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Development of a solid phase extraction method for the simultaneous determination of steroid hormones in H295R cell line using liquid chromatography–tandem mass spectrometry

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ABSTRACT

The H295R in vitro cell line produces the majority of the steroidogenesis, for which reason it is commonly used as a screening tool for endocrine disrupting chemicals. Simultaneous determination of the precursor cholesterol and key steroid hormones could give a broad insight into the mechanistic disruption of the steroidogenesis. Steroid hormones have primarily been extracted from H295R incubation medium by means of liquid–liquid extraction (LLE) and the obtained recoveries and matrix effects have typically not been stated or assessed. In the present study a solid-phase extraction (SPE) method was developed and validated for the simultaneous extraction of cholesterol and five key steroid hormones pregnenolone, 17-hydroxyprogesterone, testosterone, cortisol and aldosterone from H295R incubation medium, and finally detected by LC–MS/MS. Cholesterol was recovered at a level of 55.7%, while steroid hormone recoveries ranged from 98.2 to 109.4%. Matrix effects varied between −0.6% and 62.8%. Intra-day precision was deemed acceptable, but the inter-day precision for pregnenolone and aldosterone exceeded the precision limit of 15% RSD. Although LLE has been the most frequently used extraction method in H295R studies, however, our investigation has shown that SPE may relatively easily extract and recover steroid hormones, potentially replacing LLE.

1. Introduction

The adrenal cortex of the adrenal gland has the capability of producing steroid hormones from the precursor cholesterol [1], and the enzyme catalyzed reactions in the formation of these steroid hormones are collectively called the steroidogenesis [2]. Human adrenocortical carcinoma cells (H295R cells) have the same physiological characteristics as fetal cells of the adrenal cortex and thus can produce steroid hormones from all zones in the adult, healthy adrenal cortex [3–5]. The H295R human adrenocortical carcinoma cell line has therefore been presented as a rapid in vitro tool to determine the effects of endocrine disruption chemicals on the steroidogenesis [6]. By exposing these cells to possible endocrine disrupting chemicals, and thereafter determine the level of secreted steroid hormones relative to control incubations, allows for a more mechanistic understanding of steroidogenesis disruption [7].

Recently, the Organization for Economic Cooperation and Development (OECD) guideline for testing of various chemicals ability to disrupting the steroidogenesis was released [8], focusing on the expression of estradiol and testosterone as endpoints. Endocrine disrupting chemicals may however affect multiple parts of the steroidogenesis, and an effect on one part of the steroidogenesis may potentially lead to disruption of another part of the pathway. Consequently development of analytical methods capable of simultaneously analyzing multiple steroid hormones is crucial [9,10].

Extraction of organics in water samples has classically been performed by means of liquid–liquid extraction (LLE) and is also the most common sample preparation method for steroid hormones in H295R incubation medium [7,11–25]. However, LLE methods

Abbreviations: 17OHProg, 17-hydroxyprogesterone; ALD, aldosterone; Chol, cholesterol; Chol IS, d_6-cholesterol; F, cortisol; F IS, d_4-cortisol; GC, gas chromatography; H295R, human adrenocortical carcinoma cell line; HPLC, high performance liquid chromatography; IS, internal standard; LC, liquid chromatography; LOD_{instr}, instrumental limit of detection; LOQ_{instr}, instrumental limit of quantification; LLE, liquid–liquid extraction; MS/MS, tandem mass spectrometry; OECD, organization for economic cooperation and development; Preg, pregnenolone; RSD, relative standard deviation; SPE, solid phase extraction; T, testosterone; T IS, d_3-testosterone.

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are often labor-intensive, and time-consuming, and require strict control of experimental conditions and generally involve large volumes of solvents (typically ether in the case of steroid hormones) [26,27]. Solid-phase extraction (SPE) often offers a more sophisticated approach, with faster extraction time, lowering of hazardous solvent consumption, and no emulsion formation [28]. Yet the number of publications utilizing SPE for the extraction of steroid hormones from H295R incubation medium has been limited [9,29–31]. Furthermore these studies do not clearly state the SPE recoveries of steroid hormones of the developed method. Consequently there is a lack of knowledge concerning the performance of SPE during sample preparation and extraction of steroid hormones from H295R media. Liquid chromatography (LC–MS/MS) [11–15,19–22,29–31] and gas chromatography–tandem mass spectrometry (GC–MS/MS) [9,10,29] have been utilized to simultaneously determine several extracted steroid hormones secreted by H295R cells. LC–MS/MS is generally preferred over GC–MS/MS due to time-consuming derivatization processes often being required prior to analysis by GC–MS/MS combined with longer times of analysis in GC–MS/MS [10,32].

