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Analytical Performance of LC-MS/MS Method for Simultaneous Determination of Five Steroids in Serum

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Abstract

The accurate measurement of steroid hormones is the fulcrum of modern endocrinology. Our aim was to develop a mass spectrometry coupled with a liquid chromatographic method for 17-hydroxyprogesterone, androstenedione, 11-desoxycortisol, 21-desoxycortisol and cortisol serum quantification simultaneously. The samples were submitted to isotopic internal standards addition, protein precipitation and two dimensional liquid chromatography consisting of trapping column and reverse-phase C18 analytical column following atmospheric pressure chemical ionization and mass spectrometry detection. For all compounds tested functional sensitivity was less than 0.5 ng/mL, precision was less than 15%, recovery ranged from 93% to 120%, linearity ranged from 89% to 111%, and accuracy was considered adequate. In conclusion, we developed a suitable, fast and reliable method for simultaneous routine measurement of 17-hydroxyprogesterone, androstenedione, 11-desoxycortisol, 21-desoxycortisol and cortisol in serum.

Keywords: Mass spectrometry; Liquid chromatography; Steroid measurements

Introduction

The reliable and simultaneous analysis of steroid hormones panel is a powerful tool for investigation of steroid hormone status, which is relevant for the diagnosis and treatment of a variety of clinical disorders like adrenal insufficiency and congenital adrenal hyperplasia (CAH) [1-5]. Due to the low levels of serum steroids metabolites and chemical similarity, analysis of this kind of compounds has been a challenge [1]. Historically androgens were tested in urine using colorimetric assays [2]. In the 1960s, more specific tritiated radioimmunoassay (RIA) was developed, and although RIA reached a better sensitivity it has the disadvantage of radioactive materials usage and laborious extraction. Until 1990s tritiated RIA methods were considered the state-of-the-art methods for measuring serum steroids [3]. Nowadays, most methods for determination of steroids are based on immunoassays, which are rapid and easy but lack specificity and are single analyte methods. Liquid chromatography coupled with mass spectrometry (LC-MS/MS) is an increasingly common tool in the clinical laboratory and has the potential to overcome the limitations of immunoassays.

Tandem mass spectrometry methods with atmospheric pressure chemical ionization (APCI) ionization were developed in 1990s [4] and during the past two decades, LC-MS/MS techniques showed an increased usage in clinical laboratories, mainly to small molecules analyses [5,6]. The accurate measurement of hormones is the pivot of modern endocrinology and so lays the importance of method validation to demonstrate characteristics of reprodutibility, accuracy and specificity [7,8]. Advantages to measure five serum steroids simultaneously in the same sample includes the need of less blood sample volume allowing the diagnosis in newborn and premature babies with adrenal diseases. The analyses of 21-desoxycortisol in addition with 17-OHP analysis can improve the accuracy of CAH diagnosis independent of prematurity and birth weight [9].

Materials and Methods

Materials

7-hydroxyprogesterone(17OHP),androstenedione,11-desoxycortisol, 21-desoxycortisol and cortisol were purchased from Sigma-Aldrich (St. Louis, MO, USA). 17-hydroxyprogesterone-*d*8, 11-desoxycortisol-*d*2, Cortisol-*d*4, 21-desoxycortisol-*d*8 and 4-androstene-3,17-dione *d*7 were purchased from CDN Isotopes (Pointe-Claire, Canada). LCMS grade solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was prepared from MilliQ system (Millipore, Billerica, MA, USA). Albumin (BSA) was purchase from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of standard solutions, calibrators and quality control samples

