Talanta 143 (2015) 353–358

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

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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ARTICLE INFO

Article history:
Received 16 January 2015
Received in revised form
28 April 2015
Accepted 2 May 2015
Available online 9 May 2015

Keywords:
Estradiol
Steroid hormones
Saliva
Mass spectrometry
On-line solid phase extraction

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.

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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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http://dx.doi.org/10.1016/j.talanta.2015.05.004
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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

Table 2
MS/MS conditions for the steroid hormone compounds.

Compound	MRM (Da)	DP (V)	EP (V)	CE (eV)	CXP (V)
Estradiol*	255.2→159.1	116.0	10.0	23.0	10.0
Estradiol	255.2→133.1	116.0	10.0	25.5	12.0
Estradiol-d_3	258.2→159.1	116.0	10.0	23.0	10.0
Cortisol*	363.2→121.1	130.0	10.0	36.0	20.0
Cortisol	363.2→327.1	130.0	10.0	22.5	22.0
Cortisol-d_4	367.2→121.1	130.0	10.0	36.0	20.0
Cortisone*	361.2→163.0	132.0	10.0	35.0	10.0
Cortisone	361.2→121.2	132.0	10.0	45.0	19.0
Cortisone-d_8	369.2→169.0	132.0	10.0	35.0	10.0
Testosterone*	289.3→109.0	121.0	10.0	33.0	18.0
Testosterone	289.3→097.1	121.0	10.0	33.0	16.0
Testosterone-d_5	294.3→100.1	121.0	10.0	33.0	18.0
Progesterone*	315.3→109.0	126.0	10.0	37.0	18.0
Progesterone	315.3→097.0	126.0	10.0	33.0	16.0
Progesterone-d_9	324.3→113.0	126.0	10.0	37.0	18.0
Corticosterone*	347.3→120.9	126.0	10.0	35.0	18.0
Corticosterone	347.3→091.0	126.0	10.0	77.0	14.0
Corticosterone-d_8	355.3→124.9	126.0	10.0	35.0	18.0
DHEA*	271.2→213.2	151.0	10.0	19.0	14.0
DHEA	271.2→090.9	151.0	10.0	61.0	14.0
DHEA-d_6	277.2→219.2	151.0	10.0	19.0	14.0

For each species, the most sensitive transition (marked as *) was used for quantitation (quantifier) and the second one was used for confirmation (qualifier). MRM: multiple reactions monitoring; DP: declustering potential; EP: entrance potential; CE: collision energies; CXP: collision cell exit potential.

2.5. Mass spectrometric conditions

The LC eluent was introduced into the mass spectrometer through a turbo ion spray (APCI) probe which was operated in the positive ionization mode to obtain better signal quality. Scheduled multiple reactions monitoring (MRM) mode was utilized for the monitoring of target compounds. All main working parameters of the mass spectrometer were optimized with flow injection analysis (FIA) optimization. The main operational parameters of the mass spectrometer are summarized as follows: curtain gas at 25 psig, collision assisted dissociation gas at 7 psig, nebulizer current at 4.0 μA, sheath gas at 55 psig, resolution at unit and temperature at 500 °C.

The respective conditions of MRM detection for the mass spectrometry method of estradiol, cortisone and DHEA are summarized in Table 2. The specifications for the other examined steroid hormones are identical to those stated in our previous report [35] and may be derived from this manuscript. The ion pairs of precursor ions to product ions (Q1→Q3) with rich structure features were chosen for MRM detections of each compound. For each species, two ion pairs were chosen with the most sensitive transition being used for quantitation (quantifier) and the other transition being used for confirmation (qualifier). Compound quantitative optimization wizard was used to optimize the declustering potential (DP), entrance potential (EP), collision energies (CE) and collision cell exit potential (CXP) of these compounds. The MRM detection window time was 20 s.

2.6. Specimen collection and pretreatment

Saliva samples were collected using *Salivette* devices (Sarstedt, Rommelsdorf, Germany). Samples were stored at −20 °C in a laboratory freezer. After thawing, samples were centrifuged for 10 min at 4000 rpm. 100 μL saliva was added to a tube together with 50 μL internal standard and 100 μL methanol/water containing 50 mg/mL $ZnSO_4$ in a ratio of 50:50 (v/v). After vortexing for 1 min, the tube was centrifuged at 12,000 r/min for 5 min with centrifuge (Hettich, MIKRO 22R). Afterwards 200 μL supernatant was injected into the LC–MS/MS system.

