An LC-MS/MS method for the panelling of 13 steroids in serum

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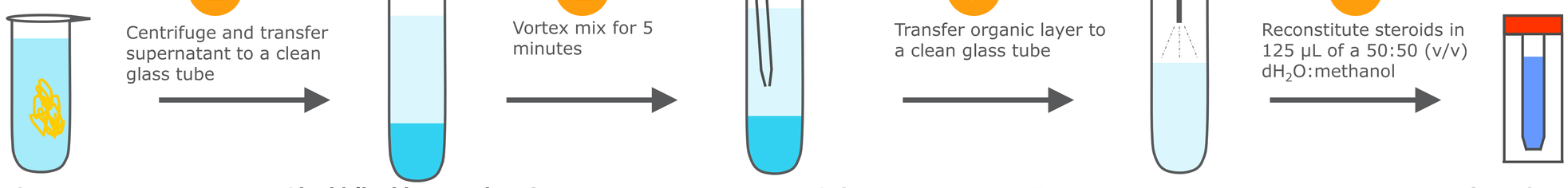
Introduction:

Analysis of serum steroids by LC-MS/MS is increasingly replacing immunoassay, especially for those most subject to cross-reaction. However, much more is possible. By using a cocktail of deuterated internal standards, multiple steroids can be simultaneously quantified to provide a ´serum steroid panel´. This also enables measurement of steroids not usually available, either due to low demand or lack of immunoassay specificity. Examples include 21-deoxycortisol, a more specific marker of 21-hydroxylase deficiency than 17-hydroxyprogesterone, and pregnenolone and 17-hydroxypregnenolone, steroid hormone precursors which may be increased in cases of adrenocortical carcinoma. In this study we describe a method for measurement of testosterone, progesterone, androstenedione, DHEAS, pregnenolone, 11-deoxycorticosterone, corticosterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, cortisol and cortisone from 250 µL serum.

Sample preparation:



Protein precipitation: 250 µL serum + 750 µL acetonitrile (containing deuterated internal standards for each compound)

Liquid-liquid extraction: Supernatant + 300 µL dH_2O + 1 mL ethyl acetate

Remove organic layer: using a glass pipette

Evaporation: evaporate ethyl acetate under nitrogen at 60°C

Transfer to insert glass vial: place in autosampler ready for analysis

• Schematic of sample preparation for LC-MS/MS. Each step has been extensively optimised to maximise recovery and minimise ion suppression. Combining protein precipitation with liquid-liquid extraction results in reproducible extraction across the range of the polarities of the steroids quantified.

LC-MS/MS conditions:

LC-MS/MS analysis was performed using a TLX-II Transcend LC system and TSQ Vantage triple quadrupole mass spectrometer (both Thermo Scientific). Separation of steroids was achieved using an Accucore RP-MS column (100 mm x 2.1 mm, 2.6 µm particle size). Mobile phases were (A) deionised water and (B) methanol, each containing 0.1% (v/v) formic acid. Steroids were eluted using the chromatography conditions shown in Table 1. Steroids were ionized using an APCI probe maintained at the conditions described in Table 2 and operated in positive ionisation mode. Parameters for each individual steroid quantified by the method are summarised in Table 3.

Start time (min)	Length (sec)	Gradient	% A	% B
0	30	Step	65	35
0.5	900	Ramp	20	80
15.5	150	Step	0	100
18	100	Step	65	35

Table 1. Chromatography gradient conditions. Flow rate was kept constant at 0.4 mL/min throughout.

Discharge current (µA)	5
Vaporiser temperature (°C)	500
Sheath gas pressure (Arb)	20
Aux gas pressure (Arb)	5
Capillary temperature (°C)	400

Table 2. APCI source conditions. Sheath and aux gas was nitrogen.

