Development of a Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS) method for determination of 9 steroids in human serum for diagnosis of Congenital Adrenal Hyperplasia (CAH) and steroidogenic biosynthesis defects

Xiaolin Wu, Chris W Sies, Ian J Phillips, Richard King, Chris M Florkowski

Canterbury Health Laboratories, Christchurch, New Zealand. Email: Xiaolin.Wu@cdhb.health.nz

Canterbury Health Laboratories
www.chl.co.nz | 0800THELAB

Background

Congenital adrenal hyperplasia (CAH) is an inherited disorder of the adrenal gland that affects the production of one or more of the steroid hormones. There are two main types of CAH: classical CAH, the more severe form, and a milder form called non-classic CAH.

The most common cause of CAH is a deficiency in 21-hydroxylase (95%), the enzyme involved in the biosynthesis of the Aldosterone and Cortisol. Other rarer forms of CAH include: 11β hydroxylase deficiency, 17-hydroxylase deficiency, and 3β hydroxysteroid dehydrogenase type 2 deficiency (3β-HSD).

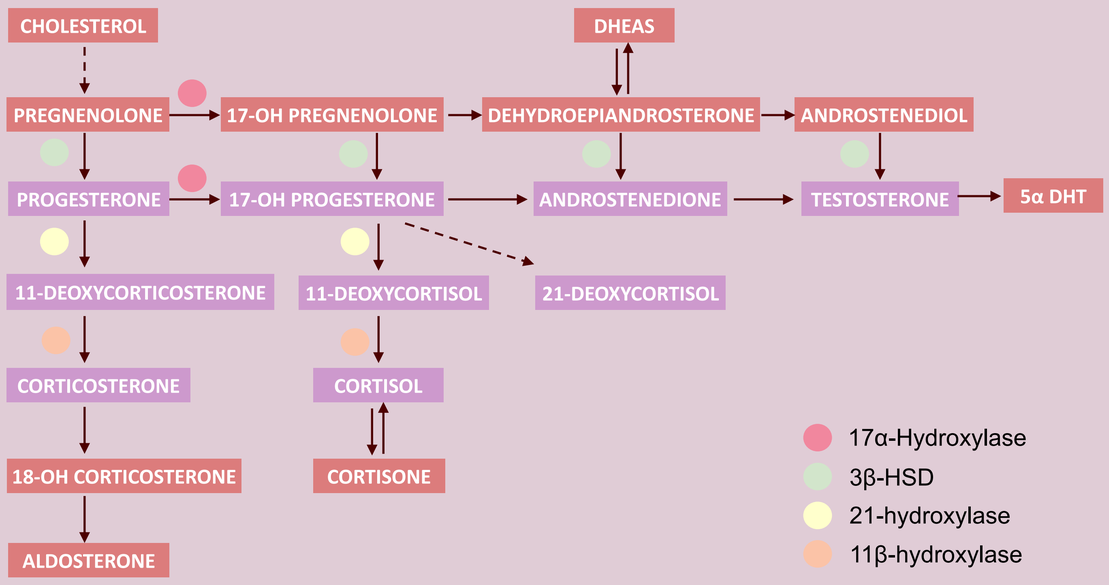


Fig 1. Steroid Biosynthesis Pathway. Steroids in purple are targeted analytes in this method (Only the relevant enzymes for CAH are shown).

Highlights

- We demonstrate that serum steroid profiling by LC-MS/MS can be used for diagnosis of CAH
- Our method can distinguish Classic and Non-classic 21-hydroxylase deficiency and other forms of CAH, along with rarer defects of steroidogenesis
- We established a new LC-MS/MS method to measure all nine steroids within a single run, and validated the method for routine clinical application
- Improved selectivity is achieved by a combination of chromatography and carefully selected MRM transitions
- Improved sensitivity is achieved by setting of time segments to increase dwell time for analytes

Materials and methods

A sensitive and selective analytical method was developed and validated for the determination of 17-hydroxyprogesterone, androstenedione, corticosterone, 11-deoxycortisol, 21-deoxycortisol, cortisol, 11-deoxycorticosterone, progesterone and testosterone in human serum.

