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Quantification of steroid hormones in human serum by liquid chromatography-high resolution tandem mass spectrometry☆

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ABSTRACT

A limited specificity is inherent to immunoassays for steroid hormone analysis. To improve selectivity mass spectrometric analysis of steroid hormones by liquid chromatography-tandem mass spectrometry (LC–MS/MS) has been introduced in the clinical laboratory over the past years usually with low mass resolution triple-quadrupole instruments or more recently by high resolution mass spectrometry (HR-MS). Here we introduce liquid chromatography-high resolution tandem mass spectrometry (LC–MS/HR-MS) to further increase selectivity of steroid hormone quantification. Application of HR-MS demonstrates an enhanced selectivity compared to low mass resolution. Separation of isobaric interferences reduces background noise and avoids overestimation. Samples were prepared by automated liquid–liquid extraction with MTBE. The LC–MS/HR-MS method using a quadrupole-Orbitrap analyzer includes eight steroid hormones i.e. androstenedione, corticosterone, cortisol, cortisone, 11-deoxycortisol, 17-hydroxyprogesterone, progesterone, and testosterone. It has a run-time of 5.3 min and was validated according to the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines. For most of the analytes coefficient of variation were 10% or lower and LOQs were determined significantly below 1 ng/ml. Full product ion spectra including accurate masses substantiate compound identification by matching their masses and ratios with authentic standards. In summary, quantification of steroid hormones by LC–MS/HR-MS is applicable for clinical diagnostics and holds also promise for highly selective quantification of other small molecules.

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1. Introduction

It is a well-known fact that immunoassays for the determination of steroid hormones lack sometimes specificity due to minor structural differences of steroid hormones and related pharmaceuticals [1,2]. Therefore, during the last years numerous methods based on liquid chromatography-mass spectrometry tandem mass spectrometry (LC–MS/MS) have been proposed demonstrating superior selectivity compared to immunoassays [2–6]. Typically, these methods apply low mass-resolution triple quadrupole instruments providing a high sensitivity [7,8].

Recently, instruments with high mass-resolution such as time-of-flight and Orbitrap analyzer have been introduced for analysis of steroids and related compounds in biological material. Thus, LC-HR-MS using an Orbitrap was applied to profile derivatives of estrogens [9], fecal glucocorticoid [10] and steroid hormones in blood and urine [11,12]. Recently, hybrid analytical instruments with high mass resolution, i.e. QExactive and QqTime-of-Flight (QqToF) have been advantageously exploited to increase specificity. Tandem mass spectrometric analysis with HR-MS of the product ions was applied for detection of anabolic steroids in bovine muscle tissue [13], synthetic hormones in animal urine [14], quantification of 25-OH-vitamin D [15,16] and oxysterols [17]. The enhanced selectivity of HR-MS compared to low-mass resolution could be demonstrated by separation of isobaric interferences [16,17]. Up to now LC–MS/HR-MS has not been applied for quantification of steroid hormones in clinical diagnostics.

Here, we present a reliable, fast and sensitive LC–MS/HR-MS method to quantify eight steroid hormones from human serum.

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2. Materials and methods

2.1. Chemicals and reagents

Methanol LC–MS Chromasolv was purchased from Riedel-de-Haën (Seelze, Germany). MTBE, phosphoric acid and sodium chloride were purchased from VWR GmbH (Darmstadt, Germany). Androstenedione, corticosterone, cortisol, cortisone, 11-deoxycortisol, 17-hydroxyprogesterone (17-OHP), progesterone, testosterone, cortisol-9,11,12,12-D_4 and cortisone-2,2,4,6,6,12,12-D_7 were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Corticosterone-2,2,4,6,6,17α,21,21-D_8 was obtained by CDN Isotopes (Quebec, Canada). Androstenedione-2,3,4-$^{13}C_3$, 17-hydroxyprogesterone-2,3,4-$^{13}C_3$, progesterone-2,3,4-$^{13}C_3$, testosterone-2,3,4-$^{13}C_3$ and 11-deoxycortisol-2,2,4,6,6-D_5 were obtained from Cerilliant Corporation, distributed by Sigma-Aldrich Chemie GmbH. Charcoal stripped serum was purchased from Golden West Biologicals Inc., Temecula, Canada.

2.2. Calibration and control samples

A combined standard solution was prepared from stock solutions of standards by dilution with methanol. Charcoal stripped serum has been used as Cal 1. Cal 2 was charcoal stripped serum spiked with the combined standard solution. Pooled serum samples from two healthy female volunteers (45 and 54 years old) were supplemented with the combined standard solution to obtain the Cal 3–7. This results in two stripped serum-based and five matrix-based calibrators in appropriate concentration ranges. The concentration range of the calibration samples used in this method was estimated based on clinical data to cover the endogenous levels of steroid hormones. The concentration ranges in the calibrators are given in Suppl. Table 1.

These in-house calibrators were adjusted by repeated quantification (n = 6) using 6PLUS1® Multilevel Serum Calibrator Set MassChrom® Steroid Panel 1 and Panel 2 obtained from Chromsystems (München, Germany).

