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Quantitative analysis of steroid hormones in human hair using a column-switching LC–APCI–MS/MS assay

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ABSTRACT

The analysis of steroid hormones in hair is increasingly used in the field of stress-related research to obtain a retrospective index of integrated long-term hormone secretion. Here, most laboratories have so far relied on immunochemical assays originally developed for salivary analyses. Although these assays are fast and easy to perform, they have a reduced reliability and specificity due to cross-reactivity with other substances and are limited to the detection of one hormone at a time. Here, we report the development of a LC–MS/MS-based method for simultaneous identification of endogenous concentrations of seven steroid hormones (cortisol, cortisone, testosterone, progesterone, corticosterone, dehydroepiandrosterone (DHEA) and androstenedione) in human hair. Hair samples were washed with isopropanol and steroid hormones were extracted from 10 mg whole, nonpulverized hair by methanol incubation. A column switching strategy for on-line solid phase extraction (SPE) was applied, followed by analyte detection on an AB Sciex API 5000 QTrap mass spectrometer. Results indicated linearity of the method for all steroids over ranges of 0.09–90 pg/mg (0.9–900 pg/mg for DHEA) with correlation coefficients ranging between 0.9995 and 0.9999. Intra- and inter-assay coefficients of variation were between 3.7 and 9.1%. The limits of quantification (LOQ) were below (or equal to) 0.1 pg/mg for all steroids, except of DHEA for which the LOQ was 0.9 pg/mg. An analysis of 30 natural hair samples (15 men/15 women) using this method confirmed that all steroid hormones could be quantified at endogenous levels in each individual. In addition, the use of whole hair samples and on-line SPE resulted in a significant reduction in sample throughput times, increasing the applicability of this method for research questions where a larger number of samples needs to be processed.

1. Introduction

The measurement of steroid hormones in hair is increasingly recognized as an important tool for the assessment of integrated hormone secretion over prolonged periods of time. While initially envisaged as a method for the detection of exogenous steroids, e.g. androgens in doping-related research [1,2]; over the past years, hair analyses for endogenous hormones, particularly glucocorticoids, have been widely applied in fields of psychiatric and stress-related research [3]. However, despite an increasing use of hair analyses in such research, the analytical procedures for the detection of steroid hormones in hair have received comparatively little attention.

As most previous researches have focused on cortisol in hair, the mostly commonly employed methods have been immunoassays with chemiluminescence detection (CLIA) or enzyme-linked immunosorbent assays (ELISA) which were originally designed for the measurement of salivary cortisol [4,5]. However, given cross-reactivity of other steroid hormones with the antibodies used in these assays, the specificity of these methods is relatively low which may result in an overestimation of the actual steroid content in hair samples. Moving away from immunoassay procedures, Gao et al. [6] reported the use of high performance liquid chromatography with fluorescence detection (HPLC-FLU) for the measurement of cortisol in human hair. However, the protocol employed by these authors requires a relatively large amount of hair matrix and extensive, time consuming pretreatment procedures. In addition, the protocol involves derivatization which is possible for cortisol but not for other steroid hormones. Besides HPLC-FLU, gas chromatography mass spectrometry (GC–MS) has been used to analysis steroid hormone concentrations in human hair samples [7]. However, while

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GC–MS methods show good specificity, they are characterized by time-consuming work and derivatization steps, long throughput times and require relatively large sample volumes. Besides these methods, other research has reported the use of high performance liquid chromatography tandem mass spectrometry (LC–MS) for the detection of different corticosteroids in hair [1,2,8]. While the employed methodologies provide good specificity and sensitivity and are thus preferable to the above methods, the respective protocols all use selected ion monitoring (SIM) mode. The limited data obtained in SIM mode make it more difficult to identify potential contaminants in biological matrices which may interfere with the determination of analytes. Even though this issue is also given in full scan mode, here more detail is provided which allows a better identification of endogenous contaminants and analytes.

Recently, the utility of on-line solid phase extraction (SPE) has been improved as an extraction technique for this type of analysis [9–13]. The on-line SPE method seems to be the best choice for improving method sensitivity, shortening pretreatment and analysis times as well as increasing the number of the samples that can be analyzed simultaneously. To our knowledge, no on-line SPE LC–MS/MS method for the simultaneous identification and quantitation of different steroids hormones in human hair has been described. Here, we thus present a novel method achieving high specificity and sensitivity at decreased throughput times with a column-switching mechanism for on-line SPE LC–MS/MS analysis.

