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Multiplexed steroid profiling of gluco- and mineralocorticoids pathways using a liquid chromatography tandem mass spectrometry method

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ABSTRACT

Serum steroid assays are major tools in the clinical evaluation of adrenal disorders. The main adrenal steroids are routinely measured with immunoassays. However, chromatographic methods are known to offer better specificity. We report a liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for simultaneous quantification of 15 adrenal steroids targeting the mineralo- and gluco-corticosteroid pathways.

Serum steroids combined with deuterated internal standards were extracted using successive protein precipitation and solid phase extraction steps. Cortisol, cortisone, 11-deoxycortisol, 17-hydroxyprogesterone, 21-deoxycortisol, progesterone, 11-deoxycorticosterone, corticosterone, 11-dehydrocorticosterone, 18-hydroxycorticosterone, 18-hydroxy-11-deoxycorticosterone, aldosterone, dehydroepiandrosterone sulfate, testosterone and androstenedione were resolved in fourteen minutes using a BEH C18 column coupled to a methanol-ammonium formate gradient. Detection was performed using multiple reaction monitoring quantitation. Routinely determined steroid levels by immunoassays were compared to those measured by LC–MS/MS. This method was applied to assess steroid profiles in congenital adrenal hyperplasia (CAH) patients with 21-hydroxylase deficiency.

Low quantification limits depending on each steroid (ranging from 0.015 ng/mL for aldosterone to 20 ng/mL for DHEAS) are adapted to the clinical use. Recoveries of steroids range from 64% for 21-deoxycortisol to 101% for cortisol and are fully corrected by internal standards. A good linearity with $R > 0.989$ is obtained for each compound. The inter-day variation coefficients ranged from 4.7% for cortisol to 16.3% for 11-deoxycorticosterone. The immunoassay for cortisol (Immulite 2000, Siemens) showed

Abbreviations: 17OHP, 17-hydroxyprogesterone; 18OHB, 18-hydroxycorticosterone; 18OHDOC, 18-hydroxy-11-deoxycorticosterone; 21DF, 21-deoxycortisol; A, 11-dehydrocorticosterone; Aldo, aldosterone; B, corticosterone; CAH, congenital adrenal hyperplasia; D4, androstenedione; DHEAS, dehydroepiandrostenedione sulfate; DOC, 11-deoxycorticosterone; E, cortisone; F, cortisol; HSD, hydroxysteroid dehydrogenase; LC–MS/MS, liquid chromatography tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple-reaction monitoring; MS, mass spectrometry; PG, progesterone; S, 11-deoxycortisol; SPE, solid phase extraction; T, testosterone.

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acceptable agreement with LC–MS/MS (bias +7.2%). However, Bland-Altman plots revealed large negative bias for aldosterone (−33.4%, AldoCT, CisBio international), for 17-hydroxyprogesterone at concentrations below 2 ng/mL (−74.1%, OHP-CT MP Biomedical), for androstenedione (−80.3%, RIA D4, Beckman Coulter) and for 11-deoxycortisol (−125.3%, Diasource Immunoassays). Finally, the analysis of samples from 21-hydroxylase defective patients demonstrated the potential usefulness of multiplexed steroid profiling for the diagnosis and/or monitoring of different forms of congenital adrenal hyperplasia.

This LC–MS/MS method provides highly sensitive and specific assessments of mineralo- and glucocorticoids pathways from a small volume sample and is therefore a promising potent tool for clinical and experimental endocrine studies.

1. Introduction

Adrenal steroid hormones are synthesized from cholesterol through coordinated reactions of multiple enzymes. According to enzymatic zonal and subcellular localization, steroids are produced through three major pathways, mineralocorticoids, glucocorticoids and adrenal androgens. The circulating end-product concentrations are often detectable within the nanomolar range whereas synthesis intermediaries are present at extremely low levels. Because of these low levels and the chemical similarity among analogues, analysis of endogenous steroids is a bioanalytical challenge, especially in infants and children as the blood sample volume is limited [1]. The accuracy of steroid assays is of major concern in the clinical evaluation of congenital or acquired adrenal disorders associated with over- or under-production of steroids [2]. Such global or partial disorders include different forms of congenital adrenal hyperplasia, adrenal salt wasting or global adrenal insufficiency. Currently, most clinical laboratories use easily available immunoassays for routine endogenous steroid quantitation. These methods have been proven to be rapid and highly sensitive, but their reliability has been shown to be questionable because of the lack of specificity and matrix effects. Although immunoassays with extraction steps improve specificity, all interfering molecules cannot be completely eliminated [3].

