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Analysis by LC–MS/MS of endogenous steroids from human serum, plasma, endometrium and endometriotic tissue

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ABSTRACT

An LC–MS/MS method was developed and validated to analyze simultaneously estrogens (estradiol, E2; estrone, E1), androgens (testosterone, T; androstenedione, A4; dehydroepiandrosterone, DHEA), progestagens (17a-hydroxypregnenolone, 17OHP5; 17a-hydroxyprogesterone, 17OHP4; progesterone, P4), glucocorticoids (cortisol, F; cortisone E; corticosterone, B; 11-deoxycortisol, S; 21-hydroxyprogesterone, 21OHP4), and mineralocorticoids (aldosterone, A) from 150 μl of human serum, plasma, or endometrium and endometriotic tissue homogenates. Samples spiked with isotope-labeled steroids as internal standards were extracted with toluene prior to LC–MS/MS analysis. The chromatographic separation of underivatized steroids was achieved on a biphenyl column with 0.2 mM NH_4F as the eluent additive and a water-methanol gradient to improve E2 and E1 ionization. Method validation was performed with human plasma samples, and analysis of certified E2, T, F, and P4 reference serums (BCR-576, ERM-DA346, ERM-DA192, ERM-DA347), as well as homogenates of endometrium and endometriotic tissue. A total of 27 steroids were included in the method development to ensure the specificity of the method. After validation, the method was found suitable for quantitative analysis of 11 steroids: E2 (6.7 pM-13 nM), E1 (1.3 pM-6.6 nM), T (3.3 pM-13 nM), A4 (13 pM-33 nM), 17OHP5 (32 pM-65 nM), 17OHP4 (33 pM-13 nM), F (33 pM-133 nM), E (13 pM-130 nM), B (33 pM-134 nM), S (13 pM-129 nM), and A (32 pM-32 nM). In addition, DHEA (333 pM-32 nM), P4 (13 pM-13 nM) and 21OHP4 (13 pM-13 nM) can be analyzed semi-quantitatively.

1. Introduction

Steroids are present in low pM to nM quantities in human serum and tissues, and thus require highly sensitive methods for their measurements. Despite the low concentrations of these compounds, they are involved in various central physiological processes in both embryonic development and adulthood as well as playing an important role in the pathophysiology of several diseases, including endometriosis. In addition to the wide range of endogenous steroids and their numerous metabolites, there are steroid-based compounds used as doping agents in sports, but more importantly various exogenous and often synthetic steroids are utilized in the treatment of various diseases, and are widely used as contraceptives. Thus, it is challenging to develop reliable, specific and sensitive methods to quantify endogenous steroids

Abbreviations: A, aldosterone; A4, androstenedione; B, corticosterone; E, cortisone; E1, estrone; E2, estradiol; F, cortisol; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; P4, progesterone; P5, pregnenolone; S, 11-deoxycortisol; T, testosterone; 17OHP4, 17a-hydroxyprogesterone; 21OHP4, 21-hydroxyprogesterone; 17OHP5, 17a-hydroxypregnenolone; ACN, acetonitrile; CE, collision energy; CID, collision-induced dissociation; dMRM, dynamic multiple reaction monitoring; ESI, electrospray ionization; IS, internal standard; LLE, liquid-liquid extraction; LLOQ, lower limit of quantitation; ME, matrix effect; MTBE, *tert*-butyl methyl ether; QC, quality control; QL, qualifier ion transition; QT, quantifier ion transition; RSD, relative standard deviation; tR, retention time.

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in body fluids and tissues [1–4]. Typically steroid concentrations are measured by using mass spectrometry (MS), connected to liquid chromatography (LC) or gas chromatography (GC), or by using immunometric methods. Immunoassays have a high sample throughput, but chromatographic methods connected to MS are more specific. This is due to the close structural similarities between many steroids, and the possible interference caused by other endogenous and exogenous compounds affecting the immunoassays. MS-based measurements may incorporate different derivatization strategies to improve specificity and sensitivity, or they may be performed without derivatization [1–3,5].

Every method has both advantages and disadvantages, and when several steroids are to be measured with the same method from one sample simultaneously, compromises may be needed for some lower priority compounds, whereas other compounds can be measured reliably even at very low concentrations. For example, the ionization efficiency is poor for some steroids, and a collision-induced dissociation (CID) may provide only nonspecific and/or low-intensity fragment ions, potentially reducing sensitivity and specificity. Although derivatization can be used to enhance the detection characteristics of steroids, in the derivatization procedure, several isomers may be formed, and the rate of derivatization may vary, leading to quantification problems if multiple derivatizable functional groups are present in the analyte [1,2]. Thus, the optimal conditions are always analyte-dependent.

In the present study, we developed and validated an LC–MS/MS method for the simultaneous analysis of steroids including estrogens (estradiol, E2; estrone, E1), androgens (testosterone, T; androstenedione, A4; dehydroepiandrosterone, DHEA), progestogens (17a-hydroxypregnenolone, 17OHP5; 17a-hydroxyprogesterone, 17OHP4; progesterone, P4), glucocorticoids (cortisol, F; cortisone, E; corticosterone, B; 11-deoxycortisol, S; 21-hydroxyprogesterone, 21OHP4) and a mineralocorticoid (aldosterone, A) in human serum, plasma and endometrium or endometriosis tissue homogenates. Analytical conditions were selected such that the E2 measurement would be as sensitive and reliable as possible.