Steroid	Q1 m/z	Q3 quantifier m/z (Collision energy, V)	Q3 qualifier m/z (Collision energy, V)	S lens (Arb)	Low standard (nmol/L)	High standard (nmol/L)	LLOQ (nmol/L)	Internal standard
Testosterone	289.2	97.0 (20)	109.0 (25)	61	0.17	69.0	0.17	Testosterone-d3
Androstenedione	287.1	97.0 (22)	109.1 (21)	57	1.75	698.0	0.88	Androstenedione-d7
DHEAS	271.1	105.0 (30)	91.0 (38)	30	35.0	13580.0	35.0	DHEAS-d2
Pregnenolone	299.2	105.1 (33)	91.0 (43)	58	8.00	316.0	4.00	Pregnenolone-d4
17-hydroxypregnenolone	297.1	105.0 (34)	91.1 (45)	60	3.00	1203.0	1.50	17-hydroxypregnenolone-d3
Progesterone	315.1	97.0 (20)	109.0 (26)	66	0.40	159.0	0.40	Progesterone-d9
17-hydroxyprogesterone	331.2	97.0 (26)	109.0 (26)	67	2.30	908.0	1.15	17-hydroxyprogesterone-d8
11-deoxycorticosterone	331.2	97.0 (26)	107.0 (26)	67	0.30	30.0	0.30	11-deoxycorticosterone-d8
Cortisol	363.2	121.0 (26)	91.0 (47)	65	3.45	1379.0	3.45	Cortisol-d4
Cortisone	361.1	163.1 (21)	121.0 (30)	74	0.69	277.0	0.69	Cortisone-d2
11-deoxycortisol	347.1	109.0 (27)	97.0 (25)	72	0.72	289.0	0.36	11-deoxycortisol-d2
21-deoxycortisol	347.1	105.1 (27)	121.1 (41)	66	0.72	289.0	0.36	21-deoxycortisol-d8
Corticosterone	347.1	105.1 (27)	121.1 (41)	66	0.72	289.0	0.72	Corticosterone-d8

Table 3. Optimised method parameters for each steroid in the panel. Calibration range, lower limit of quantification (LLOQ) and deuterated internal standard used for each steroid are also listed.