2.7. Validation parameters

2.7.1. Standard calibration curves

Calibration standards were prepared by spiking blank saliva samples with standard solutions to the final concentrations of 0, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 ng/mL for all steroids except for DHEA, for which final concentrations for DHEA were 0, 0.01, 0.1, 0.5, 1, 5, 10, 15 and 20 ng/mL. These standard saliva samples were prepared in the same way as the normal saliva samples described in 2.6. To build calibration curves, the following equation was used: $y=Ax+B$, where x is the compound concentration and y is the ratio of the compound peak area to the IS peak area. Correlation coefficients for the determination of the linear fit of the curve were obtained by plotting peak area ratios against the nominal concentrations. To identify a 'blank' saliva sample, we capitalized on the fact that our laboratory routinely runs assays for steroid hormones in saliva. From a large database of previous analyses, we were able to identify a case whose saliva contained no detectable steroid hormone levels (below the limit of detection). This sample was used to derive 'blank' aliquots for our analyses.

2.7.2. Limit of quantification

The limit of quantification (LOQ) was based on the criteria that variability in accuracy and precision (%CV, see below) was less than 20% and that the corresponding signal-to-noise ratio was greater than 10. The LOQ was finally determined by the lowest amount of spiked standards in the saliva matrix that still provided a 10-fold or greater signal to noise ratio.

2.7.3. Inter-assay and intra-assay variabilities

Inter- and intra-assay variabilities were determined by calculating precision and accuracy estimates for saliva samples with five replicates each on five separate days. Accuracy and precision were obtained as the percentage of relative error (%RE) and the percentage coefficient of variation (%CV), respectively. Accuracy and precision batches comprised five replicates of quality control (QC) samples at three different concentrations (low quality control (LQC), middle quality control (MQC), and high quality control (HQC)) for all tested compounds: 0.01, 1 and 10 ng/mL for estradiol, cortisol, cortisone, testosterone, progesterone, corticosterone as well as 0.05, 2 and 20 ng/mL for DHEA.

2.7.4. Evaluation of matrix effect and method recovery

To evaluate the matrix effect (ME) and method recovery (R), three blank saliva samples with five replicates each were spiked with pure standard samples at three different concentrations (i.e., the LQC, MQC and HQC values specified above). The response of the analyte was compared to the response of a neat standard solution. The matrix effect value for each sample was calculated as: $ME(\%)=((B/A)-1)\times 100$, where A is the analyte peak area of the pure standard sample and B is the analyte peak area of saliva spiked with the standard sample. The method recovery value was calculated as: $R(\%)=C/D\times 100$, where C is the concentration value quantitated from blank saliva spiked with the standard sample with our LC–MS/MS method, while D is the concentration value of the respective standard sample.

3. Results and discussion

3.1. LC–MS/MS method development

Fig. 1A and B illustrates exemplary chromatograms of a blank and a spiked saliva sample, respectively. Chromatograms of blank saliva samples did not show any relevant peaks or interferences which confirmed that the method was able to differentiate the analytes of