The relevance of chromatographic methods for steroid assay is increasing with the improvement of specificity and detection. Gas chromatography is highly effective for separating steroids and mass spectrometry (MS) allows high specificity in identification of steroid metabolites. Because pre-analytical steps are often more complex and time-consuming, gas chromatography–mass spectrometry is restricted mostly to the characterization of urinary steroids and is performed in only a few specialised laboratories [4,5].

In recent years, liquid chromatography tandem mass spectrometry (LC–MS/MS) performed in multiple-reaction monitoring (MRM) mode has emerged as the most accurate method for measuring small molecules [1]. LC–MS/MS mostly provides high analytical sensitivity and specificity and has the ability to quantify multiple analytes simultaneously from small volume samples. Moreover, it has a wide dynamic range and typically requires limited sample preparation [2]. The measurement of aldosterone using LC–MS/MS has been proven to be difficult because of its poor ionisation in the mass spectrometer source and low concentrations encountered in plasma [6]. Previous reports, however, have detailed methods for the quantification of aldosterone using LC–MS/MS [7–11]. The development of steroidomic profiles is particularly helpful for differential diagnosis of non-classical forms of congenital adrenal hyperplasia (CAH) [12]. Previous reports have described LC–MS/MS methods for simultaneous determination of the main four steroids on newborn blood spots [13], eight steroids in children between birth and 18 years [14] and nine glucocorticosteroids and androgens in Addison's disease [15]. Steroid hormone profile-analyses, including aldosterone, were reported after a simple protein precipitation step [16], using the Biocrates extraction kit [17] or after a solid phase extraction from culture supernatants of human adrenocortical H295R cells [18]. None of these methods, however, was sensitive enough to accurately determine low circulating aldosterone concentrations, nor to fully explore its biosynthesis pathway. More recently, using the ABSciex 5500 triple quadrupole coupled to a solid phase extraction (SPE) step, Peitzsch et al. developed and applied a sensitive method for the investigation of primary aldosteronism before and after Synacthen test [19].

In the present study, we describe a reliable ultra-performance liquid chromatography tandem mass spectrometry (LC–MS/MS) method for plasma profiling of both mineralo- and gluco-corticoid pathways using a reduced sample preparation procedure based on SPE extraction. We focused in particular on the minor steroid precursors *i.e.* 18-hydroxy precursors of aldosterone, 11-dehydrocorticosterone and 21-deoxycortisol. Clinical validation was performed by comparison of the steroid profiles of children with or without 21-hydroxylase deficiency.

2. Materials and methods

2.1. Reagents and standards

Cortisol (F), cortisone (E), 11-deoxycortisol (S), 17-hydroxprogesterone (17OHP), progesterone (PG), 11-deoxycorticosterone (DOC), androstenedione (D4), testosterone (T) and dehydroepiandrostenedione sulfate (DHEAS) were purchased from Sigma-Aldrich, France. 18-hydroxycorticosterone (18OHB), 18-hydroxy-11-deoxycorticosterone (18OHDOC), corticosterone (B), aldosterone (Aldo), 21-deoxycortisol (21DF) and 11-dehydrocorticosterone (A) were obtained from Biovalley, France.

Stock standards were prepared in methanol at a 1 mg/mL concentration and stored at −20 °C. Working solutions diluted at 100 pg/μL were used to optimize fragmentation conditions. Calibration standards (Multilevel Serum Calibrator Set) and quality control serum containing Aldo, F, E, S, B, 17OHP, P, D4 and DHEAS were purchased from Chromsystems, France. Dilutions of working solutions of DOC, 18OHB, 18OHDOC, 21DF and A were added to calibration standards and quality controls.

Combined working internal deutered standards containing aldosterone-d4, cortisol-d4, cortisone-d8, corticosterone-d8, 11-deoxycortisol-d5, 17-hydroxyprogesterone-d8, progesterone-d9, androstenedione-d7, dehydroepiandrostenedione sulfate-d6 and testosterone-d3 were obtained from Chromsystems, France. LC–MS grade water, methanol, acetonitrile, ammonium formate, formic acid and zinc sulfate heptahydrate were purchased from Sigma Aldrich, France. Oasis® HLB 30 mg extraction cartridges were obtained from Waters (Milford, Mass., USA).

2.2. Sample preparation

All steroid hormones were extracted by SPE after a protein precipitation step. Aliquots of 250 μL of calibrators, controls and