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Quantitative analysis of estradiol and six other steroid hormones in human saliva using a high throughput liquid chromatography–tandem mass spectrometry assay

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ABSTRACT

The aim of the current research was to develop a fast and sensitive analytical strategy that allows for the simultaneous measurement of estradiol and other steroid hormones in human saliva. For this purpose, we established an assay using high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) with Atmospheric Pressure Chemical Ionization (APCI) coupled with on-line solid phase extraction (SPE). The protocol was designed for the simultaneous identification of estradiol, cortisol, cortisone, testosterone, progesterone, corticosterone and dehydroepiandrosterone (DHEA) from samples of 100 μL saliva. After protein precipitation, the sample was injected into the LC–MS/MS system for direct measurement. The protocol involved minimal sample preparation and could be run with throughput times of 5.20 min.

Results indicated linearity of the method for all steroid hormones over ranges of 0.001–10 ng/mL (0.01–20 ng/mL for DHEA) with linear correlation coefficients of $r=0.999$ for each steroid. Intra- and inter-assay coefficients of variance were between 4.3% and 10.8%. The lower limits of quantification (LOQ) were below (or equal to) 5 pg/mL for all steroids, except for DHEA for which the LOQ was 10 pg/mL. A re-analysis of 16 saliva samples that had produced unusual estradiol values when quantified by immunoassay showed normal, endogenous concentrations for all samples when measured by the current method.

In conclusion, we describe an LC–MS/MS method which can be routinely employed in physiological and psychological research laboratories allowing high analytical specificity and sensitivity despite minimal sample processing and short throughput times.

1. Introduction

Endogenous steroid hormones are of high clinical importance and are frequently assessed as biological markers in psychoneuroendocrine research [1,2]. Traditionally, glucocorticoids have been most investigated in such research works due to their close relation to psychosocial stress exposure [3]. However, other sex steroids and particularly estrogens are also receiving considerable research attention [4–6]. Estradiol, the major estrogen in humans, is important for the development of a female phenotype, germ cell maturation and pregnancy as well as in non-sex-specific processes, such as nervous system maturation, bone metabolism/remodeling, and endothelial responsiveness [7–9]. Clinical monitoring of estradiol levels is further desirable for the study and treatment of hormone-dependent carcinomas [10] or for investigating ovarian function [11]. Research has also linked estradiol levels to psychological processes including resilience, working memory or cognition [12–14]. To assess endogenous estradiol concentrations, besides those of other steroid hormones, saliva is often used as the biological matrix of choice as it can be sampled non-invasively and may easily be collected, even at ambulatory settings. Further, salivary steroid hormone concentrations are closely correlated with the free, unbound hormone fraction in blood and thus provide an easily obtainable measure of the biologically active hormone fraction [15–17].

With a raised interest in the assessment of salivary steroid hormones [18,19,20], there is an increasing need for sensitive and valid laboratory methods for their detection. To date, immunoassays are most commonly used for the quantification of steroid hormones which is likely due to the fact that they are relatively cheap, easy to conduct and allow a high throughput of samples. However, a major drawback in their use is an overestimation of actual steroid content due to cross-reactivity with other substances. It has been shown that this constitutes a particularly severe problem for estradiol which may result in an

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overestimation of estradiol content by more than 60% [21,22]. In addition, there is a considerable lack of agreement between the results of different estradiol immunoassays, with individual assays showing insufficient analytical accuracy and a rather high detection limit [22,23]. Further to these problems, as immunoassays are restricted to the measurement of only one analyte at a time, no other steroid hormones may be assessed besides estradiol, thus potentially limiting the interpretation of data.

Chromatography-based assays can address these limitations of immunoassays, allowing higher sensitivity and specificity as well as the simultaneous measurement of several steroid hormones [24]. Previously, gas chromatography–mass spectrometry (GC–MS) methods have been used to quantify endogenous steroid hormone levels [25]. However, GC–MS is characterized by time-consuming workup including derivatization steps, long throughput times and the requirement of larger sample volumes. This limits its usability in research contexts in which a high number of samples need to be examined, e.g. epidemiological or population-based research. Besides GC–MS, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has emerged as another highly accurate method for measuring steroid hormones [23,26–30]. LC–MS/MS has been shown to provide superior sensitivity and sample throughput compared to GC–MS in many situations [31–33]. Derivatization has also been used to increase the sensitivity of LC–MS/MS assessments for samples with low steroid hormone levels [24]; however, again, the high expenditure of time associated with this method severely limits its practical applicability.