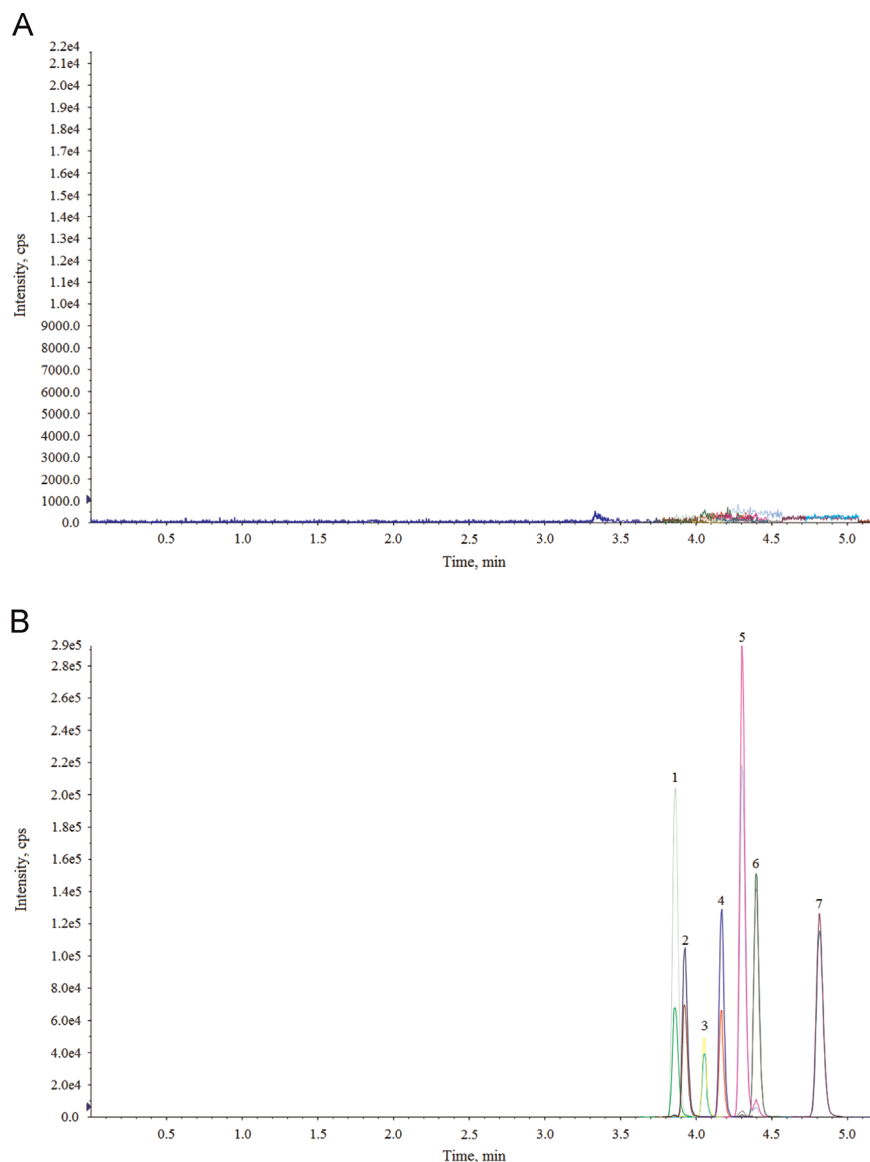

Fig. 1. High performance liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry multiple reaction monitoring (positive ion mode) chromatographic profiles of cortisol, cortisone, corticosterone, estradiol, testosterone, DHEA and progesterone corresponding to (A) a blank saliva sample and (B) a spiked blank saliva sample (standard solutions added at concentrations of 1 ng/mL for cortisol, cortisone, corticosterone, estradiol, testosterone and progesterone, 10 ng/mL for DHEA). Note: 1=cortisone; 2=cortisol; 3=corticosterone; 4=estradiol; 5=testosterone; 6=DHEA; 7=progesterone.

Table 3
Linearity, detection limit and retention time for the examined steroid hormones.

Compound	Calibration curve	*r*	LOQ (pg/mL)	*R*-time (min)
Estradiol	$y=0.541x+0.00442$	0.9999	1	4.17
Cortisol	$y=0.249x-0.00501$	0.9998	5	3.93
Cortisone	$y=0.430x-0.00337$	1.0000	3	3.86
Testosterone	$y=0.696x-0.00181$	0.9999	1	4.30
Progesterone	$y=0.280x-0.00684$	0.9998	5	4.82
Corticosterone	$y=0.395x-0.00669$	0.9999	5	4.05
DHEA	$y=0.057x-0.04180$	0.9998	10	4.40

LOQ: limit of quantification, *R*-time: retention time.

interest and IS from other components of the saliva matrix. It can be seen in Fig. 1 that the method yielded a low background signal from the biological matrix which indicates good selectivity and sensitivity for all analytes and the internal standard (IS).

To increase the sensitivity, previous research has recommended the use of derivatization methods to measure estradiol levels in saliva or blood samples [24,33]. The sample pretreatment of these methods involves several work-intensive and time consuming steps, such as careful pH and temperature control. The pH changes during pretreatment may also result in erroneous and falsely high results for the measurement of estradiol, because of the hydrolysis of conjugated estrogens in samples [30]. Our results indicate that the present method working with an API 5000 spectrometer with on-line SPE method produced sufficiently sensitive results without a need for derivatization methods. Mass spectra obtained by quantitative optimization showed a protonated molecular ion $[M+H]^+$ for all steroids except for estradiol and DHEA for which these were $[M-H_2O+H]^+$. Moreover, compared with conventional selected ion monitoring (SIM) mode, a purer background could be obtained with less interference when using MRM mode. The APCI ionization source was chosen as pilot experiments by our group had shown that ESI ionization source suppresses the signal of estradiol and DHEA (data not shown). Therefore, the LC–MS/MS with APCI positive mode and MRM mode in our present method

Table 4
Inter and intra-assay variabilities, matrix effects and extraction recovery for all steroids.