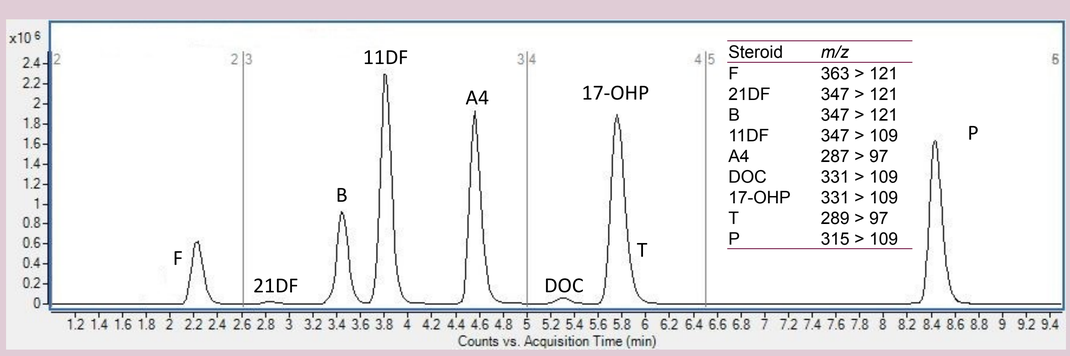
The extraction method is similar to that described in Taylor et al.[1] 500 µL serum was transferred into a 1.5-mL centrifuge tube. 50 µL internal standard mix was added. Prepare ice-cold acetonitrile and add 500 µL into the sample and vortex-mix for 1 min. Centrifuge at 12000g for 10 min, and transfer the supernatants to a 100- × 16-mm, 10-mL glass tube containing 300 µL of Milli-Q water. 1mL ethyl acetate was added, and the tube was vortex-mixed for 5 min. Following centrifugation (160g, 1 min), 900 µL of the top layer was removed to a clean 75- × 12-mm, 5-mL glass tube. Extracts were evaporated to dryness under nitrogen or compressed air at 60 ˚C and reconstituted using 100 µL reconstitution buffer (65% water: 35% MeOH) and transferred to a LC vial. Liquid chromatography conditions and mobile phase gradient were shown below and in Table 1.

Liquid Chromatography Conditions:
Mobile Phase A: Water+2% Methanol+0.2% Formic Acid
Mobile Phase B: 100% Methanol
Injection Volume: 20 µL
Column Temperature: Room Temperature

Table 1. LC Mobile Phase Gradient

Time [min]	A [%]	B [%]	Flow [mL/min]
0.00	53	47	0.475
9.50	30	70	0.475
9.51	0	100	0.475
10.50	0	100	0.475
10.51	53	47	0.475
11.50	53	47	0.475

Agilent 1290 Infinity LC System coupled with the Agilent 6490 Series Triple Quadrupole Mass Spectrometer used in this method enabled monitoring selective MRM transitions in defined time segments.



Steroid	*m/z*
F	363 > 121
21DF	347 > 121
B	347 > 121
11DF	347 > 109
A4	287 > 97
DOC	331 > 109
17-OHP	331 > 109
T	289 > 97
P	315 > 109

Fig 2. Chromatography of steroids. F, cortisol; 21DF, 21-deoxycortisol; B, corticosterone; 11DF, 11-deoxycortisol; A4, androstenedione; DOC, 11-deoxycorticosterone; 17-OHP, 17-hydroxyprogesterone; T, testosterone; P, progesterone. (Qualifiers not shown)

Method Performance

This LC-MS/MS method has been validated according to the combination of European Medicine Agency guideline on bioanalytical method validation[2] and CLSI C62-A guideline[3]. Chromatographic resolution of nine steroids was achieved within 11.5 min (Fig.2). The isobaric steroids 21-deoxycortisol, corticosterone and 11-deoxycortisol, and 11-deoxycorticosterone and 17-hydroxyprogesterone are well separated. LLoQ, ULoQ and recoveries at midpoint with calibration range of nine steroids were shown in Table 2.

Table 2. Calibration curve range, recoveries, and linearity.

	Calibration Range nmol/L	Recoveries %	LLOQ nmol/L	ULOQ nmol/L
F	24.9-800	83.99-95.63%	0.13	800
21DF	0.20-13.5	90.44-101.69%	0.20	13.5
B	1.31-139	93.93-96.70%	0.50	139
11DF	0.25-39.5	92.48-104.70%	0.25	39.5
A4	0.67-48.9	93.34-102.93%	0.50	48.9
DOC	0.14-8.51	87.82-101.29%	0.14	8.51
17-OHP	0.29-65.3	80.09-103.27%	0.05	65.3
T	0.19-39.8	82.35-98.08%	0.10	39.8
P	0.52-78.1	86.01-102.44%	0.40	78.1

Selectivity was achieved by using proper MRM transition for each steroid and separation by liquid chromatography, e.g. testosterone and 17-OHP have same retention time but are distinguished by different transitions (*m/z*).