2. Material and methods

2.1. Reagents

Chemicals were purchased as follows: Methanol (LC–MS Ultra chromasolv, tested for UHPLC–MS, ≥99.9%) from Riedel-de Haën, acetonitrile (ACN, LC–MS grade, min 99.9%) from BDH Prolabo Chemicals, VWR, ammonium fluoride (NH_4F, eluent additive for LC–MS, ≥98.0%) from Fluka, toluene (Chromasolv plus for HPLC, ≥99.9%) from Sigma-Aldrich and NaCl solution (9 mg/ml) from Braun Medical Oy, Finland.

Steroids were purchased as follows: A, androsterone, E, E1, E2, estriol, etiocholanolone, pregnenolone (P5), 17OHP5, DHEA, 17OHP4, and 21OHP4 from Sigma, A4 from Riedel-de Haën, androstanedione, 5-androstenediol, F, B, dihydrotestosterone (DHT), and P4 from Steraloids, S from Toronto Research Chemicals, and T from Fluka. The specificity of the developed method was also tested with the following compounds: 11-ketoandrostenedione from Sigma, and 11-ketotestosterone, 11-ketodihydrotestosterone, 11a-hydroxyandrostenedione, 11b-hydroxyandrostenedione, 11b-hydroxytestosterone from Steraloids.

Isotope-labeled steroids were purchased as follows: d8-B and d8-E from Toronto Research Chemicals, d7-A4, d3-DHT, d4-E2, d4-E1, d9-P4, d8-17OHP4 from Steraloids, d4-P5, and d3-17OHP5 from C/D/N Isotopes, d2-13C2-17OHP5 and d8-A from IsoSciences, d6-DHEA from Isotec, d5-S and d4-F from Aldrich and d3-T from Fluka. The reference serum samples for E2 (BCR-576), F (ERM-DA192) and P4 (ERM-DA347) were from Sigma-Aldrich, and reference serum for T (ERM-DA346) was from LGC standards. The reference serum for E2 was lyophilized material, which was diluted with water according to the manufacturer's instructions.

2.2. Plasma and tissue homogenate samples

The study on the endometriosis specimens was approved by the Joint Ethics Committee of Turku University and Turku University Central Hospital in Finland (The study approval number ETMK 34/180/2012). A written informed consent was provided by all study subjects prior to sampling. Samples of endometriosis and eutopic endometrial biopsies were collected from endometriosis patients, and endometrial biopsies from women undergoing laparoscopic tubal ligation. Patients were diagnosed and endometriosis samples collected during laparoscopy or laparotomy, and endometriosis was confirmed by histopathological evaluation. In the endometrium samples, endometriosis was excluded by laparoscopy during tubal sterilization. All samples were homogenized in cold 9 mg/ml NaCl (700 mg tissue/7 ml saline) with an ultra-turrax on ice for 2–3 min. The homogenates were centrifuged and the supernatant was transferred to another tube, except for 100 μl, which was left with the pellet. The pellet with the 100 μl supernatant was further homogenized with TissueLyzer steel beads (50 Hz, 2 × 1 min) after which the homogenate was pooled with the supernatant. Samples were then carefully mixed, aliquoted to 1 ml aliquots and stored at −80 °C until used. Pooled samples of endometrium from 4 subjects, deep endometriosis from 7 patients and ovarian endometriosis from one patient were used in all studies, except for when testing the effect of homogenization solution on the assay of tissue steroids. In this experiment, deep endometriotic lesions from 6 patients were divided into two parts. One half was homogenized in water and the other half in 9 mg/ml NaCl. Homogenization was conducted as described above. The plasma used for method validation was FFP8 plasma obtained from the Finnish Red Cross.

2.3. LC–MS/MS instrumentation and analytical conditions

The LC separation was performed using an Agilent 1290 Rapid Resolution LC System (Agilent Technologies) and the mass analysis was carried out with an Agilent 6495 Jet Stream (AJS) ionization triple quadrupole mass spectrometer (Agilent Technologies). Data acquisition and quantification were conducted with Agilent MassHunter Workstation software (Agilent Technologies). The column used was Kinetex biphenyl (100 × 2.1 mm, 1.7u), protected with a SecurityGuard Ultra biphenyl guard cartridge for 2.1 mm ID columns (Phenomenex). The eluent flow rate was 300 μl/min and the eluents were 0.2 mM NH_4F in water (eluent A) and 0.2 mM NH_4F in methanol:water 95:5 (v/v) (eluent B). The following gradient profile was used: 0–3.5 min 40 → 68% B; 3.5-9.5 min 68 → 71% B; 9.5-13.5 min 71 → 80% B; 13.5-14.5 min 80 → 100% B; 14.5-19 min 100% B; 19-19.1 min 100 → 40% B; 19.1-21 min 40% B. The column temperature was held at 35 °C, and the autosampler temperature at 10 °C. The injection volume was 40 μl. The injection was performed using a 5 s needle wash with ACN in water 1:1 (v/v).

A divert valve was used to direct the eluent flow into the mass spectrometer from 3 min to 17 min after injection. The following ionization conditions were used: Electrospray ionization (ESI), drying gas (nitrogen) temperature 210 °C, drying gas flow rate 16 l/min, nebulizer gas pressure 23 psi, sheath gas temperature 400 °C, sheath gas flow 11 l/min, capillary voltage 3000 V (ESI+) and 4000 V (ESI-), and nozzle voltage 0 V (ESI+) and 1500 V (ESI-). The ion funnel parameters were as follows: High-pressure ion funnel RF voltage (HPRF) 110 V (ESI+) and 210 V (ESI-), and low-pressure ion funnel RF voltage (LPRF) 100 V (ESI+) and 160 V (ESI-).