In this study six steroid hormones were selected as endpoints. Structures and physicochemical properties of these can be seen in Table 1. Cholesterol, pregnenolone, 17-hydroxyprogesterone, testosterone, cortisol and aldosterone were chosen, as they cover the majority of the adrenal steroidogenesis. They vary in physiochemical properties as shown in Table 1, and each endpoint represents a different steroid class: pregnenolone for progestagens, 17-hydroxyprogesterone for hydroxylated progestagens, testosterone for androgens, cortisol for glucocorticoids and aldosterone for mineralocorticoids. Another endpoint selection criterion was the physiological significance of the steroid. Cholesterol and pregnenolone are suitable endpoints, as they are implied in the rate limiting steps of the steroidogenesis [1,33]. The level of 17-hydroxyprogesterone is a frequently determined endpoint in the clinical settings, as its blood level can be used to assess the functionality of the adrenal gland, which is typically done for babies [34]. Furthermore 17-hydroxyprogesterone is an important endpoint in the screening for congenital adrenal hyperplasia in infants [34]. Testosterone is essential for the development and maintenance of the male phenotype [35]. Cortisol was selected, as it regulates the metabolism, stress and immune response [36], and is commonly analyzed in clinical settings to diagnose adrenal hyperfunction or insufficiency [37]. Finally aldosterone was selected, as it is essential for the regulation of the extracellular fluid volume by increasing sodium reabsorption and stimulating potassium excretion by the kidneys [38]. Although included in the OECD guideline [8] estradiol, an estrogen, was not analyzed as it would complicate LC–MS/MS analysis which was desired to be kept as simple as possible. Estrogens if nonderivatized are usually analyzed in negative mode while the remaining steroid hormones if nonderivatized typically are analyzed in positive mode [27].

The aim of this study was to develop and validate a SPE method capable of simultaneously extracting cholesterol and five key steroid hormones from H295R incubation medium; viz. cholesterol, pregnenolone, 17-hydroxyprogesterone, testosterone, cortisol and aldosterone. To achieve this goal a simple LC–MS/MS method capable of simultaneously analyzing the six steroid hormones also had to be established as part of the investigation, which is described in some detail in the first part of this paper.

2. Materials and methods

2.1. Materials and reagents

Cholesterol, pregnenolone, 17-hydroxyprogesterone, testosterone, cortisol and aldosterone were all purchased from Sigma–Aldrich (Glostrup, Denmark). The level of purity was above 99% for cholesterol, while it was above 96% for the remaining steroid hormones. Deuterated analogs were used as internal standards (IS); d_6-cholesterol, d_4-cortisol and d_3-testosterone were obtained from Toronto Research Chemicals (North York, ON, Canada), all with a isotopic purity above 98%. Ammonium acetate had a purity level above 98% and was purchased from Sigma–Aldrich (Glostrup, Denmark). All utilized solvents were of analytical grade and obtained from Lab-scan analytical sciences (Fisher Scientific Biotech Line, Slangerup, Denmark). Stock solutions (in the range 100–500 μg/mL) of each internal standard were prepared in methanol. From the stock solutions a methanol mixture was prepared containing the internal standards at a concentration of 37.5, 4.5 and 15.0 μg/mL for d_6-cholesterol, d_3-testosterone and d_4-cortisol, respectively. Each sample of H295R incubation medium was spiked with 20.0 μL IS mixture, i.e. 750, 90, and 300 ng of d_6-cholesterol, d_3-testosterone and d_4-cortisol, respectively. The concentration of each non-labeled steroid hormone in a mixture dissolved in methanol was 37.6, 37.7, 3.2, 4.5, 15.0 and 15.0 μg/mL for cholesterol, pregnenolone, 17-hydroxyprogesterone, testosterone, cortisol and aldosterone, respectively. Each sample was either pre- or post-spiked with 20.0 μL mixture, i.e. an absolute amount of 752, 754, 64, 90, 300 and 300 ng of cholesterol, pregnenolone, 17-hydroxyprogesterone, testosterone, cortisol and aldosterone, respectively. H295R incubation medium composed of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture medium (GibcoBRL Life Technologies, Paisar, UK) supplemented with 10 mL/L of ITS+ premix (BD Bioscience, Brøndby, Denmark) and 25 mL/L Nu-serum (BD Bioscience, Brøndby, Denmark).

2.2. Chromatographic and mass spectrometric conditions

The modular HPLC (High Performance Liquid Chromatography) system (Agilent 1100 Series; Agilent Technologies, Palo Alto, CA, USA) comprised of an autosampler held at 4 °C (model G1367A), a column compartment oven kept at 30 °C (G1316A), a quaternary pump (G1311A) and a degasser (G1322A). Chromatographic separation was carried out on a XTerra MS C18 analytical column (100 mm × 2.1 mm, 3.5 μm particles with 125 Å pore size). Mobile phases A and B composed of methanol:water at a v/v-ratio of 50:50 and 99:1, respectively, both with 2.5 mM ammonium acetate. Gradient elution of the steroid hormones was performed by pumping 100% mobile phase A isocratically from minutes 0 to 14 which over the course of 1 min was changed to 100% mobile phase B that then was pumped isocratically from minutes 15 to 24. After the steroid hormone elution mobile phase A was pumped again isocratically from minutes 25 to 35 for system re-equilibration. Flow rate was set to 250 μL/min and an injection volume of 10 μL was used. For detection an API-2000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray source was operated in positive mode under multiple reaction monitoring conditions during analysis. The ion transitions for the MS/MS analysis of each analyte and internal standard are listed in Table 2, which also includes the tandem mass spectrometer working parameters. Obtained chromatographic peak areas were acquired by the Analyst 1.4 software package (MDS Sciex) and processed in the same software, along with Microsoft Office Excel 2007 and GraphPad Prism v. 5.0 (GraphPad Software, San Diego, CA, USA).

2.3. Sample preparation

The final SPE method contained the following procedural steps. In order to stabilize the steroid hormones 1.5 mL H295R incubation medium was initially pH adjusted to pH 3.0 ± 0.1 with diluted