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Protocol status: Working

We use this protocol and it's working

Extraction and LC-MS/MS analysis of four steroids from mouse plasma and bone marrow

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Clinical Mass Spectrometry



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ABSTRACT

Steroid hormones, including progesterone, testosterone and corticosterone, play a critical role in

growth, development, reproductive function and sexual differentiation. Steroid production is controlled in the adrenals via the hypothalamic-pituitary-adrenal axis and in the gonads by the hypothalamic-pituitary-gonadal axis, but steroids are also produced and metabolised in peripheral tissues such as adipose tissue. Circulating steroids (blood levels) do not always reflect local steroid tissue levels. In studies of caloric restriction there are observed changes in steroids and there is interest in the role that bone marrow adipose tissue plays¹. Thus, to understand mechanisms of caloric restriction it is valuable to measure tissue levels of steroids as well as circulating levels of steroids.

Immunoassays can be used to measure steroids but they lack specificity, limited to one or two steroids, and suffer from cross-reactivity at low concentrations. Tandem mass spectrometry methods coupled with chromatographic separation are considered the gold standard analytical technique for steroid analysis, such as LC-MS/MS, with the added benefit of enabling simultaneous analysis of multiple steroids.

Here we have improved upon existing murine steroid liquid chromatography tandem mass spectrometry (LC-MS/MS) methods² for the quantitation of four steroids in small samples to investigate the role of steroids in caloric restriction - specifically corticosterone, 11-dehydrocorticosterone, testosterone and progesterone - in bone marrow and plasma. Bone marrow is homogenised and then homogenate samples and plasma samples (~50 µL) were extracted by automated 96-well supported liquid extraction (SLE), using dichloromethane and isopropanol as an organic solvent, carried out 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 x 3 mm; 2.6 µm) and a mobile phase of methanol and water (0.1% formic acid). The run time was 16 minutes, followed by mass spectral analysis on an AB Sciex 6500+ tandem quadrupole mass spectrometer operated in positive ionisation mode.

This automated SLE-LC-MS/MS method has been used to analyse 4 steroids - corticosterone, progesterone and testosterone - in mouse plasma and bone marrow. Validation demonstrates that this method is sensitive, specific, and suitable for steroid measurement in mouse bone marrow (4-8 bones) and a low volume of mouse plasma (50 µL), enabling investigation into tissue specific steroid levels and corresponding assessment of circulating steroid levels.

ATTACHMENTS

EXAMPLE_PLATE
MAP_NZMH.xlsx

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
0.5 mL Microtube plastic	Eppendorf		50
2 mL Microcentrifuge SafeLock Tubes	FisherBrand	05-408-138	50
Stainless Steel bead	Qiagen	69989	50
Glass culture tubes - 13 x 100 mm	Sarstedt	72.690.001	96
Isolute SLE+ 400 96 well plate	Biotage	820-0400-P01	1
2 mL deep well 96 well collection plate	Waters	186002482	1
96-well plate sealing film	VWR	391-1250	1
Adhesive Plate Seal	Waters	186006336	1
Kinetex C18 (150 x 3 mm; 2.6 um)	Phenomenex		1
Kinetex KrudKatcher, 0.5 um	Phenomenex	AFO-8497	1
Deep well 96 well collection plate	Biotage	121-5203	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
Isopropanol	VWR	20880.320
Dichloromethane	Fisher Scientific	C-23373320-X
Corticosterone	Sigma-Aldrich/Cerilliant	C-107
11-dehydrocorticosterone	Steraloids	CA3690-000, powder
Testosterone	Sigma-Aldrich/Cerilliant	(T-037) 1 mg/mL in acetonitrile (certified)
Progesterone	Sigma-Aldrich/Cerilliant	(P-069) 1 mg/mL in acetonitrile (certified)
D8-Corticosterone	Sigma-Aldrich/Cerilliant	C-159
13C3-testosterone	Sigma-Aldrich/Cerilliant	(T-070) 100 ug/mL in acetonitrile (certified)
D9-progesterone	Sigma-Aldrich/Cerilliant	P-070 100 ug/mL in acetonitrile
Formic acid	Fisher Scientific	10596814
Ammonium hydroxide	Sigma-Aldrich	221228

Chemicals and analytical standards

Solutions Required

- 0.5 M ammonium hydroxide (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
Dri-block	DB.3A	Techne
Microtube centrifuge	Heraeus Freso 21 D- 37520 LR56495	Sigma
TissueLyser II for cell lysis	II	Qiagen
Liquid Chromatography Pump	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
Analytical Balance	PS-100	Fisher Scientific
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

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 mouse studies were approved by the University of Edinburgh Animal Welfare and Ethical Review Board and were done under project licenses granted by the UK Home Office.

