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Simultaneous quantification of 17-hydroxyprogesterone, androstenedione, testosterone and cortisol in human serum by LC-MS/MS using TurboFlow online sample extraction

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ABSTRACT

Background: The simultaneous quantification of a steroid hormones panel provides more clinical information than a single steroid assay. Traditionally, steroids have been quantified with immunoassays which are characterized by high rate of positive results. Aim of this work, was to develop a TurboFlow-LC-MS/MS method for the simultaneous quantification of 17-hydroxyprogesterone, androstenedione, cortisol and testosterone in serum.

Methods: To 100 μL of serum, 100 μL of internal standard solution in methanol were added; after centrifugation the supernatant was injected in the TurboFlow for further purification. Steroids were determined using a TSQ Vantage operating with an atmospheric pressure chemical ionization source. Method was fully validated and results compared with immunoassay methods.

Results: Limit of quantification ranged from 0.02 ng/mL to 1 ng/mL. The precision was lower than 11% and accuracy ranged from 93.5 to 121.6%. The correlation was acceptable for all analytes except for low levels of testosterone. However, the Bland-Altman plots display a positive bias for androstenedione and 17-hydroxyprogesterone, and a negative bias for cortisol and testosterone.

Conclusions: TurboFlow analysis provides a simple and effective clean-up procedure minimizing the interference of the matrix. The presented method is selective, precise, and sensitive being suitable in a clinical laboratory.

1. Introduction

The clinical management of endocrine diseases relies on the sensitive and accurate steroid hormone determination. The simultaneous quantification of a steroid hormones panel is a powerful tool for hormone status investigation, because provides more valuable clinical information than single steroid assay. For this reason, steroid profiles as diagnostic method, play an important role for the clinical evaluation of endocrine disorders, such as the congenital adrenal hyperplasia and other steroid synthesis defects [1,2].

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disease characterized by disorders in the biosynthesis of steroid hormones. A defect of the 21-hydroxylase (CYP21A2) is the cause of >90% of cases of CAH, blocking production glucocorticoids (cortisol decreased) and causing buildup of 17-hydroxyprogesterone (17-OHP) and androgens (androstenedione and testosterone) [3]. Traditionally, 17-OHP have been the major indicator of CAH, because it is the immediate precursor to the enzymatic block [4]. However, considering clinical phenotypes of CAH exhibit significant variability, depending on the extent of the enzyme defect, often quantification of steroid profiles in serum are necessary, compared to the single determination of 17-OHP. For this reason, many authors discuss the importance of using steroid profiles assay for the simultaneous analysis of 17-OHP, androstenedione, and cortisol to improve the diagnosis of CAH [5,6].

Historically, steroid hormones have been measured using immunoassays. However, these methods are subject to interferences by other similar steroids or metabolites, which result in falsely elevated levels [7–12].

Furthermore, immunoassay measurements tend to exhibit high variability at low concentrations with possible erroneous and misleading results. The use of more selective techniques like gas chromatography combined with mass spectrometry (GC–MS) or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) are generally considered the gold standard for steroid hormone measurements because of their accuracy and sensitivity [13–17]. However, steroids analysis using GC–MS methods usually require extraction and purification steps, as well as derivatization prior to analysis, which is less convenient and more time-consuming when applied in a clinical laboratory to

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routine analysis. Furthermore, LC-MS/MS shows major advantages respect to GC–MS in terms of throughput and minimized sample pretreatment.

In the last years several LC-MS/MS procedures have been developed for measuring single steroids or steroid profiles in human serum [18–23].

However, in steroid analysis by LC-MS/MS both sample manipulation and chromatographic separation should be very efficient in order to reduce matrix effect and to separate structurally similar molecules.

Matrix effects due mainly to salts and phospholipids can alter ionization efficiency and have deleterious effects on the analysis [24]. In addition to the chromatography, the sample preparation is also a critical aspect to support the MS/MS method. Several sample treatment strategies have been developed such as protein precipitation, liquid liquid extraction, solid phase extraction and online solid phase extraction.

Among different approaches online solid phase extraction gives more precise and sensitive results in addition to a decrease of analysis time thus this is the best solution for high routine laboratories [25–27].

Turbulent flow is a chromatographic technique that, combining diffusion, chemistry and size exclusion, performs selective sample clean up, prior to HPLC separation and MS analysis, offering more efficient removal of potential interferences and allowing direct injection of biological fluids [28–30].

In this paper, we report the validation data of a rapid and sensitive method for the routine analysis of 17-OHP, androstenedione, cortisol and testosterone in human serum, performing an online sample purification using a TurboFlow system with subsequent analysis by LC-MS/MS.

2. Materials and method

2.1. Chemicals and reagents

Water, acetonitrile, methanol, isopropyl alcohol, acetone (LC-MS grade) were purchased from Merck (Merk KGaA, Darmstadt, Germany) and formic acid (98%) was purchased from Baker (Mallinckrodt Baker Italy, Milan, Italy).