Stock solutions of all steroids standards, deuterated and non-deuterated, were prepared in dimethyl sulfoxide (DMSO) at a concentration of 1 g/L and stored at -80°C. Steroid calibrators were prepared in a 5% BSA at different concentrations 0.5, 1.0, 2.5, 5.0, 10.0 and 25.0 (ng/mL) for androstenedione; 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 for 11-desoxycortisol; 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 150.0 for 21-desoxycortisol and 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 150.0 and 300.0 for 17-hydroxyprogesterone and cortisol. The results were analyzed using linear least square regression according to the equation y=a+bx, were y is the area ratio of substance to internal standard and x the analyte concentration of the calibrator sample. The assay acceptance criteria for each back calculated standard concentration was 20% deviation from the nominal value. The calibration curve was required to demonstrate a coefficient of correlation of 0.990. Lyphochek Immunoassay Plus, commercial controls from Bio-Rad Laboratories, Inc (CA, USA) was used for androstenedione, cortisol and 17-hydroxyprogesterone. High and low control levels for 11-desoxycortisol and 21-desoxycortisol samples were prepared by a pool of patients' serum. Calibrators, controls and samples were stored at -20°C.

Sample preparation: It consists of a deproteinization step in a 96 deep-well plate. Two thousands µL of calibrators, quality controls

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and serum samples were combined with 200 µL of precipitant solution containing cortisol-*d*4 at 40.0 ng/mL, 11-desoxycortisol-*d*2, 21-desoxycortisol-*d*8 and 17-hydroxyprogesterone-*d*8 at 20.0 ng/mL and androstene-3,17-dione *d*7 at 5.0 ng/mL prepared in $ZnSO_4$ 0.2 mol/L: methanol (20:80) solution. The plate was mixed in an automatic shaker for 10 min, centrifuged for 15 min at 4000×g. and placed in a Waters 2777 sample manager equipped with a cooling stack set at 8°C.

Liquid chromatography and extraction on-line

Twenty microliters of the supernatant were injected in a Onyx monolithic C18 10 mm × 4.6 mm cartridge (Phenomenex, Torrance, CA) with 5% methanol as mobile phase at 0.35 mL/min flow rate pumped by a Waters 1515 pump (Millford, MA) for steroid extraction during 3.2 min. This trapping column is connected to a 2-position, 6-port valve (Rheodyne) which every 3.2 min switch the position and the pre-columm was connected with the analytical column (Kinetex core-shell 5 µ C18 100A 50 × 2.1 mm, Phenomenex, Torrance, CA) kept at 40°C in a Acquity column oven (Waters, Millford, MA). The analytical column was eluted with a multistep binary gradient pumped by Acquity (UPLC), binary pump (Waters, Millford, MA, USA). The elution mobile phase consisted initially of a mixture of 55% (v/v) methanol in 0.5 mmol/L pH 3.0 ammonium formate at a flow rate of 0.35 mL/min. The methanol content was increased to 70% in 2 min and to 95% 2.5 min and then returned to initial condition in time of total run of 3.2 minutes.

Triple quadrupole mass spectrometry detection and quantification

Detection was performed on a triple quadrupole TQ-S Xevo mass spectrometer (Waters, Manchester, UK) with atmospheric pressure chemical ionization (APCI) probe operating at positive mode. The mass spectrometer operating conditions were as follows: desolvation temperature 550°C, desolvation nitrogen gas flow 900 L/h, cone gas flow (nitrogen) 150 L/h, with corona current set at 5 µA. Collision-induced dissociation was performed using argon as the collision gas at 4×10^{-3} mbar. Unit resolution was maintained for both parent and product ions for multiple reaction monitoring (MRM) analyses. Quantifier and qualifier transitions used are described in Table 1.

Data acquisitions were achieved with the MassLynx™ data processing and quantification were performed by the TargetLynx™ Application Manager. Internal standards were used for all analytes. Calibration was performed using 6 to 9 points through linear regression.

