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Simultaneous Measurement of Endogenous Cortisol, Cortisone, Dehydroepiandrosterone and Dehydroepiandrosterone Sulfate in Nails Using UPLC-MS/MS

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Abstract

Steroid hormone concentrations are mostly determined by using different body fluids as matrices and applying immunoassay technologies. However, usability of these approaches may be restricted for several reasons, including ethical barriers for invasive sampling. Therefore, we developed an ultra-performance LC-MS/MS method for high-throughput determination of concentrations of cortisol, cortisone, dehydroepiandrosterone (DHEA), and DHEA sulfate (DHEAS) in small quantities of human nails.

The method was validated according to linearity, limits of detection and quantification, recovery, intra- and interassay precision, accuracy, and matrix effect. Samples from 10 adult women were analyzed to provide proof-of-principle for the method's applicability.

Calibration curves were linear (r^2>0.999) in the ranges: 10-5000 pg/mg (for cortisol, cortisone, and DHEAS) and 50-5000 pg/mg (for DHEA). Limit of quantification amounted to 10 pg/mg for cortisol, cortisone, and DHEAS and 50 pg/mg for DHEA. The method showed good sensitivity and specificity without interferences for the analytes. The mean recoveries of cortisol, cortisone, DHEA and DHEAS were 90.5%, 94.1%, 84.9% and 95.9%, respectively, with good precision (coefficient of variation <14% for all analytes) and accuracy (relative error, %: -8.3% to 12.2% for all analytes).

The median (pg/mg, range) hormone concentrations were 69.5 (36-158), 65 (32-133), 212 (50-1077), and 246 (115-547) for cortisol, cortisone, DHEA, and DHEAS, respectively.

This method allows measuring cortisol, cortisone, DHEA, and DHEAS in small quantities of human nails, opening the way to develop applications in the field of endocrinology and beyond.

Keywords: *ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS); hypothalamic-pituitary-adrenal (HPA) axis; keratin tissue; steroid*

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is a key regulator of numerous physiological processes [1]; dysfunction of this system's hormones and related enzymes is associated with a variety of endocrinological diseases and syndromes, including Cushing's syndrome [2], Addison's disease [3] and metabolic syndrome [4], and has also been found to play a role in mental [5], immunological [6] and cardiovascular diseases [7], thereby placing a major challenge for public health [1]. Therefore, the assessment of HPA axis hormones is clinically and scientifically important in different disciplines, including endocrinology and psychiatry, and has come into focus in fields such as obstetrics and neonatology [8].

Blood, urine, saliva, and hair are target tissues for the assessment of HPA axis hormones, and most approaches make use of immunoassay technologies, such as enzyme-linked immunosorbent assay or radioimmunoassay [9,10]. However, these methods face several limitations, including disadvantages of single point measurements (e.g. in blood, urine, saliva) reflecting only momentary hormone concentrations at the time of sampling, thereby covering only short periods of time, and being subject to intraindividual fluctuations, rapid degradation of analytes, risk of cross-reactivity, rather high quantification limits and, therefore, a relatively large amount of sample required; moreover, some of the methods are invasive (i.e. blood and cord blood sampling) and health personnel is required for the sample collection.

Therefore, there is a strong need for non-invasive, easily applicable procedures overcoming previous limitations and for cumulative HPA biomarkers for research as well as diagnostic purposes. Addressing this need is in line with the strategic goal of major health initiatives, such as The Biomarkers Consortium managed by the Foundation for the National Institutes of Health. However, as yet, respective methods are scarce.

There are several lines of evidence that support the use of nail as target tissue for measuring HPA axis hormones: First, cortisol and cortisone have previously been reported to be quantifiable in hair, another keratinized tissue [11]. Second, it has been shown previously that exogenous substances such

as arsenic [12], lead [13] and drugs [14] are incorporated into nail tissue, and that, third, also endogenous substances like testosterone and pregnenolone are quantifiable in human nail tissue [15].

Besides the immunoassay techniques mentioned above, hyphenated techniques were validated for the measurement of cortisol and cortisone in human and animal biological matrices with GC-MS and LC-MS [16,17], as well as for the measurement of DHEA and DHEAS [18,19]. LC-MS/MS technology provides the opportunity to accurately determine hormone concentrations even in small amounts of sample. It has been shown previously that adrenal hormones can be assessed in biological fluids via LC-MS/MS [20-23].

The main objective of this study was to develop an ultra-performance LC-MS/MS (UPLC-MS/MS) analytical method for the simultaneous measurement of cortisol, cortisone, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) in human nails, and provide proof-of-principle for the method's applicability.