17-OHP, androstenedione, cortisol and testosterone were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and 17-OHP-d_8, androstenedione-d_3, cortisol-d_4 and testosterone-d_3 were purchased from Cambridge Isotope Laboratories (Cambridge Isotope Laboratories, Andover, MA, USA). Stock solutions of single steroid hormones were prepared in methanol at a concentration of 1 mg/mL. The stock solutions were used to prepare calibration curves. Likewise, internal standard (IS) stock solutions of single steroid hormones were prepared in methanol at concentrations of 1.0 mg/mL for androstenedione-d_3 and testosterone-d_3, 2.0 mg/mL for 17-OHP-d_8 and 10 mg/mL for cortisol-d_4. Stock solutions were kept at −80 °C until use.

An eight-point calibration curve (0, 0.03, 0.1, 0.3, 0.6, 1, 5 and 10 ng/mL for 17-OHP, androstenedione and testosterone; 0, 3, 10, 30, 60, 100, 500 and 1000 ng/mL for cortisol) were prepared in water/methanol (50/50, volume/volume) and kept at −80 °C until use. Quantification was achieved by plotting standard/deuterated IS standard peak area ratio versus nominal concentration.

IS mix solutions were prepared by mixing each IS stock solutions to obtain the following concentrations: 2000 ng/mL for cortisol-d_4 and 20 ng/mL for 17-OHP-d_8, androstenedione-d_3 and testosterone-d_3. The IS mix solutions were kept at −80 °C until use.

Lyophilized serum quality controls (QC) purchased from Biocrates (AbsoluteIDQ® Stero17 Kit, BIOCRATES Life Sciences AG, Innsbruck, Austria) were reconstituted according to the manufacturer's instructions. QC were treated according to the sample preparation described below. The QC were kept at −80 °C until use.

2.2. Subjects and treatment

Serum samples from 80 "healthy" blood donors were collected. The study was approved by the institutional Ethics Committee, and an informed written consent was obtained from each subject in accordance with the principles of the Declaration of Helsinki.

IS mix solutions were diluted (1:20), daily, with methanol to obtain an IS working solutions with following concentrations: 1 ng/mL for 17-OHP-d_8, androstenedione-d_3 and testosterone-d_3 and 100 ng/mL for cortisol-d_4.

To 100 μL of serum samples, calibrators and quality controls were added 100 μL of IS working solution in order to deconjugate steroids from their transport proteins.

After vortex mixing and centrifugation (14.000 rpm at 8 °C for 5 min) 100 μL of the supernatant was directly injected into a TurboFlow system.

2.3. Instrumentation

The TurboFlow-LC-MS/MS system consisted of a Transcend™ TLX system, a CTC PAL autosampler, Turboflow pump and triple quadrupole mass spectrometer TSQ Vantage™ equipped with an atmospheric pressure chemical ionization (APCI) operating in the positive ion mode (Thermo Fischer, Palo Alto, CA, USA).

The TurboFlow-LC-MS/MS system was controlled by Aria OS 1.6.3 and Xcalibur™ 2.0.7 software (Thermo Fischer, Palo Alto, CA, USA).

Data acquisition and quantitative analysis were carried out using the mass-spectrometer software, Xcalibur™ 2.0.7. Statistical analysis was performed using Microsoft® Excel® 2010 (Microsoft, USA).

2.4. TurboFlow-LC and MS methods

The TurboFlow-LC method contains a series of steps that control pump flow rate, valve positions, step duration, and mobile phase composition. Mobile phases consisting of solvent A (water containing 0.05% formic acid), B (methanol containing 0.05% formic acid) and C (Acetonitrile:Isopropanol:Acetone 45:45:10 v/v). TurboFlow Cyclone column (50 × 0.5 mm) was equilibrated with water at 0.05% formic acid and delivered at a flow rate of 2.000 mL/min. Analytical column Kynetex C-18 XB column (100 × 2.1 mm; particle size 1.7 μm) (Phenomenex, Torrance, CA, USA) was equilibrated with water/methanol (80:20 v/v) at 0.05% formic acid and delivered at a flow rate of 0.350 mL/min. The chromatographic parameters used in the method and valve-switching profile are shown in Table 1. The injection volume was 100 μL. Total run time, including the online extraction and the elution phases, was 9 min.

Steroid hormones were detected using the highly selected reaction monitoring (H-SRM) mode. Table 2 shows the optimized MS/MS parameters for each steroid and IS and steroid ion transitions. The APCI source parameters were as follows: discharge current, 4.5 V; vaporizer temperature, 350 °C; capillary temperature, 350 °C; sheath gas (N_2) pressure, 50 units; auxiliary gas (N_2) pressure, 30 units; ion sweep gas (N_2) pressure, 0.0 units; collision gas (Ar) pressure, 1.5 mTorr; declustering voltage, 0.0 V.

2.5. Method validation

The method was validated according to the bioanalytical method validation guidelines of the European Medicines Agency [31] testing the following parameters: matrix effect, limit of quantification (LOQ), linearity, imprecision, accuracy, recovery, carry-over and stability.

Matrix effect was evaluated comparing the peak areas of each deuterated IS dissolved in water/methanol and each deuterated IS dissolved into the deproteinezed serum matrix. After the precipitation step, serum and aqueous samples were both added with deuterated IS and subjected to the complete TurboFlow procedure. Results of peak areas