Assay validation

Method validation was assessed for functional sensitivity, linearity, precision, accuracy and recovery. Precision was determined with 10-20 intra and inter assays of at less two different sample. Precision was expressed as the coefficient of variation (CV%). Functional sensitivity was defined as the lowest sample concentration with inter-assay variation less than 20%. Linearity was assessed by a high level sample dilution with 5% BSA solution at 1/5, 1/10 and 1/20. Recovery was determined by comparing four replicate injections samples spiked with standards in low and high levels. Carry-over was evaluated when potential preceding elevated concentration of analyte interferes in the following concentration and was investigated by assaying two analyte specimens with low and high concentrations and comparing SD (standard deviation) of 11 samples with low concentration and 10 samples with high concentration a the following order: 3 low/ 2 high specimens, specimens, 1 low/2 high specimens, 4 low/ 2 high specimens, 1 low/2 high specimens, 1 low/2 high specimens, and 1 low specimen. Samples containing estriol, estrone, β-estradiol, aldosterone, corticosterone were tested for potential interference in this method. Results were analyzed using EP evaluator (David G. Rhoads Associates, Inc, Kennet Square, PA) or EXCEL® software (Microsoft Corporation, USA) [9,10].

Compound	Quantifier m/z	Qualifier m/z
Androstenedione	287.2>97.5	287.2>109.5
Cortisol	363.22>121.5	363.22>327.6
11-desoxycortisol	347.3>97.5	347.3>109.5
21-desoxycortisol	347.3>97.5	347.3>121.5
17-hydroxyprogesterone	331.3>97.5	331.3>109.5

Table 1: Quantifier and qualifier transitions.

Results

Performance characteristics

Chromatographic separation and detection was achieved for all analytes tested and calibration curves were linear (r^2>0.99) for the five steroids (Figures 1 and 2).

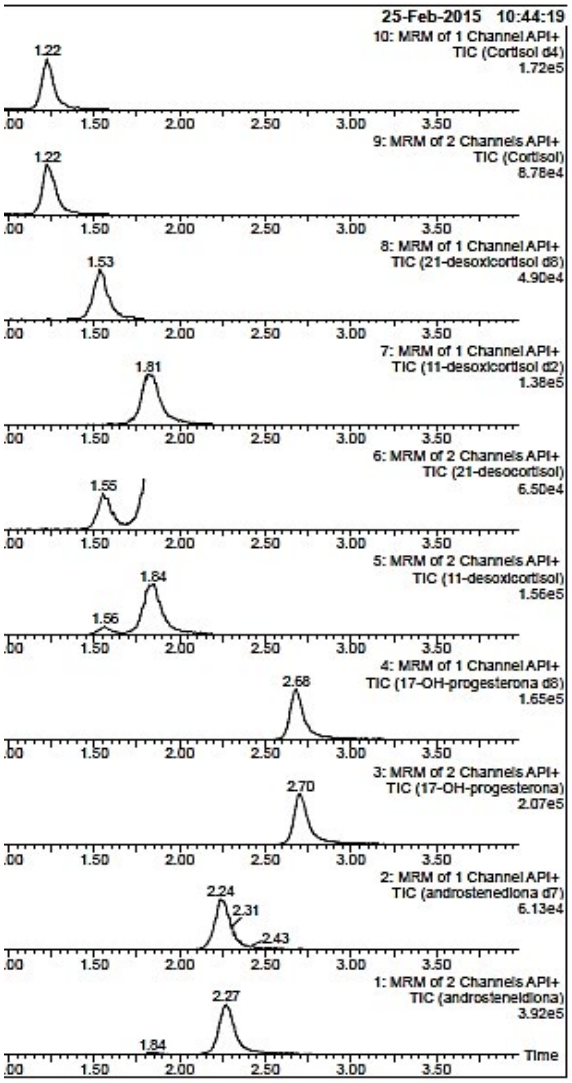

Figure 1: Total ion chromatogram (TIC) obtained for androstenedione, 11 deoxycortisol, 21-deoxycortisol, 17OHP and cortisol (10 ng/mL) and the corresponding deuterated compound.