Experimental

Chemicals

Cortisol, cortisone and DHEA were obtained from Sigma (Saint-quentin Fallavier, France). DHEAS was purchased from Steraloids (Newport, USA). All analytical standards were purchased as crystal powder with a purity of > 98%. Deuterated cortisol (cortisol-d3) was obtained from Cambridge Isotope Laboratories (Andover, USA).

HPLC-grade methanol (MeOH), isopropanol, formic acid (HCOOH) and ammonium hydroxide (NH_4OH) were purchased from Prolabo (Fontenay-sous-bois, France). HPLC-grade acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany), Oasis® HLB (30mg/30µm) solid-phase microextraction plate from Waters (Milford, USA). Water was purified by a Milli-Q system obtained from Millipore (Molsheim, France).

Solution preparation

Cortisol, cortisone, DHEA and DHEAS solutions were prepared in MeOH at a final concentration of 1, 0.1, 0.01, and 0.001 mg/L. Cortisol-d3 was prepared in MeOH at a final concentration of 1 mg/L. These solutions were refrigerated at 4°C until use and were stable at least for 6 months.

Sorensen buffer was prepared by adding 38.8 mL KH_2PO_4 (9.07 g/L) to 6.12 mL Na_2HPO_4 (11.87 g/L); pH value was adjusted to pH 7.6.

Sample collection

Calibration standards were prepared with nail samples from six members of our laboratory. To provide proof-of-principle of the method's applicability, nails of the right thumbs from 10 adult women, recruited from the general community in the Basel region, were collected and analyzed. Participants gave written informed consent. Appropriate institutional ethics approval was obtained from the Ethics Committee of the States of Basel, Switzerland.

Calibration standards and quality controls

Nail clippings for calibration standards were washed ten times with warm deionized water and ten times with MeOH (~10 min for every cycle) in order to eliminate endogenous steroids for obtaining a pool of blank samples. Blank samples were analyzed to make sure that the four analytes were not detectable. Calibration was performed using 10 mg nails. Calibration standards were prepared at concentrations ranging from 10 to 5000 pg/mg of each analyte.

To determine accuracy and intra- and interassay precision, quality controls were prepared by enriching 10 mg blank nails with the analytes tested at concentrations of 50, 200 and 1000 pg/mg.

Sample preparation

Based on previous reports on successful decontamination of nails with double methanolic wash [24], nail clipping were washed once with warm deionized water and twice with methanol (1 mL) by vortexing 10 seconds.

Samples were dried in an oven (45°C, 2 h) and cut into small pieces of 1-2 mm. Two to 15 mg of nails were incubated in 0.7 mL Sorensen buffer (pH 7.6) for 15 minutes in an ultrasonic bath, followed by incubation for 2 hours at 45°C, in the presence of 10 ng cortisol-d3 as internal standard. Based on previous reports on solid phase extraction for steroid analysis in keratin substance [11], for further purification, SPME Oasis® HLB extraction plates were used. Activation was operated with 0.2 mL MeOH, followed by 0.2 mL deionized water. The incubation medium was centrifuged and the supernatant was removed and deposited on the activated plate, then rinsed with 0.2 mL deionized water/MeOH (95:5, v/v). The plate was allowed to dry for 5 minutes at room temperature. Analytes were eluted with 35 µL ACN/isopropanol (40:60, v/v) with 2% of concentrated NH_4OH, followed by 35 µL deionized water. Ten µL of this extract were directly injected into the UPLC-MS/MS system.

Instruments

A Waters Acquity UPLC system (Milford, MA, USA) with a column heater, autosampler and a 10 µL injection loop was used. Analytes were separated at 30°C on a Waters Acquity UPLC BEH C18 column (1.7 µL, 100 x 2.1-mm, i.d.). Separation was achieved using a gradient elution with 0.1% HCOOH (pH 2.6) and ACN at a flow rate of 0.4 mL/min (0 to 5 minutes: 90% HCOOH and 10% ACN; 5 to 6 minutes: 60% HCOOH and 40% ACN; 6 to 9.5 minutes: 90% HCOOH and 10% ACN). The total run time was 9.5 minutes, including periods required for injection and equilibrating the column before the next injection.

Detection was performed using a Quattro Premier XE tandem Mass Spectrometry (MS/MS) (Waters-Micromass, Manchester, UK) equipped with an electrospray ionization probe and operated switching

between positive and negative ionization mode. The capillary voltage was set to 3.5 kV in positive mode and 2.5 kV in negative mode. The ion source temperature was 120°C and the desolvation gas was heated to 350°C at a flow rate of 800 L/h. The cone gas was set at 50 L/h and collision gas (argon) pressure was maintained at 3.7 mbar. Multiple reactions monitoring (MRM) was employed to detect quantification and confirmation ions, as listed in Table 1. Data acquisitions were done using Masslynx® 4.1 software (Waters).