2. Materials and methods

2.1. Chemicals and reagents

Cortisol, cortisone, testosterone, progesterone, corticosterone, dehydroepiandrosterone (DHEA) and androstenedione were purchased from Sigma–Aldrich (Hamburg, Germany). Deuterated internal standard samples (cortisol-d_4, cortisone-d_7, testosterone-d_5, progesterone-d_9, corticosterone-d_8, DHEA-d_4, androstenedione-d_3) were obtained from Biocrates Life Sciences AG (Innsbruck, Austria). LC–MS grade methanol was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Acetic acid and ammonium acetate, also of LC–MS grade, were 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

Cortisol, cortisone, testosterone, progesterone, corticosterone, DHEA and androstenedione were prepared in methanol at final concentrations of 1 mg/mL as respective stock solutions. Internal standard mixture was prepared in methanol at the final concentrations (cortisol-d_4: 60 ng/mL, cortisone-d_7: 3 ng/mL, testosterone-d_5: 3 ng/mL, progesterone-d_9: 1.8 ng/mL, corticosterone-d_8: 6 ng/mL, DHEA-d_4: 2.1 ng/mL, androstenedione-d_3: 1.8 ng/mL). Deuterated internal standards were chosen as the internal reference for LC–MS/MS quantitation as they had similar structures as the analytes and were absent in the biological samples. The stock solutions were further individually diluted with the same diluents to give working standard solutions of all agents. The concentrations of working standard solutions were 0, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 ng/ml for all steroids except for DHEA for which concentrations were 0, 0.1, 0.5, 1, 5, 10, 50 and 100 ng/ml. All stock solutions and working standard solutions were stored at 4 °C when not in use.

2.3. Instrumentation

The HPLC–MS/MS system consisted of a Shimadzu LC-20AD HPLC unit, a Shimadzu SIL-20AC autosampler and a Shimadzu CTO-20AC column temperature oven (Shimadzu, Canby, OR, USA). This was coupled to an AB Sciex API 5000 Turbo-ion-spray® triple quadrupole tandem mass spectrometer equipped with atmospheric pressure chemical ionization (APCI) ion source (AB Sciex, Foster City, CA, USA). The system was controlled by AB Sciex Analyst® software (version 1.5.1). A Chromolith® Speed ROD RP-18e HPLC 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 from Phenomenex (Aschaffenburg, Germany).

2.4. Chromatographic conditions

2.4.1. On-line SPE methodology

The chromatographic program was accomplished by an on-line SPE method. A 6-port switch valve in column temperature oven controlled by the HPLC system was used for on-line solid phase extraction (Fig. 1). A binary gradient with a single injection sequence table was used. The valve was at position 0 when loading the sample to the SPE cartridge with pump C-A. After washing the sample on the SPE cartridge with pump C-A, the valve was switched to position 1 for back eluting the sample from the SPE cartridge onto the analytical column with pumps A and B (binary gradient module). After the analytes were loaded onto the analytical column for separation, the valve was switched back to position 0 for cleaning up the SPE cartridge, the autosampler and the sample loop with pump C-B. Then the pump C-A started to re-equilibrate the autosampler and the SPE cartridge for the next sample injection while analytes were still being eluted from the analytical column.

2.4.2. Liquid chromatography methodology

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 0.4 mL/min. Mobile phase C-A was a mixture of methanol–water in a ratio 10:90 (v:v). Mobile phase C-B was a mixture of methanol–water in a ratio 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. The details of the HPLC running conditions are listed in Table 1.

2.5. Mass spectrometric conditions

The LC eluent was introduced into the mass spectrometer through a turbo ion spray (APCI) probe. Multiple reactions monitoring (MRM) mode was utilized for the detection of target compounds. The mass spectrometer was operated in the positive ionization mode. The main operational parameters of the mass spectrometer are summarized as follows: curtain gas at 15 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 450 °C.

The precursor ions to the product ions (Q1 → Q3) with rich structure features were chosen for MRM detections of each compound. Compound quantitative optimization wizard was used to optimize the desolvation potential (DP), entrance potential (EP), collision energies (CE) and collision cell exit potential (CXP) of these compounds. The respective conditions are summarized in Table 2. The MRM dwell time was 80 ms.

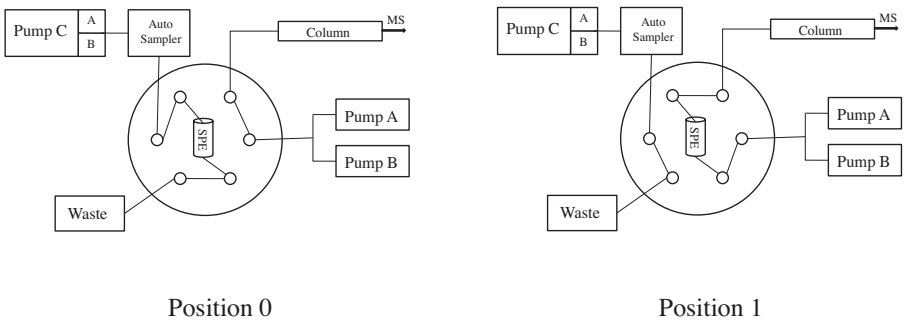

Fig. 1. Illustration of the setup of the on-line SPE/HPLC system. Position 0: sample loading to the SPE column. Position 1: eluting to the analytical column.