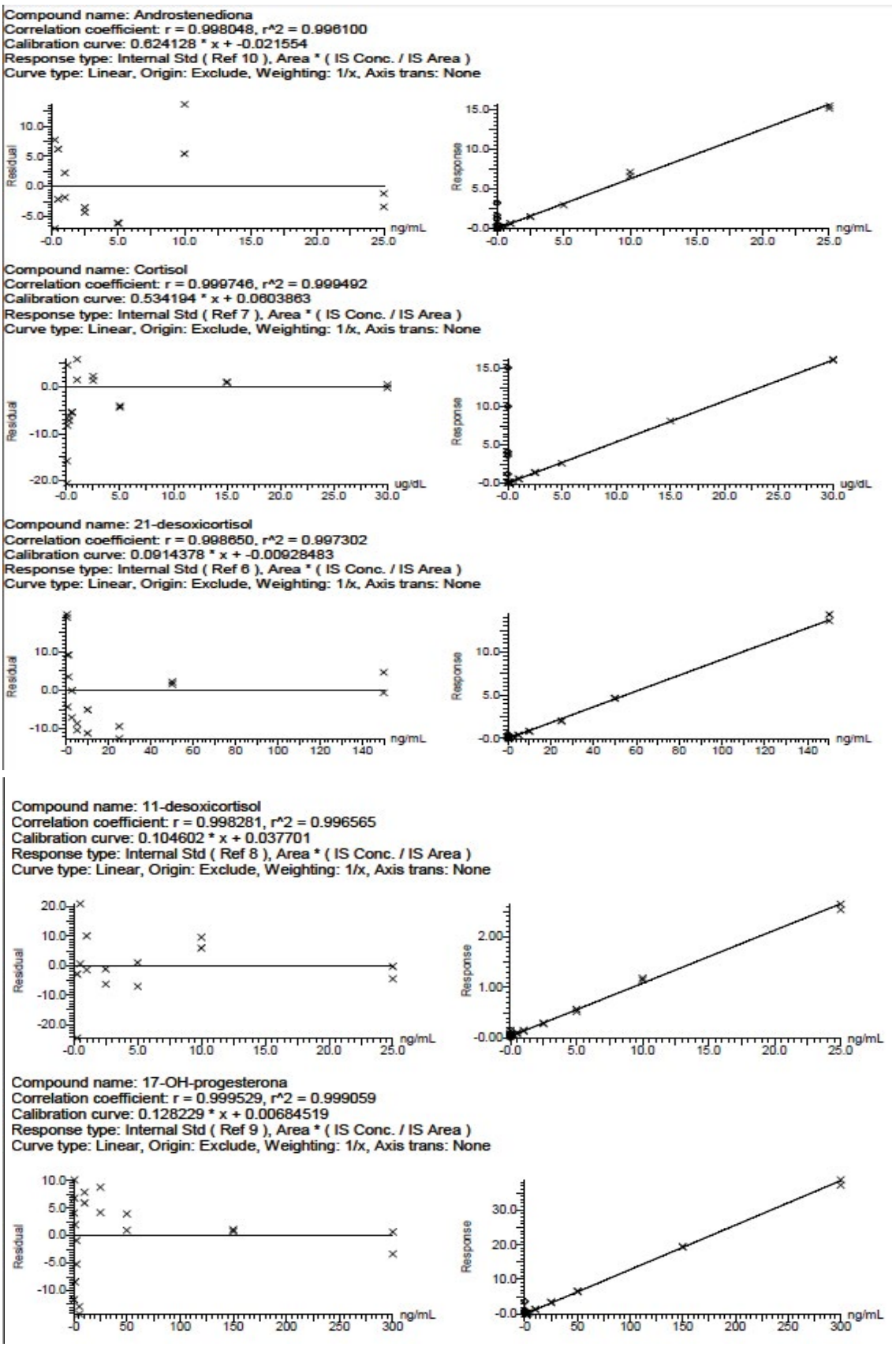

Figure 2: Calibration curves for each steroid showing prefect data fitness for all steroids.

