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The comprehensive characterization of adrenocortical steroidogenesis using two-dimensional ultra-performance liquid chromatography – electrospray ionization tandem mass spectrometry

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ABSTRACT

The perturbation of the homeostasis of adrenocortical steroids plays a fundamental role in several pathological conditions. Currently, only a few of the substances involved in steroidogenesis are routinely analysed in clinical laboratories for the diagnosis of these conditions.

Recently, interest has grown over the development of clinical assays of endogenous steroids using liquid chromatography-tandem mass spectrometry (LC–MS/MS). However, no approaches have assessed the adrenocortical steroidogenesis comprehensively. Here, a novel LC–MS/MS assay is presented for evaluating the serum levels of all respective major substances (aldosterone, androstenedione, dehydroepiandrosterone, dehydroepiandrosterone sulfate, 11-deoxycorticosterone, 11-deoxycortisol, 21-deoxycortisol, dihydrotestosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, corticosterone, cortisol, cortisone, pregnenolone, progesterone and testosterone). The analysis time was 5.5 min following highly efficient solid phase extraction conducted on a novel polymer phase with *N*-polyvinylpyrrolidine branches.

The method was validated in accordance with the respective guideline of the European Medicines Agency. The cross-validation of 8 analytes with immunoassays was also accomplished.

Two-dimensional chromatography allowed the elution of the 16 analytes between 2.3–4.6 min and with a sufficient resolution of isobaric compounds. Quantitation was performed throughout the clinically relevant concentration ranges. Within-run accuracy was 87.1–115%, 90.0–109%, 87.2–111% and 87.6–107% at spiking levels 1 thru 4, while the precision was 4.7–27.9%, 2.9–17.7%, 5.6–13.9% and 1.9–15.0%, respectively. Between-run accuracy was 81.0–119.5, 85.2–113, 87.4–113 and 93.1–113%, respectively, while the precision was 3.4–13.5%, 2.0–10.2%, 2.1–15.0%, and 1.5–6.6%, respectively. In cross-validation studies, the mean percentage differences ranged between −51.4% (dehydroepiandrosterone sulfate) and 17.5% (dehydroepiandrosterone).

The approach allows the comprehensive characterization of the adrenocortical steroid homeostasis in clinical diagnostics.

1. Introduction

The adrenal cortex produces steroid hormones which have a pivotal role in a wide range of physiological processes. Pathological phenomena related to the anatomy or the physiology of the adrenal glands typically result in disorders which affect the entire organism and may even prove fatal. Therefore, patients with congenital adrenal hypoplasia and other inherited endocrine disorders, polycystic ovary syndrome, prostate cancer, adrenal tumors, a history of adrenalectomy or undergoing hormone replacement therapy, to name but a few related conditions which are especially prevalent could benefit considerably from diagnostic tools which allow the fine-tuned characterization of the homeostasis of steroidogenesis.

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There is now a growing interest in developing novel clinical assays which allow the simultaneous analysis of endogenous steroid hormones using liquid chromatography-tandem mass spectrometry (LC–MS/MS) [1–3]. At the same time, there is a hiatus of methods for the comprehensive yet straightforward assessment of the systemic levels of major adrenocortical steroids without requiring specialized instrumentation. Fanelli et al., Lindner et al. and Peitzsch et al. have offered straightforward approaches for assaying 9, 12 and 15 major steroid substances, respectively, using the most widely employed triple quadrupole mass analyzer, but lacking comprehensiveness [4–6]. Other methods are exceedingly laborious due to the requirement of the chemical derivatization of analytes, double sample preparation, or multiple assaying using separate analytical configurations [7–10]. A few works have relied on atmospheric pressure photoionization, ultra-fast or high turbulence liquid chromatography and high-resolution or MS^3 mass spectrometry, which are especially potent technologies but less likely to be accessible to clinical laboratories [9,11–15]. Finally, some authors have focused on the analysis of more specialized sets of substances such as steroid regio- or stereoisomers [10] or specific conjugates [16].

The aim of the present work is to introduce an assay method which meets all the three goals. The approach involves the simultaneous analysis of 16 major, diagnostically important adrenocortical steroids (glucocorticoids, mineralocorticoids, androgens and progestagens), allowing the comprehensive characterization of the adrenocortical steroidogenesis (Fig. 1). Sample preparation is linear and suitable for automation, and the analysis is accomplished rapidly. Finally, the method relies on the use of an ultra-performance liquid chromatograph coupled to a mass spectrometer equipped with an electrospray ion source and a triple quadrupole mass analyzer, the most common configuration found in clinical diagnostic laboratories.

The presented method offers two novel procedures for the analysis of steroid substances using LC–MS/MS. For solid phase extraction during sample preparation, the utility of an alkyl polymer phase containing modified *N*-vinylpyrrolidone branches is demonstrated. Using this phase gave rise to high and reproducible recoveries of the 16 analytes and the 7 internal standards. In addition, two-dimensional chromatographic separation is performed on an octadecylsilica and a biphenyl stationary phase, connected in series, which allows the simultaneous yet very rapid assessment of the 16 substances, as well as the separation of the isobaric analytes and the minor interferences present in all serum samples.