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

Time (min)	Binary gradient module for LC column (pump A + B)	Isocratic module for on-line SPE extraction (pump C)		Comment
	Pump B (%)	Solenoid valve	Valve position	
0	30	A	0	Start
1.00	30		1	
3.00			0	
3.20		B		
4.80	20			
5.50		A		
6.50	10			
8.50	10			
8.60	30			
9.00	30	A	0	END

2.6. Sample preparation

2.6.1. Specimen collection and blank hair samples

Hair strands were carefully cut with fine scissors as close as possible to the scalp from a posterior vertex position. A minimum of 20 mg of hair for a 3 cm segment was obtained from each participant. Steroids concentrations were determined from the 3 cm hair segment most proximal to the scalp which, based on an average hair growth rate of 1 cm/month [14], represents hair grown over the three months period prior to hair sampling.

For the determination of matrix effects and extraction recovery (see Section 2.7.4), blank hair samples were required. For these, hair samples of three individuals were used that had previously been identified as exhibiting very low steroid hormone concentrations below the limit of detection (LOD). These samples were taken from individuals with long hair and comprised hair segments distant from the scalp (27–31 cm). The fact that steroid concentrations were very low in these distant hair segments concurs with previous evidence showing that cortisol concentrations show a steady decline from proximal to more distal hair segments [3]. In line with this, chromatograms of blank hair samples did not show any relevant peaks which confirmed their suitability as blank samples. An exemplary chromatogram of a blank hair sample is provided in Fig. 2(A).

Table 2
MS/MS conditions for the compounds studied.

Compound	MRM (Da)	DP (V)	EP (V)	CE (eV)	CXP (V)
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	367.3 → 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_7	368.2 → 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_4	275.2 → 217.2	151.0	10.0	19.0	14.0
Androstenedione*	287.3 → 097.1	121.0	10.0	33.0	16.0
Androstenedione	287.3 → 109.0	121.0	10.0	33.0	18.0
Androstenedione-d_3	290.3 → 100.1	121.0	10.0	33.0	16.0

For each species, the most sensitive transition (marked as *) was used for quantitation (quantifier) and the second one was used for confirmation (qualifier).

