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A chromatography/tandem mass spectrometry method for the simultaneous profiling of ten endogenous steroids, including progesterone, adrenal precursors, androgens and estrogens, using low serum volume

Patrick Caron, Véronique Turcotte, Chantal Guillemette *

Pharmacogenomics Laboratory, Centre Hospitalier Universitaire de Québec Research Center and Faculty of Pharmacy, Laval University, Québec, Canada

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ABSTRACT

Measurement of a large set of sex steroids in clinical epidemiology and laboratory research with reliable methods providing low quantification limits and using a limited volume of blood sample represents a significant challenge. We report a new validated gas chromatography selected reaction monitoring – tandem mass spectrometry assay (GC–MS/MS) for the simultaneous quantification of ten endogenous steroids including progesterone (PROG), dehydroepiandrosterone (DHEA), androstenediol (5-diol), androstenedione (4-dione), testosterone (T), dihydrotestosterone (DHT), androsterone (ADT), 5alpha-androstan-3beta-17beta-diol (3β-diol), estrone (E1) and estradiol (E2). After addition of stable isotope internal standards, the approach involved the combination of liquid–liquid extraction, derivatization and solid-phase extraction for injection into the GC system and multiple reaction monitoring (MRM). The method presents high reproducibility for all analytical parameters in 250 µl serum samples. The lower limit of quantification (LLOQ) were of 100 pg/ml for DHEA, 50 pg/ml for PROG, 5-diol, 4-dione and ADT, 30 pg/ml for T, 10 pg/ml for 3β-diol and DHT, 5 pg/ml for E1, and 1 pg/ml for E2. The applicability of the validated method to determine the concentrations of these 10 steroids was successfully tested on serum from men ($n = 15$), premenopausal ($n = 10$) and postmenopausal women ($n = 20$), and is currently used for larger cancer-related epidemiology studies. One of the most considerable advantages over existing methods is the simultaneous determination of ten steroids in a limited volume of serum that will help conserve important clinical samples from existing biobanks.

1. Introduction

Steroids derived from cholesterol and synthesized in the adrenal cortex, the gonads, and a variety of peripheral tissues play a critical role in transmitting a vast array of biological signals in the organism. Quantitative assessment of sex-steroid hormones and their adrenal precursors is relevant to the diagnosis and treatment of a variety of diseases and conditions, including disorders of puberty, amenorrhea, infertility, polycystic ovary syndrome, osteoporosis, adrenal insufficiency, hypogonadism, cognitive dysfunction, cardiovascular diseases and hormone-related malignancies. Current methods especially those based on radioimmunoassay suffer from poor sensitivity and especially lack of specificity due to endogenous and exogenous interferences explained by a lack of specificity of antibodies claimed to measure a particular steroid [1–4]. Most particularly, high sensitivity and specificity mass spectrometry-based assays for clinically important endogenous steroids such as testosterone and estradiol are needed in most clinical situations [5,6]. For instance, at menopause, ovarian biosynthesis ceases therefore low levels of estradiol are present in circulation and as such, high-sensitivity estradiol assays are challenging [7]. High specificity is also required because of the multiplicity of related steroids and this is well exemplified for estradiol that is converted to more than 100 conjugated and unconjugated metabolites [8]. Steroid biomarkers are also used for the management of deprivation therapy such as aromatase (CYP19A1) inhibitors and 17α-hydroxylase/C17, 20-lyase (CYP17A1) inhibitors used in breast and prostate cancers, and for detection of emerging resistance [9–12]. Over the last decade, a plurality of GC–MS and LC–MS methods for the determination of

* Corresponding author at: Canada Research Chair in Pharmacogenomics, Centre Hospitalier Universitaire de Québec Research Center, R4720, 2705 Boul. Laurier, Québec G1V 4G2, Canada.
E-mail address: chantal.guillemette@crchudequebec.ulaval.ca (C. Guillemette).

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different steroids in human serum have been published [13–20]. However, several assays are often limited to one or few steroids whereas a profile of multiple steroids is often required to better comprehend androgen and estrogen exposure and because these steroids have different biological properties and even opposite biological effects [8,21–24]. Therefore, there is a necessity for more specific, sensitive, and reliable method in addition to assays providing information on multiple steroids.

The aim of this study was to develop a multiplex assay based on gas chromatography–mass spectrometry while using a small sample volume, to measure a broad spectrum of steroids in serum including progesterone (PROG), dehydroepiandrosterone (DHEA), androstenediol (5-diol), androstenedione (4-dione), testosterone (T), dihydrotestosterone (DHT), androsterone (ADT), 5alpha-androstan-3beta-17beta-diol (3β-diol), estrone (E_1) and estradiol (E_2) (Fig. 1). To reduce complexity for the simultaneous detection of numerous steroids, a solid-phase extraction (SPE) method was applied. To allow for more efficient ionization and solubility during the analytical process, steroids were chemically derivatized prior to GC–MS/MS analysis. The derivatization prevents contamination along the subsequent steps of sample preparation by endogenous contaminants. Moreover, derivatization permits better compounds volatility and improved sensitivity with GC. Finally, the method was applied to clinical samples from healthy men, premenopausal and postmenopausal women. To our knowledge, no published and validated method has been accurate and sensitive for simultaneous measurement of this panel of 10 steroids while using a small serum volume of 250 μl. Our method provides high-resolution separation of analytes, improves specificity (tandem MS), wide dynamic range and enhanced sensitivity required for clinical epidemiologic studies and laboratory research.

