma 33	F8 Plasma 41	F9 Plasma 49	F10 Plasma 57	F11 Plasma 65	F12 Plasma 73
G	G1 0.050 STD	G2 25.0 STD	G3 Plasma 2	G4 Plasma 10	G5 Plasma 18	G6 Plasma 26	G7 Plasma 34	G8 Plasma 42	G9 Plasma 50	G10 Plasma 58	G11 Plasma 66	G12 QC
H	H1 0.100 STD	H2 50.0 STD	H3 Plasma 3	H4 Plasma 11	H5 Plasma 19	H6 Plasma 27	H7 Plasma 35	H8 Plasma 43	H9 Plasma 51	H10 Plasma 59	H11 Plasma 67	H12 Solvent blank

Table S1 - Plate Map - Column-wise plate layout for automated Supported Liquid Extraction on an Extrahera liquid handling robot (Biotage, Sweden)

Preparation of calibration standard curve and samples

5 Prepare calibration standards directly into the **96-well deep well plate** using Table S2 below, for volumes of each stock concentration, into a final volume of 200 µL water.

	A	B	C	D
	Standard name	**Amount (ng)**	**STD Mix Vol (uL)**	Vol water (uL)
	0 STD	0	0	200
	0.00250 STD	0.00250	5 uL x 500 pg/mL	195
	0.00500 STD	0.00500	10 uL x 500 pg/mL	190
	0.01000 STD	0.0100	20 uL x 500 pg/mL	180
	0.0250 STD	0.0250	5 uL x 5 ng/mL	195
	0.0500 STD	0.0500	10 uL x 5 ng/mL	190
	0.100 STD	0.100	20 uL x 5 ng/mL	180
	0.250 STD	0.250	5 uL x 50 ng/mL	195
	0.500 STD	0.500	10 uL x 50 ng/mL	190
	1.00 STD	1.00	20 uL x 50 ng/mL	180
	2.50 STD	2.50	5 uL x 500 ng/mL	195
	5.00 STD	5.00	10 uL x 500 ng/mL	190
	10.0 STD	10.0	20 uL x 500 ng/mL	180
	25.0 STD	25.0	5 uL x 5 ug/mL	195
	50.0 STD	50.0	10 uL x 5 ug/mL	190
	100 STD	100.0	20 uL x 5 ug/mL	180
	200 STD	250	5 uL x 50 ug/mL	195
	400 STD	400.0	8 uL x 50 ug/mL	192

Table S2 - Calibration standard preparation table

5.1 Aliquot 200 µL plasma sample into the correct well according to the plate map design.

Supported liquid extraction of steroids from calibration standards and samples 10m

6 Using a multi-step pipette enrich the plate containing calibration standards with WIS by adding 20 µL **x 5 ng/mL Working Internal Standard** into each calibration standard, including 0 std and each sample (human plasma), except for the double blank and solvent blank.

7 Using the Extrahera liquid handling robot, set up with the batch labelled SLE400 extraction plate and the deep well extraction plate, containing the calibration standards and samples. Programme Extrahera to aliquot 200 µL 0.1% formic acid in water (v/v) into each well of the 96-well deep well plate containing the samples and standards.

8 Programme the Extrahera to transfer 400 µL of liquid from each well (containing sample and the diluent, into a 400 µL volume Supported Liquid Extraction plate (SLE400), pre-placed into the deck on the Extrahera, with a deep well Waters 2 mL deep well collection plate below, pre-labelled with the batch details and date of extraction.

9 Allow the diluted sample to adsorb onto the SLE extraction bed for 00:05:00 before eluting with 600 µL x 98:2 (v/v) dichloromethane/isopropanol and repeating twice more, each time collecting the eluent into the collection plate 5m

10 Dry down the eluent collected into the 2 mL collection plate using the SPE Dry down for 96-well plates under nitrogen.

11 Resuspend in 100 µL x 70:30 water/methanol, seal the plate with a zone-free plate seal and shake on ThermoShaker for 00:05:00 at 300 rpm 5m

12 Place the plate in the autosampler for LC-MS/MS or store at -20 °C until ready for analysis.

Steroid analysis by LC-MS/MS 16m

13 Set up an acquisition batch in Analyst software using the electronic excel file of the calibration standards and sample list. Set to inject 10 µL per sample and use a method of chromatographic separation as described in step 16 and 17 and mass spectrometer settings as outlined in steps 18 and 19.