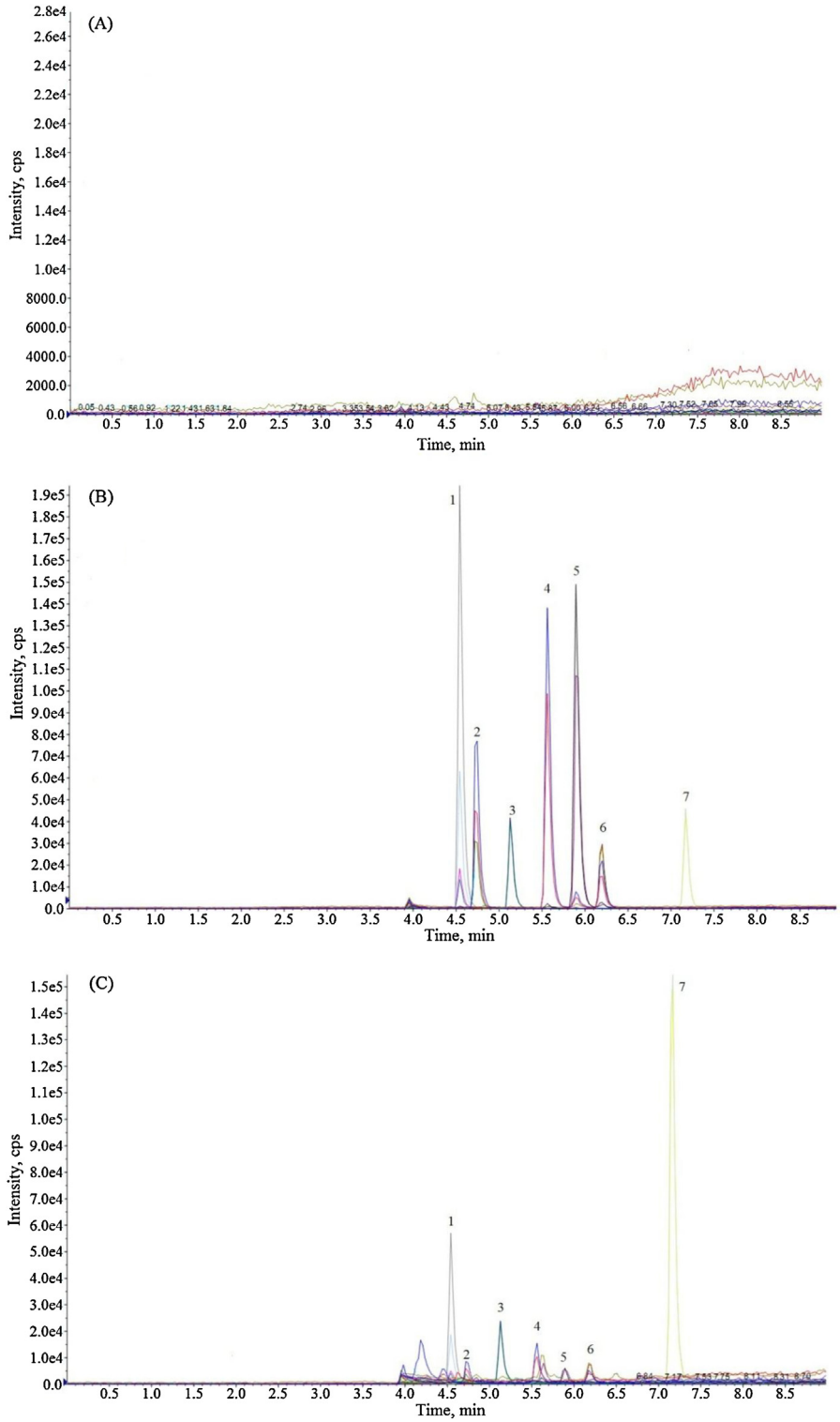

Fig. 2. Exemplary MS chromatogram of (A) a blank hair sample, (B) a spiked hair sample (standard solutions added at concentrations of 90 pg/mg for all steroids; 900 pg/mg for DHEA) and (C) a natural hair sample. *Note*: 1 = cortisone: 28.44 pg/mg; 2 = cortisol: 11.25 pg/mg; 3 = corticosterone: 9.53 pg/mg; 4 = androstenedione: 11.61 pg/mg; 5 = testosterone: 2.97 pg/mg; 6 = DHEA: 27.01 pg/mg and 7 = progesterone: 53.12 pg/mg.

2.6.2. Extraction procedure

The hair washing and steroid extraction procedures were based on the protocol previously described in Stalder et al. [15; study II] with modifications being made to allow analysis by LC–MS/MS. Specifically, hair strands were washed by shaking them in 2.5 mL isopropanol for 3 min at room temperature. They were then allowed to dry under a fume hood for at least 12 h. 10 mg of whole, nonpulverised hair was carefully weighed out and transferred into a 2 mL tube (Eppendorf, Hamburg, Germany). After this, 50 μL internal standard and 1.8 mL methanol were added and the hair was incubated for 18 h at room temperature for steroid extraction. Samples were spun in a centrifuge at 10,000 rpm for 2 min and 1 mL of

the clear supernatant was transferred into a new 2 mL tube. The alcohol was evaporated at 65 °C under a constant stream of nitrogen until the samples were completely dried (duration: approximately 20 min). The dry residue was resuspended using 250 μL distilled water, 200 μL of which were used for LC–MS/MS analysis.

2.7. Validation parameters

2.7.1. Standard calibration curves

Calibration standards were prepared by spiking 10 mg of blank hair with 90 μL standard solutions at concentration of 0, 0.09, 0.45, 0.9, 4.5, 9, 45 and 90 pg/mg of hair samples for all steroids except for DHEA. The final concentrations of DHEA were 0, 0.9, 4.5, 9, 45, 90, 450 and 900 pg/mg of hair samples. 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 the peak area ratios against the nominal concentration.

2.7.2. Limit of quantification

The limit of quantification (LOQ) was based on the criteria that the variability in accuracy and precision (%CV, see below) was less than 20% and that the corresponding signal/noise ratio was greater than 10. The signal/noise ratio was calculated based on the peak areas of the compounds versus the peak area of the background noise in true blank samples in respective biological matrices. The LOQ was finally determined by the lowest amount of spiked standards in the hair matrix that still provided 10-fold or greater signal to noise ratio.

2.7.3. Inter- and intra-day variation

Inter- and intra-day variation were determined by calculating precision and accuracy estimates for hair samples with three replicates each on three separate days. Accuracy and precision were obtained by computing the percentage of relative error (%RE) and the percentage coefficient of variation (%CV) respectively. Accuracy and precision batches comprised three replicates of quality control (QC) samples at five different concentrations (very low quality control (VLQC), low quality control (LQC), middle quality control (MQC), high quality control (HQC) and very high quality control (VHQC)) for all tested compounds: 0.5, 4.5, 9, 45 and 90 pg/mg for cortisol, cortisone, testosterone, progesterone, corticosterone, androstenedione as well as 5, 45, 90, 450 and 900 pg/mg for DHEA.

2.7.4. Evaluation of matrix effect and extraction recovery

To evaluate the matrix effect (ME) and extraction recovery (R), five blank hair samples were extracted with the routine method and spiked with pure standard samples of five different concentrations (identical to the VLOC, LQC, MQC, HQC and VHQC 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: $\mathrm{ME}\ (\%) = ((B/A) - 1) \times 100$, where A is the analyte peak area of the pure standard sample (i.e., in the absence of hair matrix) and B is the analyte peak area of hair spiked with the standard sample after extraction. The extraction recovery value was calculated as: $R\ (\%) = C/B \times 100$, where C is the analyte peak area of hair spiked with the standard sample before extraction and B is the analyte peak area of hair spiked with the standard sample after extraction.