Recently the use of on-line solid phase extraction (SPE) methods has led to a further improvement of method sensitivity as well as to shorter pretreatment and analysis times [34]. The combination of on-line SPE and LC–MS/MS methodology may thus provide a highly specific and sensitive analytical strategy for the endogenous salivary steroid quantification that is also applicable in an applied or clinical research context. To our knowledge, no on-line SPE LC–MS/MS method without derivatization for the simultaneous identification and quantification of estradiol and other steroids hormones in saliva has been described to date. Here, we thus set out to develop such an analytical protocol.

2. Materials and methods

2.1. Chemicals and reagents

Estradiol, cortisol, cortisone, corticosterone, testosterone, progesterone, dehydroepiandrosterone (DHEA) were purchased from Sigma-Aldrich (Hamburg, Germany). Deuterated internal standard samples (estradiol-d_3, cortisol-d_4, cortisone-d_8, testosterone-d_5, progesterone-d_9, corticosterone-d_8, DHEA-d_6) were purchased from Toronto Research Chemicals Inc. (North York, Canada). LC–MS grade methanol was purchased from Fisher Chemical (Leics, UK). LC–MS grade ammonium acetate was obtained from Sigma-Aldrich (Hamburg, Germany). Distilled water was deionized by using a Simplicity® Water Purification system (Millipore, USA).

2.2. Preparation of stock and standard solutions

Standards for each hormone were prepared in methanol at final concentrations of 1 mg/mL. The stock solutions were further individually diluted with methanol to give working standard solutions of all agents. Internal standard mixtures were prepared in methanol at the final concentrations (estradiol-d_3: 5 ng/mL, cortisol-d_4: 5 ng/mL, cortisone-d_8: 50 ng/mL, testosterone-d_5: 1.25 ng/mL, progesterone-d_9: 5 ng/mL, corticosterone-d_8: 5 ng/mL, DHEA-d_6: 50 ng/mL). All stock solutions and working standard solutions were stored at 4 °C when not in use.

2.3. Instrumentation

The LC system consisted of three Shimadzu LC-20AD pumps, a Shimadzu SIL-20AC autosampler and a Shimadzu CTO-20AC column temperature oven (Shimadzu, Canby, OR, USA). The LC system was coupled to AB Sciex API 5000 Turbo-ion-spray® triple quadrupole tandem mass spectrometer equipped with Atmospheric Pressure Chemical Ionization (APCI) Source (AB Sciex, Foster City, CA, USA). The system was controlled by AB Sciex Analyst® software (version 1.5.1). Nitrogen and zero grade air were produced by a high purity nitrogen generator (cmc Instruments GmbH, Eschborn, Germany). A Chromolith® Speed ROD RP-18e LC column (4.6 mm × 50 mm) from Merck KGaA (Darmstadt, Germany) was used as on-line SPE column for cleaning up the samples prior to the injection of analytes to the analytical column. The analytical column was a Shim-pack XR-ODS LC column (3.0 mm × 75 mm, 2.2 μm) from Shimadzu (Shimadzu, USA) equipped with a security guard column (security guard cartridge: C18 4 × 2.0 mm^2 ID) from Phenomenex (Aschaffenburg, Germany).

2.4. Chromatographic conditions

2.4.1. On-line SPE methodology

The chromatographic program was accomplished by a similar on-line SPE method as we have previously described for the analysis of steroid hormones in hair [35]. In short, a 6-port switch valve in a column temperature oven controlled by the LC system was used for on-line solid phase extraction. A binary gradient with a single injection sequence table was used.

2.4.2. Liquid chromatography methodology

Details of the LC running conditions are listed in Table 1. Mobile phase A was a mixture of methanol and water (containing 2.0 mM ammonium acetate) in a ratio of 90:10 (v:v). Mobile phase B was a mixture of methanol and water (containing 2.0 mM ammonium acetate; pH value adjusted to 4.5 with acetate acid) in a ratio 5:95 (v:v). The total flow rate of the first binary gradient module was maintained at 0.5 mL/min. Mobile phase C–A was a mixture of methanol and water in a ratio of 10:90 (v:v). Mobile phase C–B was a mixture of methanol and water in a ratio of 90:10 (v:v). The flow rate of the second isocratic module was maintained at 3 mL/min. The column temperature was set at 40 °C. The injection volume was 200 μL.

Table 1
HPLC conditions for the on-line SPE/HPLC analysis of steroids in saliva.

Time (min)	Binary gradient module for LC column (Pump A+B) Pump B (%)	Isocratic module for on-line SPE extraction (Pump C)			Comment
		Flow rate mL/min	Solenoid valve	Valve position	
0	30	3	A	0	Start
1.00				1	
1.20	30				
3.00	10			0	
3.20			B		
4.20	10		A		
4.23	0				
5.00	0				
5.01	30				
5.20	30	3	A	0	End