2. Materials and methods

2.1. Chemicals and reagents

Aldosterone (ALDO, 0.1 mg/mL in acetonitrile), androstenedione (AD, 1.0 mg/mL in acetonitrile), corticosterone (17DF, 1.0 mg/mL in acetonitrile), cortisol (F, 1.0 mg/mL in methanol), cortisone (CN, 0.1 mg/mL in methanol), dehydroepiandrosterone (DHEA, 1.0 mg/mL in methanol), dehydroepiandrosterone sulfate sodium salt (DHEAS, 1.0 mg/mL in methanol), 11-deoxycortisol (11DF, 1.0 mg/mL in methanol), 21-deoxycortisol (21DF, 0.1 mg/mL in methanol), 11-deoxycorticosterone (DOC, 0.1 mg/mL in methanol), 5α-dihydrotestosterone (DHT, 1.0 mg/mL in methanol), 17α-hydroxypregnenolone (OHPREG, 0.1 mg/mL in methanol), 17α-hydroxyprogesterone (OHP, 1.0 mg/mL in methanol), pregnenolone (PREG, 0.1 mg/mL in acetonitrile), progesterone (P, 1.0 mg/mL in acetonitrile), testosterone (T, 1.0 mg/mL in acetonitrile), $^{13}C_3$-androstenedione (C_3AD, 0.1 mg/mL in acetonitrile), D_5-dehydroepiandrosterone sulfate (D_5DHEAS, 0.1 mg/mL in methanol), D_8-21-deoxycortisol ($D_8$21DF, 0.1 mg/mL in methanol), D_3-5α-dihydrotestosterone (D_3DHT, 0.1 mg/mL in methanol), and $^{13}C_3$-testosterone (C_3T, 0.1 mg/mL in acetonitrile) were certified reference materials purchased from Sigma-Aldrich Kft (Budapest, Hungary). D_8-cortisone (D_8CN, 99% pure) and D_9-progesterone (D_9P, 99% pure) were obtained from Bio-Kasztel Kft (Budapest, Hungary). Acetonitrile, formic acid, methanol and water, all LC–MS grade, and NORIT GSX Ultra steam activated, acid washed charcoal were purchased from VWR International Kft (Debrecen, Hungary).

2.2. Calibrators, quality control samples and internal standards

Chromsystems MassChrom Steroids 6PLUS1 Multilevel Serum Calibrator Set MassChrom® Steroid Panels 1 and Panel 2 (lots 2016 and 3016, respectively), and trilevel Chromsystems MassCheck® Steroid Panel 1 (level I: lot 3815, levels II and III: lot 2016) and Panel 2 (lot 0616) Serum Controls were purchased from ABL&E-JASCO Magyarország Kft. (Budapest, Hungary).

The internal standard (IS) mix contained C_3AD, $D_8$21DF, D_3DHT, D_9P and C_3T at 2.6 ng/mL, D_8CN at 9.4 ng/mL and D_5DHEAS at 24 ng/mL in acetonitrile. DOC, 21DF, OHPREG and PREG were spiked to Panel 2 calibrators and controls. Calibrator ranges and quality control levels are displayed in Supplementary Table 1.

2.3. Sample preparation

Human samples used for method development, validation and cross-validation were collected in accordance with the effective legislation. Leftover human serum samples, collected for routine hormone assays, were selected, anonymised and provided for use by the Central Laboratory and the Endocrine Department of our Institute. 0.2 mL serum sample was mixed with 0.05 mL internal standard solution. 0.6 mL ice-cold methanol was added, followed by brief vortexing and centrifugation at 13000*g* for 5 min 0.65 mL supernatant was separated and diluted with 1.3 mL water.

Solid phase extraction was carried out on Phenomenex Strata™-X 33 μm, 60 mg, 2 mL polymeric reversed phase 96-well plates (Gen-Lab Kft, Budapest, Hungary). The phase was conditioned with 1.95 mL methanol followed by 1.95 mL water-methanol (3:1, v/v). The diluted sample supernatants were applied and allowed to drip through. The wells were washed 3 times with 1.95 mL water-methanol (3:1, v/v). After drying the wells under full vacuum for 2 min, the analytes were eluted by applying 2 × 0.85 mL acetonitrile-methanol (1:1, v/v). The eluates were evaporated to dryness, reconstituted with 0.045 mL methanol-water (1:1, v/v) and submitted for analysis.

2.4. 2D-UHPLC–MS/MS conditions

Analysis was performed on a Shimadzu in vitro diagnostics (IVD) certified ultra-performance liquid chromatograph-triple quadrupole tandem mass spectrometer (UHPLC–MS/MS) consisting of the following modules: two Nexera LC30AD pumps, a SIL-30AC automated liquid sampler, a CTO-20AC column oven, an LCMS-8060 triple quadrupole mass spectrometer, a Peak Genius 1051 nitrogen and dry air generator and the LabSolutions MS version 5.89 software (Simkon Kft., Budapest, Hungary). Two-dimensional chromatographic separation was performed by connecting a Phenomenex Kinetex XB-C18 to a Phenomenex Kinetex Biphenyl (both 50 × 2.1 mm, particle size: 1.7 μm) analytical column, thermostated at 40 °C. The mobile phase consisted of water-formic acid (99.9:0.1, v/v, A) and methanol-formic acid (99.9:0.1, v/v, B). The following gradient program was used (% B): initial, 60%, 1.00 min, 60%, linear ramp to 4.00 min, 100%, 4.50 min, 100%, 4.51 min, 60%. The run time was 5.50 min, the cycle time was approximately 7.5 min, and the injection volume was 5.0 μL. The mass spectrometer, equipped with an electrospray ionization

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