Trueness was tested with serum controls MassCheck® Steroid Panel 1 und Panel 2 Serum Controls Level I, II, III purchased from Chromsystems (München, Germany) and AbsoluteIDQ® Quality Controls from Biocrates (Biocrates Life Sciences AG, Innsbruck, Austria). Additionally, pooled serum was used as an in-house quality control. Serum samples were collected from residual patient's material after immunological steroid hormone analysis in clinical routine diagnostics.

European Reference Materials ERM®-DA192 cortisol in serum and ERM®-DA347 progesterone in serum were purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany):

2.3. Sample preparation

All samples were extracted by liquid–liquid extraction with MTBE. Briefly, 100 μl sample/control/calibrator were placed into 96-well deep-wells (2 ml Costar Assay Block; Corning, Amsterdam, The Netherlands). 10 μl of a methanolic solution of internal standards was added and incubated for 10 min under slight movement followed by 40 μl 1 M NaCl solution and 5 μl 50% (w/w) H_3PO_4. Extraction was performed with 1000 μl MTBE. After vigorous shaking and centrifugation, 10 min at 3000 × g, 600 μl of the upper phase were recovered using a Tecan Genesis (Männedorf, Switzerland) robot and transferred to another 96-well deep-well plate. Solvent was removed by vacuum-centrifugation. The samples were re-dissolved in 50 μl methanol/water (70/30 v/v) and sealed with Corning Sealing Mats (Omnilab-Laborzentrum, Bremen, Germany). Calibrators and controls underwent the same sample preparation procedure as the samples.

2.4. LC–MS/HR-MS

Steroid hormones analysis was performed by liquid chromatography-high resolution tandem mass spectrometry (LC–MS/HR-MS). The LC consisted of an UltiMate 3000 XRS quaternary UHPLC pump, an UltiMate 3000 RS column oven and an UltiMate 3000 isocratic pump (Thermo Fisher Scientific Waltham, MA USA) connected to a PAL HTS-xt autosampler (CTC Analytics, Zwingen, CH) and a hybrid quadrupole-Orbitrap mass spectrometer QExactive (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source.

10 μl of the dissolved samples were injected and separated on a Kinetex™ 2.6 μm Biphenyl column (50 × 2.1 mm, Phenomenex, Aschaffenburg, Germany) at a temperature of 40 °C. Mobile phase A consisted of methanol/water (5/95; v/v), mobile phase B was 100% methanol, both containing 0.1% formic acid and 2 mmol/L ammonium acetate.

Gradient elution started at 100% A with a flow rate of 500 μl/min, followed by a linear increase to 68% B in 0.1 min, to 71% B in 2 min and to 82% in 2 min. For column cleaning the methanol percentage and flow were increased to 100% and 800 μl/min within 0.3 min. After flushing for 0.6 min, the solvent composition was changed to 100% A within 0.1 min and hold until 5.3 min at a flow rate of 800 μl/min. To minimize contamination of the mass spectrometer, the column flow was directed only from 1.3 to 4.5 min into the mass spectrometer using a diverter valve. Otherwise methanol with a flow rate of 200 μl/min was delivered into the mass spectrometer using an isocratic pump.

The ion source was operated in the positive ion-mode using the following settings: Ion spray 3500 V, sheath gas 53, aux gas 14, sweep gas 3 and aux gas heater temperature of 450 °C. Capillary temperature was set to 269 °C and the S-lens RF level to 55. Data were collected in PRM mode with the following settings: Resolution 35,000, AGC target: 5e5, maximum IT 100 ms with a multiplex of 2 and quadrupole isolation window of 0.8 *m*/*z*.

Data acquisition and analysis was performed with TraceFinder 3.3 Clinical (Thermo Fisher Scientific), a software module that extracts target ions, generates calibration lines, checks quality controls and ion ratios of quantifier to qualifier ions.

2.5. Immunoassays

Cortisol, testosterone and progesterone were measured by chemiluminescence immunoassays on Siemens ADVIA Centaur XP (Siemens Health Care GmbH, Germany). 17-Hydroxyprogesterone was measured by ELISA from IBL (IBL International GmbH, Hamburg, Germany). All assays were applied according to the instructions of the manufacturers, test definitions from 2016.

3. Results

3.1. Optimization of chromatography

Aim of the current study was to develop an accurate and fast LC–MS/HR-MS method for clinical relevant endogenous steroid hormones in human serum. LC separation has to provide selectivity for isomeric compounds with similar product ions such as corticosterone, 11- and 21-deoxycortisol (precursor mass *m*/*z* 347.2217) or prednisolone, cortisone and aldosterone (precursor mass *m*/*z* 361.2010). We tested three different core-shell materials i.e. XB-C18, pentafluorophenyl (PFP) and biphenyl. The biphenyl phase was selected due to its excellent selectivity concerning separation of isomers (Suppl. Fig. 3). This is in accordance with our recent

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