2.7.5. Comparison between whole and milled/pulverized hair

As previous hair analysis protocols had frequently involved the milling of hair prior to incubation, we conducted a comparison between the above stated extraction method using whole, nonpulverized hair (see Section 2.6.2) and analyses carried out on milled hair. For this comparison, five different hair samples were used which were divided into eight parts with the same weight (10 mg). Four of these samples were incubated as whole, nonpulverised hair as stated above. Hair samples in the milled condition were powdered using a Retsch ball mill (5 min at 30 Hz) prior to incubation. Hair samples in both conditions were subsequently spiked with four different concentrations of the examined steroids (0, 4.5, 9 and 45 pg/mg for cortisol, cortisone, testosterone, progesterone, corticosterone, androstenedione; 0, 45, 90 and 450 pg/mg for DHEA) and with the internal standard mixture. No other changes were made to the protocol. To evaluate the quality of these different protocols, the ratio of the peak area of whole hair (WPA) and the peak area of milled hair (MPA) were used (WPA/MPA).

3. Results and discussion

3.1. LC–MS/MS method development

Mass spectra obtained by quantitative optimization showed a protonated molecular ion $[M+H]^+$ for all steroids except for DHEA for which this was $[M-H_2O+H]^+$. Formation of a single dominant precursor ion proved to be superior for enhancing sensitivity compared to the formation of multiple ions for the same analyte. Fragmentation of these precursor ions yielded some product ions, the strongest ones were chosen for quantitation respectively. All main working parameters of the mass spectrometer were optimized with flow injection analysis (FIA) optimization.

Low background noise from the biological matrix showed good selectivity and sensitivity for all analytes and the internal standard (IS) as seen from the chromatograms of a spiked and a natural hair samples (Fig. 2 (B) and (C)). The retention times (R-time) for all steroids are shown in Table 3.

The column switching strategy for on-line SPE achieved an analysis time of 9 min/sample by allowing the simultaneous performance of sample cleaning and HPLC analysis. The average analysis time required by previous SPE protocols was considerably longer than this, e.g. 50 min [9]. Hence, the current on-line SPE method was able to considerably shorten sample preparation times and to increase hair analysis throughput.

A mobile phase with pH 4.5 was found to be best suited for the formation of molecular ions in steroids analysis and for avoiding potential peak tailing during separation. This acidic condition could also help to minimize the formation of sodium adducts. The HPLC conditions were optimized to achieve a good baseline separation of all compounds. Separating the substrate and products completely helped to reduce the potential ion suppression of the product signals by the substrate. It also reduced the chance that the substrate would interfere with the quantitation of the product. Thus, a better LOQ could be achieved.

The LC–MS/MS with APCI positive mode and MRM mode provided a highly selective and sensitive method for the determination of these steroids. Compared with conventional selected ion monitoring (SIM) mode, a purer background could be obtained with less interference when using MRM mode.

3.2. Method validation

3.2.1. Linearity and detection limit

A linear response in the peak area ratios was observed in blank hair samples for all steroids. Detailed summary of the calibration curves and LOQ are provided in Table 3. The correlation coefficients were greater than 0.9995 for each analyte, indicating adequate linearity of the analytical procedure. In addition, the results suggested LOQ values below (or equal to) 0.09 pg/mg for all analytes except for DHEA for which the LOQ was 0.9 pg/mg. These LOQ values were

Table 3
Linearity, detection limit of the method and the retention time of all steroids.

Compound	Calibration curve	r	LOQ (pg/mg)	R-time (min)
Cortisol	$y = 0.573x + 0.0122$	0.9999	0.09	4.73
Cortisone	$y = 0.991x - 0.0623$	0.9996	0.07	4.54
Testosterone	$y = 1.46x - 0.00546$	0.9998	0.08	5.89
Progesterone	$y = 0.277x + 0.00528$	0.9998	0.09	7.15
Corticosterone	$y = 0.336x - 0.00312$	0.9998	0.08	5.12
DHEA	$y = 0.483x - 0.0127$	0.9999	0.9	6.18
Androstenedione	$y = 1.8x - 0.111$	0.9995	0.08	5.53

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

considered highly suitable for the quantification of the examined steroids in hair.

Importantly, the sensitivity of the present analytical method exceeds the sensitivity of previous published LC–MS/MS methods for cortisol, corticosterone, androstenedione [16–18], cortisone [16,18], DHEA, progesterone and testosterone [16,17]. Moreover, when directly compared to previous GC–MS and LC–MS methods for hair steroids analysis, the present method achieved a higher sensitivity [1,2,7,8]. Specifically, the LOQ values of published GC–MS methods for the measurement of androstenedione, progesterone, testosterone and DHEA in hair have ranged between 0.5 and 1.0 pg/mg [7], which is higher than the LOQ values of the present method. Even more so, previous LC–MS methods for hair cortisol, cortisone and corticosterone have reported LOQ values around 5 pg/mg [1,2,8] which are considerably higher than the LOQ values of the present method.

3.2.2. Inter- and intra-day variation

Table 4 shows results of inter- and intra-day CVs which were below 10% for QC samples in the matrices. As these results were within the acceptable range [CV < 15%; e.g., 19], the assay was considered suitable in terms of accuracy and precision.