Compound	Nominal (ng/mL)	Intra-assay		Inter-assay		ME ± SD (%) compound	*R* ± SD (%) compound
		%RE	%CV	%RE	%CV		
Estradiol	0.01	7.2	8.3	8.4	9.1	−9 ± 4	98 ± 5
	1	5.6	8.1	7.8	6.3	−7 ± 2	99 ± 4
	10	4.2	4.7	6.6	4.3	−5 ± 2	96 ± 7
Cortisol	0.01	8.2	9.2	6.4	7.7	10 ± 4	104 ± 4
	1	−4.8	9.8	−5.2	7.4	9 ± 3	100 ± 5
	10	3.7	5.7	5.9	6.8	9 ± 4	105 ± 2
Cortisone	0.01	9.6	7.2	8.5	8.3	49 ± 5	96 ± 3
	1	−6.1	5.7	4.8	7.9	42 ± 2	95 ± 5
	10	−3.0	6.7	3.9	7.1	47 ± 7	93 ± 6
Testosterone	0.01	9.2	7.2	7.9	8.6	−9 ± 4	98 ± 4
	1	6.3	9.1	−4.5	6.2	−5 ± 5	95 ± 6
	10	8.8	5.7	6.8	8.1	−2 ± 3	99 ± 3
Progesterone	0.01	2.5	10.8	5.7	9.7	−15 ± 6	97 ± 5
	1	−10.7	7.9	−6.2	8.9	−13 ± 2	95 ± 7
	10	7.3	6.6	−3.6	4.8	−16 ± 5	92 ± 5
Corticosterone	0.01	11.8	9.4	9.3	10.1	−8 ± 3	93 ± 4
	1	6.3	9.2	10.2	7.4	−6 ± 4	91 ± 6
	10	9.4	7.0	−6.3	9.9	−7 ± 2	95 ± 6
DHEA	0.05	12.1	8.1	8.8	6.0	−9 ± 6	97 ± 5
	2	8.9	9.4	7.0	5.7	−10 ± 4	103 ± 7
	20	−1.8	6.3	7.7	5.7	−7 ± 2	94 ± 3

%RE: percentage of relative error; %CV: percentage coefficient of variation; ME: matrix effect; *R*: method recovery.

Table 5
Steroid hormone concentrations in 13 saliva samples for which analyses by immunoassay had revealed no detectable estradiol concentrations.

Estradiol	**Cortisol**	**Cortisone**	**Testosterone**	**Progesterone**	**Corticosterone**	**DHEA**
2530	63.4	6770	38.3	34.2	553	134
2480	78.1	17,300	71.2	39.1	18.5	281
813	85.5	4750	55.7	41.8	39.9	288
7420	472	2530	51.9	41.7	14.2	497
588	854	10,040	14.8	27.1	15.6	41.2
7590	713	3970	47.1	31.2	20.3	396
7480	709	5190	58.9	39.3	31.5	431
2740	566	3870	47.6	29.5	0	384
131	478	6520	33.7	35.5	59.1	687
77.3	837	5430	36.1	30.7	8.7	214
329	371	6970	45.8	48.6	5.3	4860
129	399	5120	36.1	40.3	88.4	2450
77.1	431	2380	21.5	39.7	5.2	1240
14.0	148	2170	21.5	31.5	27.0	56.0
54.7	494	5450	44.6	14.0	31.4	124
103	432	8440	128.0	37.5	104	15.7

All concentrations are reported in pg/mL.

provided a highly selective and sensitive method for the determination of these steroids.

The column switching strategy for on-line SPE achieved an analysis time of 5.20 min/sample by allowing the simultaneous performance of sample cleaning and LC analysis. Hence, the current on-line SPE method was able to considerably shorten sample preparation and throughput times and to improve sample purification. This constitutes a considerable enhancement in efficiency compared to previous methods with off-line SPE [24,27] or without extraction [29,30].

The retention times (*R*-times) for all steroids are shown in Table 3. The mobile phases A and B for the LC method was found to be best suited for the formation of molecular ions in steroid analysis and for avoiding potential peak tailing during separation. The LC conditions were optimized to achieve good baseline separation of all compounds and to reduce the chance of the substrate interfering with the target products. Separating the substrate and products also helped to reduce the effects of potential ion suppression of the product signal by the substrate. Thus, a better LOQ could be achieved.

