Validation of a Reliable LC-MS/MS Method for Analysis of Five Steroids Simultaneously in Clinical Laboratory

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INTRODUCTION

The reliable and simultaneous analysis of a panel of steroid hormones is a powerful tool for investigation of hormone status, which is relevant for a variety of clinical questions and diagnoses like adrenal insufficiency in congenital adrenal hyperplasia (CAH)[1-3]. Most methods for determination of steroids are based on immunoassays, which are rapid and easy but lacks specificity. LC–MS/MS is an increasingly common tool in the clinical laboratory and has the potential to overcome the limitations of immunoassays[4-7]. Here we developed and evaluated a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to measure 17-hydroxyprogesterone (17OHP), androstenedione, 11- desoxycortisol, 21-desoxycortisol and cortisol in serum. The advantages are that the profiles can be measured simultaneously and afford the specificity, precision, accuracy and limits of quantification to help adrenal diseases diagnosis. Here we demonstrate the reliability of 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 analytical column following atmospheric pressure chemical ionization and mass spectrometry detection with a total run time of 6.4 minutes.

MATERIAL AND METHODS

Materials

17-hydroxyprogesterone (17OHP), androstenedione, 11- desoxycortisol, 21- desoxycortisol and cortisol were purchased from Sigma–Aldrich (St. Louis, MO). 17-Hydroxyprogesterone-*d*8, 11-desoxycortisol-*d*2, Cortisol-*d*4, 21-desoxycortisol-*d*8 and 4-androstene-3,17-dione *d*7 were from CDN Isotopes (Pointe-Claire, Canada). LCMS grade solvents were obtained from

Sigma–Aldrich (St. Louis, MO). Ultrapure water was prepared from MilliQ system (Millipore, Billerica, MA). Albumin (BSA) were purchase from AMRESCO (OH, USA).

Preparation of standard solutions, calibrators and quality control samples

Stock solutions of all steroids standards, deuterated and non-deuterated, were prepared in methanol at a concentration of 1 g/L and stored at -80^{0}C. Steroid calibrators were prepared in a 5% BSA at 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; 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 150.0 for 21-desoxycortisol and 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 , 150.0 and 300.0 for 17-hydroxyprogesterone and cortisol. Lyphochek Immunoassay Plus, comercial controls from Bio-Rad Laboratories, Inc (CA, USA) were used for androstenedione, cortisol and 17-hydroxyprogesterone. Two levels of control for 11-desoxycortisol and 21-desoxycortisol samples were prepared by a pool of samples of patients. Calibrators, controls and samples were stored at −20 ◦C.

Sample preparation

Sample preparation consists of a deproteinization step in a 96 well plate. Two thousands µL of calibrators, quality controls and serum samples were combined with 200µL of precipitant solution containing cortisol-*d*4 at 40.0 ng/mL, 11-desoxycortisol-*d*2 and 21-desoxycortisol-*d*8 at 5.0 ng/mL, 17-Hydroxyprogesterone-*d*8 and androstene-3,17-dione *d*7 at 2.5 ng/mL in ZnSO4 0.2mol/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 10 ◦C.

Two-dimensional liquid chromatography

Twenty microliters of the supernatant were injected in a Onyx monolithic C18 10mm×4.6mm 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) wich every 3.2 min switch the position and the precolumn was connected with the analytical column

(Kinetex core-shell 5u C18 100A 50x2,1 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). The elution mobile phase consisted initially of a mixture of 55% (v/v) methanol in 0.5 mmol/L pH3.0 ammonium formate at a flow rate of 0.35mL/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.

Mass spectrometry detection and Quantitation

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 5mA. Collision-induced dissociation was performed using argon as the collision gas at 4×10^{-3} mbar. For product ion spectra and multiple reaction monitoring (MRM) analyses, unit resolution was maintained for both parent and product ions. Quantifier and qualifier transitions used are described in Table 1.

Table 1- Quantifier and qualifier transitions

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-hidroxyprogesterone	331.3>97.5	331.3>109.5

Data acquisitions were achieved with the MassLynx™ data processing and quantitation were performed by the TargetLynx™ Application Manager. For all analytes were used internal standard. Calibration was performed using a 6 points, 8 and 9 points curve (androstenedione, 11-desoxycortisol and 21-desoxycortisol, cortisol and 17-hidroxyprogesterone respectively) through linear regression.

Method validation

Validation was assessed for functional sensitivity, linearity, precision, accuracy and recovery[7-12]. Functional sensitivity was defined as the lowest sample concentration which inter-assay variation was less than 20%. Precision was determined with 10-20 intra and inter assays of at less two different samples Recovery of the analytes was determined by comparing four replicate injections samples spiked with standards in low and high levels. Linearity was assessed by high-level sample dilution at 1/5, 1/10 and 1/20 with 5% BSA solution. Accepting values ranged between 80 and 120 %. Carry-over was investigated by assaying two specimens with low and high concentrations of the analytes. Proficiency samples from Ligand Assay and General Chemistry and Therapeutic Drugs surveys of the College of American Pathologists (CAP) were tested for accuracy verification.

RESULTS

Chromatographic separation and detection was achieved for analytes tested and calibration curves were linear (r^2>0.99) for the five steroids. Functional sensitivity were 0.5 ng/mL for androstenedione, 21-desoxycortisol, 17-hidroxyprogesterone and cortisol; 0.25 ng/mL for 11-desoxycortisol. Precision results and acceptable limits are summarized in table 2 for each analyte. Recovery and linearity were considered adequate and results are summarized in table 3.

Table 2- Results of precision

Analyte	Intra-assay variation (%) n=10	Inter-assay variation (%) N=20	Acceptable limits (%)
Androstenedione	3.23-5.59	5.96-7.64	11.25
11- desoxycortisol	6.27-13.71	10.75-15.71	16.05
21- desoxycortisol	4.74-8.55	9.29-14.68	15.0
Cortisol	2.65-4.79	3.47-8.78	11.40
17-hidroxyprogesterona	2.06-11.50	2.68-13.35	14.70

Table 3: Recovery and linearity results summary

Analyte	Recovery (%)	Linearity (%)	Acceptable limits (%)
Androstenedione	94.0-98.1	85-103	11.25
11- desoxycortisol	102.0-120.0	85-106	16.05
21- desoxycortisol	106,1-116.7	83-99	15.00
Cortisol	108.6-110.7	95-108	11.40
17-hidroxyprogesterona	93.3-109.9	93-106	14.70

Accuracy was considering adequate for all compounds tested, except 21-desoxycortisol which is not included in CAP surveys. Carry-over was not detected for any analyte.

DISCUSSION AND CONCLUSION

The LCMSMS assay methodology to steroids tested here showed adequate performance. 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. Using MRM qualifier and quantifier ion transition ratios allows sensitivity with added confidence in compound identity. The quantification limits reached by this method allowed the determination of all analytes in disease and non-disease ranges. In conclusion, we developed a suitable method for routine measurement of 17-hydroxyprogesterone, androstenedione, 11-desoxycortisol, 21-desoxycortisol and cortisol in serum which improved accuracy of adrenal diseases diagnosis.

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