3.2.3. Matrix effect and extraction recovery

The results of matrix effects and extraction recovery are also provided in Table 4. For the whole group of compounds tested, all steroids (except for progesterone) showed a negative matrix effect behavior ranging from −9% to −50%. This suggests that the responses of these analytes in hair were lower than those in the mobile phase and that, hence, severe suppression of ionization was observed for these analytes and internal standards in hair samples. Progesterone showed positive matrix effect behavior ranging from 12% to 18%, suggesting ion enhancement effects for this analyte.

Table 4
Inter- and intra-day variation, matrix effects and extraction recovery for all steroids.

Compound	Nominal (pg/mg)	Intra-assay		Inter-assay		ME ± SD (%)Compound/IS	R ± SD (%)Compound/IS
		%RE	%CV	%RE	%CV		
Cortisol	0.5	4.2	8.4	4.9	8.8	−55 ± 3/−50 ± 2	91 ± 4/93 ± 3
	4.5	3.8	5.4	4.2	6.8	−45 ± 2/−49 ± 2	89 ± 3/90 ± 2
	9	3.5	8.1	4.5	8.5	−43 ± 5/−45 ± 5	92 ± 5/90 ± 4
	45	2.7	3.7	5.3	6.3	−50 ± 3/−48 ± 3	91 ± 3/92 ± 4
	90	1.8	4.1	3.2	4.7	−47 ± 2/−47 ± 3	93 ± 2/91 ± 3
Cortisone	0.5	3.8	7.2	4.7	8.5	−39 ± 5/−37 ± 3	90 ± 4/87 ± 3
	4.5	2.1	5.4	4.3	7.4	−37 ± 4/−40 ± 4	87 ± 5/88 ± 4
	9	−2.3	5.1	1.9	5.2	−35 ± 2/−34 ± 7	89 ± 4/87 ± 2
	45	1.8	4.3	−2.7	6.1	−31 ± 5/−34 ± 6	87 ± 6/89 ± 5
	90	1.5	3.8	2.1	4.2	−35 ± 2/−33 ± 4	90 ± 2/91 ± 2
Testosterone	0.5	6.2	8.1	6.7	8.8	−11 ± 3/−13 ± 2	94 ± 5/95 ± 3
	4.5	4.1	7.2	6.3	7.8	−13 ± 2/−12 ± 3	95 ± 8/93 ± 4
	9	3.9	8.3	−4.1	6.9	−9 ± 1/−10 ± 2	93 ± 5/96 ± 6
	45	2.7	5.6	4.3	8.3	−10 ± 2/−10 ± 3	96 ± 7/98 ± 3
	90	2.5	3.1	2.7	4.2	−10 ± 3/−11 ± 2	97 ± 2/96 ± 5
Progesterone	0.5	4.7	7.1	4.9	8.3	26 ± 3/27 ± 5	84 ± 4/82 ± 5
	4.5	−3.7	5.3	−4.5	7.4	28 ± 2/29 ± 4	80 ± 3/81 ± 4
	9	3.5	5.7	−3.8	5.9	22 ± 6/21 ± 5	82 ± 5/84 ± 3
	45	4.1	6.4	−2.9	5.2	25 ± 3/27 ± 2	85 ± 2/83 ± 7
	90	3.8	5.1	−3.0	4.3	24 ± 2/24 ± 3	86 ± 2/85 ± 4
Corticosterone	0.5	5.5	8.1	6.1	7.2	−23 ± 4/−21 ± 4	102 ± 5/103 ± 3
	4.5	3.5	8.4	4.1	6.3	−18 ± 3/−20 ± 2	105 ± 7/101 ± 5
	9	4.2	7.5	3.7	8.1	−21 ± 3/−20 ± 4	101 ± 4/98 ± 7
	45	5.1	4.1	2.9	7.4	−25 ± 5/−26 ± 3	103 ± 6/106 ± 4
	90	3.4	3.1	3.9	4.2	−20 ± 3/−23 ± 3	100 ± 2/99 ± 5
DHEA	5	3.8	5.4	3.6	6.3	−18 ± 2/−17 ± 4	85 ± 4/82 ± 5
	45	−2.8	7.5	2.3	6.9	−17 ± 3/−15 ± 2	82 ± 3/81 ± 3
	90	−3.5	9.1	−2.8	8.5	−15 ± 2/−16 ± 4	79 ± 7/83 ± 6
	450	−2.7	5.7	3.2	7.3	−19 ± 3/−20 ± 3	78 ± 6/75 ± 4
	900	2.1	4.5	−3.1	6.3	−18 ± 1/−20 ± 4	80 ± 2/82 ± 3
Androstenedione	0.5	6.6	6.8	7.3	8.7	−31 ± 5/−29 ± 3	100 ± 3/98 ± 5
	4.5	3.7	7.3	5.1	9.1	−27 ± 4/−25 ± 2	99 ± 5/96 ± 7
	9	5.1	5.8	4.7	8.3	−29 ± 3/−31 ± 2	102 ± 4/98 ± 4
	45	2.7	4.9	−2.5	5.7	−31 ± 4/−28 ± 3	97 ± 8/101 ± 6
	90	3.9	5.1	4.2	6.3	−29 ± 4/−30 ± 5	98 ± 5/97 ± 2

%RE: percentage of relative error; %CV: percentage coefficient of variation; ME: matrix effect; R: extraction recovery; IS: internal standard.