Sensitivity was improved by setting up time segments which allows increased dwell time given enough separation.

The within- and between-run accuracy and precision of the method for nine steroids were within acceptable limits in Guidelines[2,3] (%CV value of QC's do not exceed 15%).

Matrix effect was evaluated for at midpoint of calibration range for each steroid and the values obtained were within acceptable limits (%CV of the matrix factors calculated from the 6 lots of matrix do not exceed 15%).

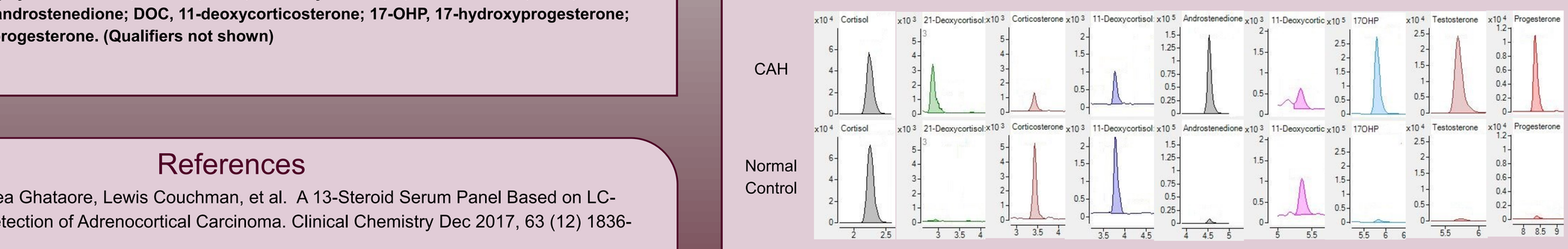
CAH and Patient Samples

21-hydroxylase deficiency including its two subtypes, and other three forms of CAH and their hormonal profile were shown in Table 3. The even rarer types such as POR (P450 oxidoreductase deficiency) and lipoid adrenal hyperplasia or SCC enzyme deficiency were not shown.

A 21-hydroxylase deficiency sample (female) and a normal patient sample (female) were analysed using this LC-MS/MS method. Changes of concentration in the 21-hydroxylase deficiency patient sample was shown in Table 4 and hormonal profiles of two samples were shown in Figure 3.

Table 3. Congenital Adrenal Hyperplasia Types and Hormonal Profile.

Form	Occurrence	Affected	Hormonal profile
21-Hydroxylase CAH (Classical)	90–95%	17-OH-Progesterone > 11-Deoxycortisol Progesterone > Deoxycorticosterone	↑17-OHP, 21DF, and A4 ↓F and aldosterone
21-Hydroxylase CAH (Non-Classical)		17-OH-Progesterone > 11-Deoxycortisol Progesterone > Deoxycorticosterone	↑17-OHP, 21DF, and A4 = F; = aldosterone.
11β-Hydroxylase CAH	5%	11-Deoxycortisol > Cortisol DOC > Corticosterone	↑DOC, 11DF, A4, and 17-OHP (mild) ↓F, aldosterone, B, and renin
3β-HSD CAH	1%	Pregnenolone > Progesterone 17-OH-Pregnenolone > 17-OHP DHEA > Androstenedione	↑17OHP ↓F, aldosterone, P, 17OHP, DOC, A4 and 11DF
17α-Hydroxylase CAH	1%	Pregnenolone > 17-OH Pregnenolone Progesterone > 17-OH Progesterone 17-OH-Pregnenolone > DHEA	↑DOC, B and P ↓F, aldosterone, 17OHP, and A4



CAH	F	21-DF	B	11-DF	A4	11-DOC	17-OHP	T	P
Concentration Change	↓	↑↑	↓	↓	↑↑	↓	↑↑	↑↑	↑↑

Fig 3. Chromatographic profiles of CAH and normal control.

References

1. David R. Taylor, Lea Ghataore, Lewis Couchman, et al. A 13-Steroid Serum Panel Based on LC-MS/MS: Use in Detection of Adrenocortical Carcinoma. Clinical Chemistry Dec 2017, 63 (12) 1836-1846.
2. European Medicines Agency. Guideline on bioanalytical method validation. 2011 Jul. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf (Accessed Sep 2019)
3. C62-A. Liquid Chromatography-Mass Spectrometry Methods; Apprved Guideline Clinical Laboratory Standards Institute, 2014. ISBN 1-56238-978-5.