BEFORE START INSTRUCTIONS

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

Bone Marrow Collection and Homogenisation

1h 41m 30s

- 1 Prepare 0.5 mL Eppendorf microtubes for all bone marrow samples by removing the bottom of a 0.5 mL microtube (Eppendorf) with a razor blade
- 2 Batch sizes of 20-40 bone marrow samples are most manageable. Isolate bone marrow (BM) from frozen (or fresh) tibia by doing the following:
 - 2.1 Remove tibiae from freezer and transfer frozen tibiae from dry ice onto wet ice prior to cutting.
 - 2.2 Use a razor blade to cut off the proximal and distal ends of each tibia
 - 2.3 Place each cut tibia into a cut-off 0.5 mL microtube, to keep the bone upright, and add this tibia in a 0.5 mL microtube into a 2 mL microtube.
 - 2.4 Centrifuge the 2 mL cap-lock microtube and its contents in a microcentrifuge for 1m 8000 rcf, 4°C, 00:01:00 . The BM pellet will collect at the base of the 2 mL microtube
 - 2.5 Record the mass of each BM pellet, using an analytical balance, prior to homogenisation. Multiple BM pellets can be combined at this point. (e.g. 2, 4 , 6 or 8 BM pellets can be homogenised together).

- 3** Add  500 µL acetonitrile w/ 0.01% formic acid to the bone marrow in the 2 mL microtube 30s
- 3.1** Add a 5 mm stainless steel metal bead into a 2 mL microtube, cap securely and homogenise in the Qiagen bead mill homogeniser at 30 Hz for  00:00:30 30s
- 3.2** Remove and transfer the homogenisation tube containing the homogenate to the freezer for  01:00:00 at  -20 °C 1h
- 3.3** Remove the homogenate tube from the freezer, centrifuge for  00:05:00 at 16,100 rcf 5m
- 3.4** Transfer the homogenate to a clean, labelled glass vial using a glass pipette
- 3.5** Add another aliquot of  500 µL acetonitrile w/ 0.01% formic acid to the bone marrow pellet, vortex and centrifuge for  00:05:00 at 16,100 rcf 5m
- 3.6** Transfer the supernatant from the homogenisation tube and add it to the first supernatant in the glass tube.
- 3.7** Reduced the supernatant to dryness under nitrogen at  40 °C 30m

- 3.8** Resuspend the dried down homogenate supernatant of  Sample by adding  200 µL water prior to preparation of calibration standards (4), working internal standard (5) and then supported liquid extraction (See point 7) of steroids

Preparation of mouse plasma for extraction

- 4** Remove mouse plasma samples from the freezer and defrost on ice. Label up glass culture tubes with mouse sample IDs and aliquot  50 µL of each  Sample into labelled glass tubes, alongside bone marrow. Record exact volume of plasma aliquoted in plate map.

Preparation of calibration standard solutions

- 5** Prepare 100 µg/mL stock solutions of each steroid - corticosterone (B), 11-dehydrocorticosterone (A), testosterone (T) and progesterone (P4) in 1.75 m
- 5.1** Prepare a mixed stock of the 4 steroids - B, A, T, P4 - by using 100 µg/mL stock solutions. Do this by adding 50 µL x 100 µg/mL B, 50 µL x 100 µg/mL A, 50 µL x 100 µg/mL T and 50 µL x 100 µg/mL P4 + 800 µL methanol to give a **5 µg/mL stock**.
- 5.2** 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**
- 5.3** 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**
- 5.4** Dilute the **50 ng/mL** mixed STOCK by 1:10 dilution (100 µL x 5 ng/mL + 900 µL methanol) to give **5 ng/mL stock**

5.5 Dilute the **5 ng/mL** Mixed STOCK by 1:10 dilution ($100 \mu\text{L} \times 5 \mu\text{g/mL} + 900 \mu\text{L}$ methanol) to give **500 pg/mL stock**

5.6 Dilute the **500 pg/mL** Mixed STOCK by 1:10 dilution ($\Delta 100 \mu\text{L} \times 5 \mu\text{g/mL} + \Delta 900 \mu\text{L}$ methanol) to give **50 pg/mL stock** \Rightarrow go to step #6

Preparation of internal standard solution

6 Prepare **100 µg/mL solutions** of each isotopically labelled internal standard (d8-corticosterone, $^{13}\text{C}_3$ -testosterone and d9-progesterone) in methanol.