14 Set up the Shimadzu Nexera X2 liquid chromatography system and fit with a Phenomenex Krud Katcher and a Phenomenex 150 × 3 mm; 2.6 µm Kinetex C18 liquid chromatography column, using mobile phase A - water with 0.1% formic acid and mobile phase B - methanol with 0.1% formic acid at 0.5 mL/min and 40 °C diverting to the mass spectrometer at 0.2 mins and

15 Set up chromatographic gradient as below (Table S3) with a run time of 00:16:00 per sample 16m

	A	B	C	D
	Time (min)	**Flow (mL/min)**	**A (%)**	**B (%)**
	Initial	0.5	45	55
	4.00	0.5	45	55
	10.00	0.5	0	100
	12.00	0.5	0	100
	12.10	0.5	45	55
	16.00	0.5	45	55

Table S3 - Chromatographic gradient details. A - water w/ 0.1% formic acid; B - methanol w/ 0.1% formic acid. 40oC. Kinetex C18 (150 x 3 mm; 2.6 µm)

16 Set up the mass spectrometer for Multiple Reaction Monitoring (MRM) method in positive mode, with electrospray ionisation as below (Table S4), with divert of LC flow into the mass spectrometer set at 1 minute and 12 minutes.

A	B
Instrument	Sciex QTrap 6500+
Source, Ionisation Mode	IonDrive Turbo V Source, ESI
Scan Mode, Polarity	MRM, Positive
Resolution (Q1/Q3)	unit/unit
Mass range	Low mass
Pause Time	5.007 ms
Acquisition time	16.0 min
Delay time	0 sec
Curtain Gas (CUR) (N2)	30 units
Collision Gas (CAD) (N2)	Medium
IonSpray Voltage (IS) (Positive)	5500 V
Temperature (TEM)	600 °C
Ion Source Gas 1 (GS1) (Air)	40 units
Ion Source Gas 2 (GS2) (Air)	60 units
Entrance Potential (EP) (Positive)	10 V
Probe position (x – axis)	5
Probe position (y – axis)	2

Table S4 - Mass Spectrometry source settings for positive ion electrospray ionsiation on QTrap 6500+ mass spectrometer

17 Set up the mass spectrometer to monitor for the following multiple reaction monitoring (MRM) transitions for each steroid and each isotopically labelled steroid (Table S5).

	A	B	C	D	E	F	G
	Q1 Mass (Da)	**Q3 Mass (Da)**	**Scan time (msec)**	**Steroid Name**	**DP (V)**	**CE (V)**	**CXP (V)**
	287.1	97.0	10	Androstenedione 1	61	27	14
	287.1	78.9	10	Androstenedione 2	61	67	10
	363.1	121.2	10	Cortisol 1	66	31	12
	363.1	91.0	10	Cortisol 2	76	83	10
	289.1	97.0	10	Testosterone 1	101	29	12
	289.1	109.2	10	Testosterone 2	101	31	6
	333.1	109.1	10	17a-hydroxyprogesterone 1	66	31	12
	333.1	96.9	10	17a-hydroxyprogesterone 2	66	29	12
	355.3	128.1	10	d8B-Corticosterone 1	37	45	14
	355.3	125.0	10	d8B-Corticosterone 2	29	56	14
	292.1	100.0	10	13C3-Testosterone	96	29	12
	290.2	100.1	10	13C3-Androstenedione	31	27	12
	324.1	100.0	10	d9-progesterone	151	31	15

	A	B	C	D	E	F	G
	367.2	121.1	10	d4-cortisol	80	29	16

Table S5 - Multiple reaction monitoring (MRM) settings for each steroid, including quantitative (1) and qualitative (2) ions for each steroid. DP - declustering potential, CE - collision energy, CXP - collision exit potential

18 Check the retention times of the steroids are as expected, as shown in the chromatogram in Fig S1:

Expected result

Retention times; cortisol at **4.0 mins**, d8-corticosterone at **6.1 mins,** androstenedione at **7.3 mins,** testosterone at **7.8 mins** and 17a-hydroxyprogesterone at **8.1 mins**