Table 5
Mean steroid hormone concentrations in 30 individual hair samples.

Sex	Cortisol	Cortisone	Testosterone	Progesterone	Corticosterone	DHEA	Androstenedione
M	13.86	47.34	1.73	3.15	2.39	8.96	7.44
M	6.26	30.42	4.18	2.49	0.84	7.36	3.31
M	3.77	13.68	1.28	2.11	0.74	9.09	3.61
M	7.44	18.72	2.24	3.12	1.11	15.30	5.49
M	17.64	52.74	2.13	7.50	0.91	29.97	4.46
M	3.18	16.92	1.16	2.67	0.88	11.70	4.97
M	5.09	19.26	1.65	2.22	0.46	12.33	3.87
M	7.12	19.26	1.58	2.54	1.22	8.96	3.71
M	7.00	31.86	2.30	3.85	0.62	9.54	6.42
M	4.23	20.61	2.21	2.70	1.16	22.05	5.45
M	12.69	24.21	2.11	7.32	1.36	12.51	5.31
M	4.77	26.55	1.47	4.78	0.49	16.20	4.55
M	2.30	20.97	1.21	5.51	0.62	12.96	5.57
M	6.87	31.05	2.41	4.01	1.29	12.06	5.40
M	4.49	24.30	1.79	5.08	0.59	8.80	5.11
F	5.67	27.18	1.35	2.39	0.60	33.39	3.03
F	4.55	19.80	0.78	8.42	0.99	17.19	3.65
F	13.41	41.85	0.77	2.35	0.93	20.43	2.32
F	5.37	18.00	1.00	6.31	0.93	21.42	2.78
F	9.99	30.24	0.70	3.64	0.44	8.18	2.18
F	2.10	10.08	1.56	3.33	1.39	8.72	2.27
F	10.89	36.81	2.82	3.38	1.21	42.48	6.44
F	1.62	6.07	0.68	6.49	1.33	26.55	3.73
F	13.86	35.19	0.76	2.23	0.40	10.35	2.56
F	4.51	17.19	0.77	9.45	0.61	5.34	3.74
F	10.71	28.98	0.88	8.87	0.73	12.60	6.16
F	3.54	16.11	0.64	3.05	0.64	6.35	2.51
F	6.25	27.72	0.79	3.14	0.87	13.77	3.52
F	5.11	21.51	0.77	7.96	0.71	7.19	2.70
F	7.52	22.14	0.76	2.39	0.36	5.57	1.98
Mean:	7.06	25.22	1.48	4.41	0.89	14.58	4.14
Min:	1.62	6.07	0.64	2.11	0.36	5.34	1.98
Max:	17.64	52.74	4.18	9.45	2.39	42.48	7.44

M = male, F = female. All concentrations are reported in pg/mg.

However, the results of Table 4 show that the use of the internal standards could correct the signal suppression by 88–113%. As this indicated that 9–50% of sensitivity were lost due to matrix effects, matrix-matched calibration plots in blank hair samples with the internal standard were built to compensate for this effect. Besides the results of matrix effects, Table 4 shows that high and consistent extraction recoveries (77–105%) and IS normalized recoveries (95–104%) were achieved for all analytes at each of the three examined concentrations.

3.3. Comparison of whole and milled hair

The comparison of peak areas of samples incubated as whole hair (WPA) and samples milled prior to incubation (MPA) indicated that the size of the respective peak areas was in a comparable range, with data showing WPA/MPA ratios between 77% and 104%. The respective pattern of WPA/MPA ratios was comparable between the different steroid hormones and the five examined concentrations. Across all comparisons, a WPA/MPA ratio below 100% was found in 27 out of the 35 analyses, indicating that steroids were extracted somewhat more efficiently from hair powder than from whole hair. Still, the extent of this difference in the obtained signal was rather small compared to the overall signal strengths and the low LODs for these substances. As a result, all analytes could be detected without any problems using either extraction method. Given that the pulverization of hair using a ball mill involves a considerable amount of manual sample handling and is thus likely to reduce sample throughput times, the extraction of whole hair samples appears to be a valuable alternative strategy for the analysis of these examined steroids, specifically when a larger number of samples need to be processed.

3.4. Analysis of natural hair samples

To further evaluate the usefulness of the developed on-line SPE LC–MS/MS analysis method for the detection of endogenous hormone levels, we used this method to analyze steroid hormone concentrations in hair samples of 30 individuals (15 men/15 women, mean age: 38.1 years, age range: 21–58 years). The respective results are summarized in Table 5. It can be seen that the obtained concentrations were comfortably above the LOQ for each examined hormone in each sample.