6.1 Prepare a **mixed 5 µg/mL Internal Standard mix stock solution** of the three isotopically labelled steroids by adding $\Delta 25 \mu\text{L} \times 100 \mu\text{g/mL}$ d8-corticosterone, $\Delta 25 \mu\text{L} \times 100 \mu\text{g/mL}$ $^{13}\text{C}_3$ -testosterone and $\Delta 25 \mu\text{L} \times 100 \mu\text{g/mL}$ d9-progesterone to $\Delta 425 \mu\text{L}$ methanol.

6.2 Prepare a **5 ng/mL Working Internal Standard** solution by taking $10 \mu\text{L} \times 5 \mu\text{g/mL}$ Int Std Mix + 1990 μL methanol.

Preparation of calibration standards

7 Prepare calibration standards directly into **labelled glass culture tubes** using the following table 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

A	B	C	D
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

Calibration standard preparation table**Supported liquid extraction of steroids from calibration standards and sa... 10m**

- 8 Transfer 200 µL of prepared calibration standards, bone marrow homogenate and mouse plasma from glass culture tubes into a 2 mL deep well 96-well collection plate (Biotage), prepared in Microsoft Excel template (see Files) and following a plate map design as below -

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1 Double Blank	A2 0.250 STD	A3 Sample 001	A4 Sample 009	A5 Sample 017	A6 Sample 025	A7 Sample 033	A8 Sample 041	A9 Sample 049	A10 Sample 057	A11 Sample 065	A12 Sample 073
B	B1 0 STD	B2 0.500 STD	B3 Sample 002	B4 Sample 010	B5 Sample 018	B6 Sample 026	B7 Sample 034	B8 Sample 042	B9 Sample 050	B10 Sample 058	B11 Sample 066	B12 Sample 074
C	C1 0.00250 STD	C2 1.00 STD	C3 Sample 003	C4 Sample 011	C5 Sample 019	C6 Sample 027	C7 Sample 035	C8 Sample 043	C9 Sample 051	C10 Sample 059	C11 Sample 067	C12 Sample 075
D	D1 0.00500 STD	D2 2.50 STD	D3 Sample 004	D4 Sample 012	D5 Sample 020	D6 Sample 028	D7 Sample 036	D8 Sample 044	D9 Sample 052	D10 Sample 060	D11 Sample 068	D12 Sample 076
E	E1 0.0100 STD	E2 5.00 STD	E3 Sample 005	E4 Sample 013	E5 Sample 021	E6 Sample 029	E7 Sample 037	E8 Sample 045	E9 Sample 053	E10 Sample 061	E11 Sample 069	E12 Sample 077
F	F1 0.0250 STD	F2 10.0 STD	F3 Sample 006	F4 Sample 014	F5 Sample 022	F6 Sample 030	F7 Sample 038	F8 Sample 046	F9 Sample 054	F10 Sample 062	F11 Sample 070	F12 Sample 078
G	G1 0.0500 STD	G2 Double Blank	G3 Sample 007	G4 Sample 015	G5 Sample 023	G6 Sample 031	G7 Sample 039	G8 Sample 047	G9 Sample 055	G10 Sample 063	G11 Sample 071	G12 Sample 079
H	H1 0.100 STD	H2 Double Blank	H3 Sample 008	H4 Sample 016	H5 Sample 024	H6 Sample 032	H7 Sample 040	H8 Sample 048	H9 Sample 056	H10 Sample 064	H11 Sample 072	Solvent blank

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

- 9 Using a multi-step pipette enrich the plate containing calibration standards with IS by adding $\text{20 } \mu\text{L} \times 5$ **ng/mL Working Internal Standard** into each calibration standard, including 0 std and each sample (bone homogenate and mouse plasma), except for the Double blank and solvent blank.
- 10 Using the Extrahera liquid handling robot, set up with an SLE400 extraction plate and a deep well extraction plate, aliquot $200 \mu\text{L}$ **[M] 0.5 Molarity (M)** ammonium hydroxide in water into each well of the 96-well plate.
- 11 Transfer $\text{400 } \mu\text{L}$ of liquid from each well (containing sample and the diluent, **[M] 0.5 Molarity (M)** ammonium hydroxide) into a $400 \mu\text{L}$ 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.
- 12 Allow the diluted sample to adsorb onto the SLE extraction bed for **00:05:00** before eluting with $\text{600 } \mu\text{L} \times 98:2 (\text{v/v})$ dichloromethane/isopropanol and repeating twice more, each time collecting the eluent into the collection plate **5m**

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

14 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

15 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

16 Set up an acquisition batch in Analyst software using the electronic 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.