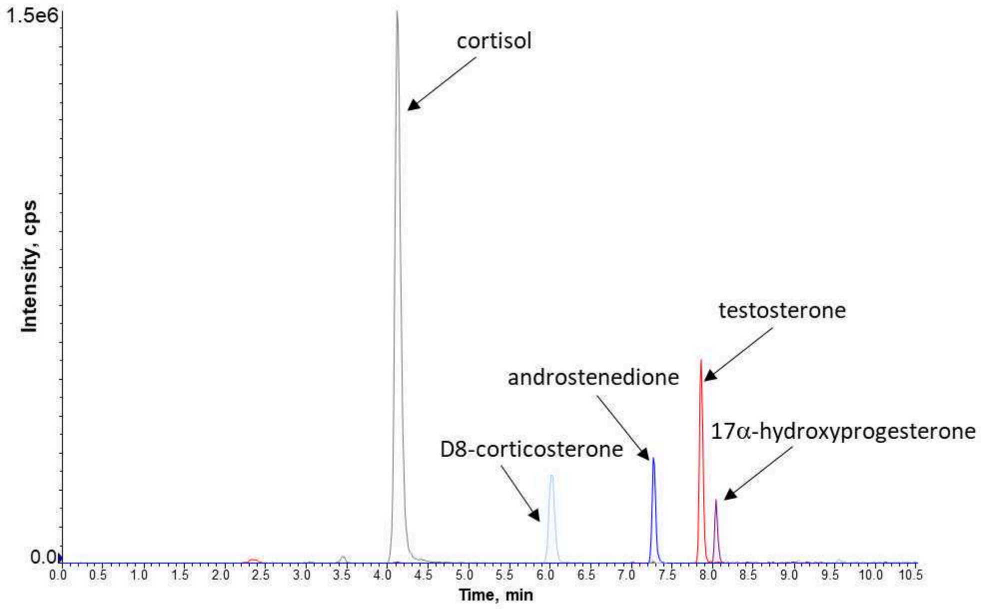


Figure S1 - Overlaid Ion Chromatogram of MRM transitions for cortisol, D8-corticosterone, androstenedione, testosterone and 17a-hydroxyprogesterone, separated by gradient on a Kinetex C18 (150 x 3 mm; 2.6 um) at 0.5 mL/min with mobile phase 0.1% formic acid in water and methanol

19 Inject a mid-level standard. Check the chromatography and each steroid retention time is consistent with expected times and peak area response is as expected. Once satisfied

then set the batch of samples to analyse, injecting 10 µL per sample.

Method specific data evaluation of LC-MS/MS data

20 Use the data analysis parameters to assess the peak area of the chromatograms for the Steroid analytes and their nominated internal standards (Table S6)

	A	B	C	D
	Steroid Name	**Abbreviation**	**Retention Time (min)**	**Internal Standard**
	Cortisol	F	4.0	d4F
	d8-Corticosterone	d8B	6.1	d4F
	Androstenedione	A4	7.3	13C3A4
	Testosterone	T	7.8	13C3T
	17a-hydroxyprogesterone	17OHP	8.1	d9P4
	Internal Standards			
	d4-cortisol	d4F	3.9	Int Std
	13C3-Androstenedione	13C3A4	7.3	Int Std
	13C3-Testosterone	13C3T	7.8	Int Std
	d9-progesterone	d9P4	9.0	Int Std

Table S6 - Method specific parameters of retention time and specific internal standard of the steroids

21 Use MultiQuant software and Microsoft Excel to evaluate the LC-MS/MS data, by defining calibration standard levels, ensuring accuracy of the calibration standards and linear regression > 0.99. Use the Table above, to calculate the concentration of steroids in each sample, as detailed in the protocol below. Remember to account for the volume of sample extracted and express as ng/mL.

Protocol

NAME

Using MultiQuant and Excel software to evaluate and report multi-analyte targeted LC-MS/MS data

CREATED BY

Natalie ZM Homer

Preview

Protocol references

1 - Claahsen-van der Grinten HL, Speiser PW, Ahmed SF, Arlt W, Auchus RJ, Falhammar H, Fluck CE, Guasti L, Huebner A, Kortmann BBM, Krone N, Merke DP, Miller WL, Nordenstrom A, Reisch N, Sandberg DE, Stikkelbroeck N, Touraine P, Utari A, Wudy SA & White PC. Congenital Adrenal Hyperplasia-Current Insights in Pathophysiology, Diagnostics, and Management. *Endocr Rev* 2022 **43** 91-159.

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