Parameter	Androstenedione		11-deoxycortisol		21-deoxycortisol		Cortisol		17OH-progesterone	
	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)	Mean (ng/ml)	CV (%)
Intra-assay precision (n=10)	1.0 2.8 6.3	5.6 4.9 3.7	0.4 0.4 1.0	10.1 13.7 6.3	0.4 1.0 9.1	4.0 8.5 4.7	40.3 71.8 293.43	4.8 2.6 4.2	1.15 3.3 6.0	11.5 7.1 4.7
Inter-assay precision (n=20)	0.7 2.3 5.6	8.7 7.9 7.6	0.4 0.4	10.1 15.7	0.8 9.2	9.3 14.7	34.4 175.4 263.6	5.0 7.0 5.9	1.0 2.7 4.9	14.4 13.1 7.2
Acceptable limits (%)	11.2		16.0		15.0		11.4		14.7	
Functional sensitivity (ng/mL)	0.5		0.25		0.5		0.5		0.5	

Table 2: Precision and recovery obtained for androstenedione, 11-deoxycortisol, 21-deoxycortisol, 17OHP and cortisol.

Analyte	Recovery (%)	Linearity (%)	Acceptable limits (%)
Androstenedione	94.0-98.0	85-103	80-120
11- desoxycortisol	102.0-120.0	85-106	80-120
21- desoxycortisol	106.1-117.0	83-99	80-120
Cortisol	108.6-111.0	95-108	80-120
17-hydroxyprogesterona	93.3-110.0	93-106	80-120

Table 3: Recovery and linearity results.

Quantification limits (LOQ) or functional sensitivity were 0.5 ng/mL for androstenedione, 21-desoxycortisol, 17OHP and cortisol and 0.25 ng/mL for 11-desoxycortisol. Precision, recovery and linearity results are summarized in Tables 2 and 3 for each analyte. Carry-over and cross reactivity were not detected for all analyte. Steroids interferent which were tested showed no cross reactivity for all steroids determinate.

Discussion and Conclusion

Novel mass spectrometry techniques hyphened with liquid chromatography has become an important tool that continues to accelerate clinical research and clinical laboratories routines since the past decade [11]. The last generation of tandem mass spectrometers has superior limits of quantification, permitting omission or previously employed derivatization steps [11]. In the present study we evaluated a method to quantify adrenal steroids by liquid chromatography tandem mass spectrometry (LC-MS/MS) We developed a method to measure five steroids using protein precipitation, isotopic internal standards and two dimensional liquid chromatography, consisting of trapping column and reverse-phase C18 core-shell analytical column following atmospheric pressure chemical ionization and mass spectrometry detection with a total run time of 6.4 minutes including on-line extraction [6]. The combination of on-line extraction and the use of the corresponding deuterated internal standards reduced variability and contributed to the good precision achieved for all compounds. Due to the porous nature of monolithic silica, it is possible to apply high flow rates resulting in efficient sample clean-up and analyte concentration. This strategy provides a low cost alternative to dedicated systems for on-line extraction and reduces off-line sample preparation. In addition, the use of core-shell analytic columns reduces band broadening in chromatographic separations with better resolution, higher sensitivity when compared to fully porous particles. The use of a core-shell columns results in efficiencies improvement. The MRM qualifier and quantifier ion transition ratios results in better specificity of the method.

Other authors achieve similar results to our method using on-line extraction and APCI ionization, and silica column for steroid separation, however need at least 600 µL of serum sample and longer analysis time [6,12]. Other methods used manual sample preparation employing solid-phase or liquid-liquid extraction, evaporation or/and derivatization steps and although adequate for steroid analysis, total time of analysis is greater and much more laborious than our method [13,14].

LCMSMS assay methodology for steroid measurement developed in the present study showed excellent performance. The combination of on-line extraction and the use of the corresponding deuterated internal standards reduced variability and increased precision for all compounds. In conclusion, we validated a suitable and fast method for routine measurement of 17-hydroxyprogesterone, androstenedione, 11-desoxycortisol, 21-desoxycortisol and cortisol in smaller volume of serum samples, especially valuable for newborns.

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