17 Set up the Shimadzu Nexera X2 liquid chromatography system and fit with a Phenomenex Krud Katcher and a Phenomenex 150 x 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 .

18 Set up chromatographic gradient as below 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

A	B	C	D
12.10	0.5	45	55
16.00	0.5	45	55

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)

- 19 Set up the mass spectrometer for Multiple Reaction Monitoring (MRM) method in positive mode, with electrospray ionisation as below, 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)	4500 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

A	B
Probe position (x – axis)	5
Probe position (y – axis)	2

Mass Spectrometry source settings for positive ion electrospray ionsiation on QTrap 6500+

- 20 Set up the mass spectrometer to monitor for the following MRM transitions for each steroid and isotopically labelled steroid.

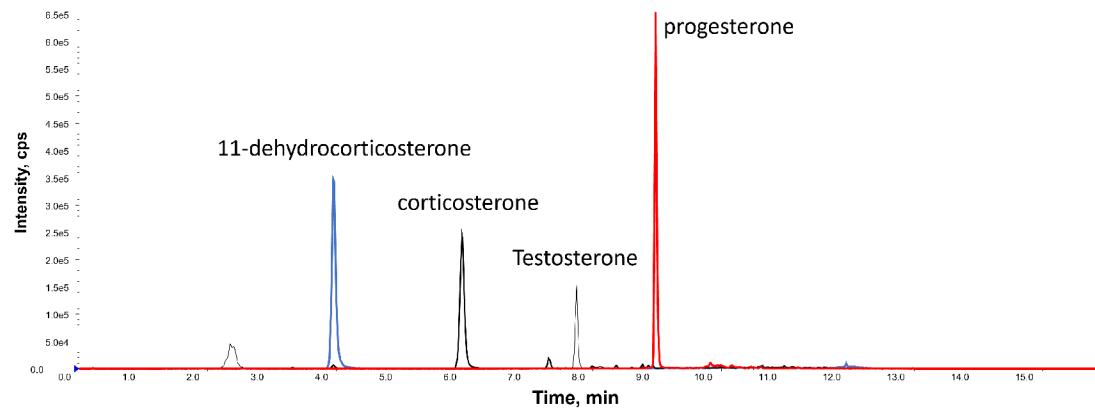
A	B	C	D	E	F	G
Q1 Mass (Da)	Q3 Mass (Da)	Scan time (msec)	Steroid Name	DP (V)	CE (V)	CXP (V)
347.1	121.1	50	Corticosterone 1	76	29	8
347.1	90.9	50	Corticosterone 2	76	75	12
345.1	121.2	50	11-Dehydrocorticosterone 1	66	31	12
345.1	91.2	50	11-Dehydrocorticosterone 2	66	83	40
289.1	97.0	50	Testosterone 1	101	29	12
289.1	109.2	50	Testosterone 2	101	31	6
315.1	97.1	50	Progesterone 1	96	23	10
315.1	109.1	50	Progesterone 2	96	27	10
355.3	128.1	50	d8B-Corticosterone 1	37	45	14
355.3	125.0	50	d8B-Corticosterone 2	29	56	14
292.1	100.2	50	13C3-Testosterone	101	29	12
324.1	100	50	d9-Progesterone	96	23	10

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

- 21 Check the retention times of the steroids are as expected, as shown in the chromatogram below:

Expected result

Retention times; 11-dehydrocorticosterone at **4.1 mins**, corticosterone at **6.2 mins**, testosterone at **8 mins** and progesterone at **9 mins**



Overlaid Ion Chromatogram of MRM transitions for 11-dehydrocorticosterone, corticosterone, testosterone and progesterone, separated on a Kinetex C18 (150 x 3 mm; 2.6 um) at 0.5 mL/min

- 22 Once the chromatography has been checked and the retention times are consistent, set the batch of samples to analyse. Use MultiQuant software and excel to evaluated the LC-MS/MS data to calculate the concentration of steroids in each sample, as detailed:

Protocol



NAME

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

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[PREVIEW](#)