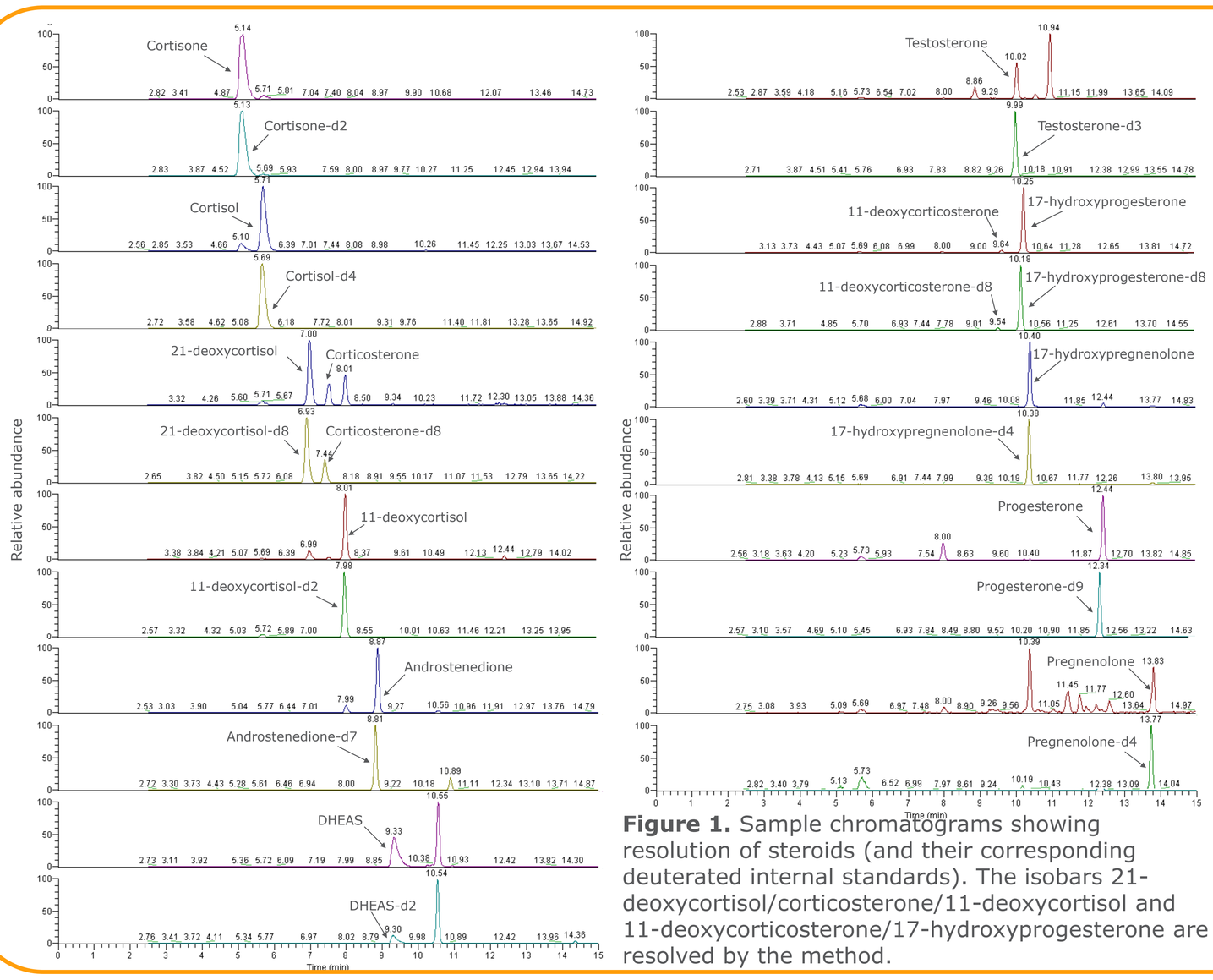


Figure 1. Sample chromatograms showing resolution of steroids (and their corresponding deuterated internal standards). The isobars 21-deoxycortisol/corticosterone/11-deoxycortisol and 11-deoxycorticosterone/17-hydroxyprogesterone are resolved by the method.

Results:

- The described method allows quantification of 13 steroids from a single 250 µL aliquot of serum. Several of the steroid are not currently routinely quantified in the UK.
- Increasing the percentage of methanol slowly from 35% to 80% (v/v) over 15 minutes allows complete resolution of steroid isobars (21-deoxycortisol/corticosterone/11-deoxycortisol and 11-deoxycorticosterone /17-hydroxyprogesterone, Figure 1). This cannot be achieved using shorter analysis time.
- The method is linear for all steroids over the calibration ranges used, with good LLOQ obtained for each steroid (Table 3).
- Intra- and inter-batch precision and UKNEQAS comparison to LC-MS groups were excellent where available (data not shown).
- No ion suppression was noted following the extensive sample clean-up (data not shown).

Conclusion:

- This method will allow specific panels to be targeted for individual diseases with disordered steroidogenesis.
- Examples include:
- **CAH panel:** 17-hydroxyprogesterone and 21-deoxycortisol (21-hydroxylase deficiency), 11-deoxycortisol and 11-deoxycorticosterone (11β-hydroxylase deficiency), 17-hydroxypregnenolone (3β-HSD deficiency), 11-deoxycorticosterone and corticosterone (17α-hydroxylase deficiency) plus cortisol, DHEAS and androstenedione
- **Hirsutism panel:** Testosterone, DHEAS, androstenedione and 17-hydroxyprogesterone.
- **Adrenocortical carcinoma:** Adrenocortical carcinoma typically produces excess steroid precursors. As such (and consistent with our experience from urine steroid profiling) preliminary data shows steroids such as pregnenolone, 17-hydroxypregenolone, corticosterone and 11-deoxycortisol may be useful markers of malignant transformation, in addition to more established markers such as androstenedione, DHEAS and 17-hydroxyprogesterone.

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