As an additional proof-of-concept, we also compared hair testosterone concentrations between male and female participants. Univariate analysis of variance indicated that testosterone levels were significantly higher in hair of men (mean ± SD: 1.96 ± 0.74 pg/mg) than in hair of women (mean ± SD: 1.00 ± 0.56 pg/mg; $F_{(1,28)} = 16.02$; $p < 0.001$; $\eta^2 = 0.364$).

4. Conclusions

Here we present a novel on-line SPE LC–MS/MS method for the simultaneous identification and quantitation of seven steroid hormones in human hair samples. Due to the low concentrations of these steroid hormones in hair, a highly sensitive method allowing complete separation of these compounds is required. Our results indicate that the presented LC–MS/MS protocol is able to achieve these objectives, providing a highly sensitive, selective and reliable method for the quantification of these steroids in hair. The protocol resulted in very low levels of quantification (LOQ) which were equal to or below 0.1 pg/mg for all steroids, except of DHEA for which the LOQ was 0.9 pg/mg. Given that the minimal values observed in the present hair data from thirty individuals were between 3.6-times (corticosterone) and 60.7-times (cortisone) higher than these

LOQ values, we are confident that the presented method is sensitive enough to quantify these hormones even at low endogenous concentrations.

Given that hair analyses for steroid hormones are increasingly used in stress-related psychoendocrinological research where often a larger number of samples have to be processed, a particular concern in our method development was to increase throughput times. Here, the coupling of the LC–MS/MS protocol with an on-line SPE led to a considerable shortening of pretreatment and analysis times. This approach allowed sample throughput times of 9 min/sample as well as simultaneous sample cleaning and analysis. In addition to optimization of the extraction method, the use of whole hair samples instead of milled hair also helped to reduce overall processing time while maintaining high analytical quality. While our comparison of whole and milled hair results indicated a tendency towards higher peak areas in chromatograms of milled compared to whole hair samples, the magnitude of this difference was rather low and virtually negligible given the overall sensitivity of the method. Taken together, our data suggest that the presented LC–MS/MS protocol with on-line SPE carried out on whole hair samples constitutes a highly sensitive, selective and fast method for the simultaneous detection of seven steroid hormones in hair.

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References

[1] V. Cirimele, P. Kintz, V. Dumestre, J. Goulle, B. Ludes, Forensic Sci. Int. 107 (2000) 381.
[2] F. Bévalot, Y. Gaillard, M.A. Lhermitte, G. Pépin, J. Chromatogr. B 740 (2000) 227.
[3] T. Stalder, C. Kirschbaum, Brain Behav. Immun. 26 (7) (2012) 1019.
[4] C. Kirschbaum, A. Tietze, N. Skoluda, L. Dettenborn, Psychoneuroendocrinology 34 (2009) 32.
[5] B. Sauvé, G. Koren, G. Walsh, S. Tokmakejian, S.H.M. Van Uum, Clin. Invest. Med. 30 (5) (2007) E183.
[6] W. Gao, Q.Z. Xie, J. Jin, T. Qiao, H.Y. Wang, L.Q. Chen, H.H. Deng, Z.H. Lu, Clin. Biochem. 43 (2010) 677.
[7] M.H. Choi, B.C. Chung, Analyst 124 (1999) 1297.
[8] J. Raul, V. Cirimele, B. Ludes, P. Kintz, Clin. Biochem. 37 (2004) 1105.
[9] L. Gao, W.J. Chiou, H.S. Camp, D.J. Burns, X.H. Cheng, J. Chromatogr. B 877 (2009) 303.
[10] L. Gao, X.H. Cheng, J. Zhang, D.J. Burns, Rapid Commun. Mass Spectrom. 21 (2007) 3497.
[11] U. Chiuminatto, F. Gosetti, P. Dossetto, E. Mazzucco, D. Zampieri, E. Robotti, M.C. Gennaro, E. Marengo, Anal. Chem. 82 (2010) 5636.
[12] M. Rauh, M. Gröschl, W. Rascher, H.G. Dörr, Steroids 71 (6) (2006) 450.
[13] T. Koal, D. Schmiederer, H. Pham-Tuan, C. Röhring, M. Rauh, J. Steroid Biochem. Mol. Biol. 129 (3–5) (2012) 129.
[14] R. Wennig, Forensic Sci. Int. 107 (2000) 5.
[15] T. Stalder, S. Steudte, R. Miller, N. Skoluda, L. Dettenborn, C. Kirschbaum, Psychoneuroendocrinology 37 (2012) 602.
[16] N. Janzen, S. Sander, M. Terhardt, M. Peter, J. Sander, J. Chromatogr. B 861 (2008) 117.
[17] T. Guo, R.L. Taylor, R.J. Singh, S.J. Soldin, Clin. Chim. Acta 372 (2006) 76.
[18] V.M. Carvalho, O.H. Nakamura, J.G.H. Vieira, J. Chromatogr. B 872 (2008) 154.
[19] D. Caruso, S. Scurati, O. Maschi, L. De Angelis, I. Roglio, S. Giatti, L.M. Garcia-Segura, R.C. Melcangi, Neurochem. Int. 52 (2008) 5.