Moreover, the sample volume of the present method (100 μL saliva) was considerably lower than the volume used by former protocols, such as 200 μL for serum [30] and 1 mL for saliva [25]. The possibility of allowing high quality steroid hormone assessment from low sample volumes is an important practical feature for applications in clinical and research laboratories.

3.2. Method validation

3.2.1. Linearity and detection limit

A detailed summary of the calibration curves and LOQs are provided in Table 3. Almost perfect linear correlation coefficients (larger than $r=0.999$) were found for each analyte indicating adequate linearity of the analytical procedure. In addition, LOQ values were found to be below (or equal to) 5 pg/mL for all steroids, except for DHEA for which the LOQ was 10 pg/mL. These LOQ values were considered highly suitable for the quantification of each of the seven examined steroid hormones.

Moreover, the present method achieved a higher absolute sensitivity (1 pg/mL) compared to previous GC–MS and LC–MS methods

for estradiol analysis in serum (2 pg/mL, LC–MS/MS) [30] or saliva (2 pg/mL, GC–MS) [25]. In line with these previous reports, the sensitivity of present method was considered well-suited for the measurement of estradiol and other steroid hormones in saliva. This was particularly important as a considerably smaller sample volume was used for the present analyses.

3.2.2. Inter- and intra-assay variabilities

Results of inter- and intra-assay CVs are shown in Table 4. CVs for all QC samples in the matrices were found to be below 11%, which are similar to our method for hair steroid hormones [35]. These results are well within the previously reported acceptable range of CVs below 15% e.g., [19]. The current method was thus considered suitable in terms of accuracy and precision.

3.2.3. Matrix effect and method recovery

For the whole group of compounds tested, cortisol and cortisone showed positive matrix effect behavior ranging from 9% to 49%, while the other steroid hormones showed negative matrix effect behavior ranging from −2% to −16% (as shown in Table 4). This suggests that the respective responses in saliva were higher or lower than those in the mobile phase, indicating enhancement/suppression effects of ionization, respectively. However, the method recovery results showed that the use of the internal standards could correct these matrix effects by 91–105%. This indicates that matrix-matched calibration plots in blank saliva samples with the internal standard could correct for the error of salivary steroid hormone levels.

3.3. Analysis of clinical saliva samples

Besides general evaluation of the present method, we aimed to examine whether our protocol would produce meaningful results for saliva samples for which a previous analysis by immunoassay had failed to produce sensible results. For this, we identified 16 natural saliva samples (chosen from a previous study, $N=565$) for which a routine immunoassay (IBL International, Hamburg, Germany) had indicated no detectable estradiol concentrations (i.e., 0 pg/mL). 8 samples of them were collected from patients who were injected with estradiol, while the other 8 samples were collected from controls who were normal persons without estradiol injection. Table 5 shows the results of the analysis of these samples by the present on-line SPE LC–MS/MS method. It can be seen that for each sample, concentrations were above the LOQ for each examined hormone. These data indicate that the present method, besides the other mentioned advantages, may also help to prevent false negative results which may be of considerable importance, e.g. in clinical research settings.

We also compared salivary estradiol concentrations between patients and controls of this sample. Univariate analysis of variance indicated that estradiol levels were significantly higher in saliva of patients (mean ± SD: 3955.1 ± 3036.4 pg/mL) than in saliva of controls (mean ± SD: 114.4 ± 94.9 pg/mL; $F_{(1,14)}=12.79$, $p=0.003$, $\eta^2=0.477$).

4. Conclusions

A novel on-line SPE LC–MS/MS method has been developed for the simultaneous identification and quantitation of estradiol and six other steroid hormones in saliva samples. To the best of our knowledge, this is the first report of an on-line SPE LC–MS/MS analysis of salivary estradiol without derivatization. The method achieved excellent levels of quantification, fast throughput times of 5.20 min/sample as well as simultaneous sample cleaning and analysis, when coupled with on-line SPE. Particularly these latter aspects highlight the usability of the present method for laboratory contexts in which larger numbers of samples need to be processed quickly. Under such circumstances, we believe that this method is an excellent alternative to commonly used immunoassay methods.

Acknowledgments

The authors would like to thank Sarah Brand and Anja Schultz for their excellent technical support of the current project.

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