2. Material and methods

2.1. Material

2.1.1. Chemicals

E_1 (99.9%) and E_2 (99.9%) were purchased from USP reference standard (Rockville, USA), ADT (99.5%), DHT (95.8%), Testo (99.9%), 4-dione (99.9%), DHEA (98.6%), 5-diol (97.3%) and Prog (99.9%) were purchased from Steraloids (Newport, USA). 3β-diol (99.6%) was purchased from Research Plus (Barnegat, USA). Purity of the reference standard has been established. Stable isotope internal standards consist of d4-E_2, d4-E_1, d7-4-dione, d3-Testo, d3-DHT, d3-5-diol, d5-ADT, d3-3β-diol and d9-Prog that were purchased from CDN isotopes (Pointe-Claire, Canada). d3-DHEA was synthesized by the Organic Synthesis Service of the CHU de Québec Research Center (Québec, Canada). Pentafluorobenzoyl chloride (PFB-Cl), pentafluorobenzoyl amine (PFB-NH_2), acetic acid, sodium bicarbonate and sodium acetate were purchased from Sigma (Oakville, Canada). Pyridine was purchased from Chromatography Specialities Inc (Brockville, Canada). Hexanes, 1-chlorobutane, ethyl-acetate and iso-octane were purchased from EMD (Mississauga, Canada). Reagent alcohol (Anhydrous Ethyl Alcohol 90% ±1% v/v; Methyl Alcohol approx. 5% v/v; 2-Propanol approx. 5% v/v) was purchased from Fisher Scientific (Ontario, Canada). The human sera (men and premenopausal women) were purchased from Bioreclamation LLC (Westbury, NY, USA). The free steroids matrix used was charcoal-adsorbed serum prepared in-house. Briefly, pooled serum (50 ml) was treated overnight with activated charcoal (6.0 g) in a horizontal shaker at 4 °C and then centrifuged for 20 min at 2000*g*. The stripped serum samples were stored at −20 °C.

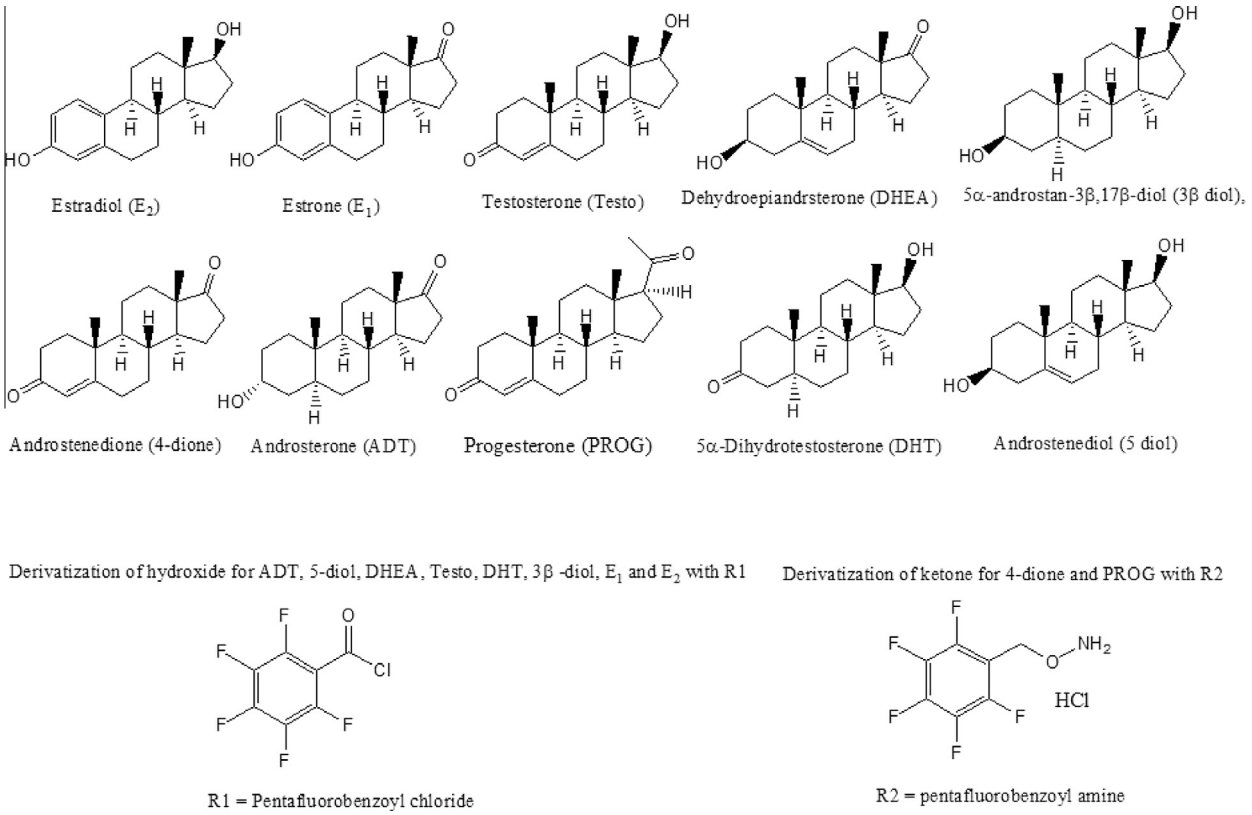

Fig. 1. Chemical structures of quantified steroid analytes.