[Insert Table 1 around here]

Method validation

The method was validated according to the following criteria: limit of detection (LOD), limit of quantification (LOQ), linearity, recovery, accuracy, intra- and interassay precision and matrix effect. Method validation was carried out with six analytical runs on five consecutive days. LOD and LOQ were determined by assaying a series of decreasing concentrations of the analytes in nails. LOD was defined as the lowest concentration where the signal of the compounds was equivalent to three times the background noise, and LOQ was defined as the lowest concentration with a signal-to-noise ratio of 10:1 and a coefficient of variation (CV) not greater than 20%. Linearity was tested by the preparation of calibration curves that ranged from LOQ to 5000 pg/mL for each analyte. A series of three curves was prepared with nine levels for cortisol, cortisone and DHEAS (10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 pg/mg), and seven levels for DHEA (50, 100, 200, 500, 1000, 2000 and 5000 pg/mg). Linearity of the methods was investigated by calculation of the regression line by the method of least squares, and expressed by the correlation coefficient (r^2). The extraction recovery was determined by comparing the peak areas of blank extracted samples spiked before extraction with the peak area of blank samples spiked after the extraction procedure. Three levels of cortisol, cortisone, DHEA and DHEAS concentrations (50, 200 and 1000 pg/mg) were tested. Intra- and interassay precision and accuracy were evaluated using three quality controls (50, 200 and 1000 pg/mg). Precision was expressed as CV, and accuracy was expressed as the relative error, defined as error

relative to the percentage of deviation between nominal and measured concentrations (relative error = (accuracy – 100) : 100). Matrix effect was evaluated using the method described by Matuszewski and colleagues [25]. Blank nails were extracted and fortified by analytes at three different concentrations (50, 200 and 1000 pg/mg; n=6) and analyzed (B). The peak areas were compared to directly injected standards at the same concentrations (A). Matrix effect was calculated using the formula: ME% = B/A x 100. Matrix effect values of 100% indicated no matrix effect, values < 100% indicated ion suppression, and values > 100% indicated ion enhancement.

Statistics

In addition to absolute hormone concentrations, we determined the cortisol/cortisone ratio, cortisol/DHEA ratio, cortisol/DHEAS ratio, and DHEA/DHEAS ratio, which have been associated with a variety of diseases, for example related to the reproductive, immune, metabolic and central nervous system [26-28], as indicators of enzymatic activity. Besides allowing the study of hormone metabolism and enzyme activity, hormone ratios facilitate the comparison of results between studies that used different analytical methods.

As indicators of mutual interdependence of the concentrations of the four analytes, we estimated the association between the concentrations of cortisol, cortisone, DHEA and DHEAS using Kendall's τ. All tests were two-tailed and we set the level of significance at 0.05. For statistical analyses, we used SPSS software (version 17.0).

Results

UPLC-MS/MS characteristics

In UPLC conditions, cortisol, cortisone, DHEA and DHEAS were sufficiently separated chromatographically (Figure 1). The mean (± standard deviation) retention times were 4.51 ± 0.09

minutes for cortisol, 4.59 ± 0.09 minutes for cortisone, 6.49 ± 0.09 minutes for DHEA and 5.35 ± 0.09 minutes for DHEAS. Analysis of six samples of blank nails, Sorensen buffer and deionized water showed no interfering peaks at the retention time of any of the steroids, nor the internal standards examined.

[Insert Figure 1 around here]

Method validation

Linearity, LOD, LOQ, recovery and sensitivity

LOQs in nails were 10 pg/mg (CV < 8.7%) for cortisol, cortisone, and DHEAS and 50 pg/mg for DHEA (CV < 10.3%), respectively (Figure 2).

[Insert Figure 2 around here]

LODs ranged from 5 pg/mg for cortisol, cortisone, and DHEAS to 20 pg/mg for DHEA. The calibration curves showed good linear responses ($r^2 > 0.999$) over the range from 10 to 5000 pg/mg for cortisol, cortisone and DHEAS and 50 to 5000 pg/mg for DHEA, using $1/x^2$ weighing factor. Table 2 shows the recovery and matrix effect tested for each analyte at three different concentrations (50, 200 and 1000 pg/mg; n=6). The extraction recoveries were > 87% for cortisol, > 91% for cortisone, > 82% for DHEA and > 93% for DHEAS. No significant matrix effect was observed in the nails tested (relative error between 90 and 105% for the different analytes).

[Insert Table 2 around here]

Precision and accuracy

Accuracy and intra- and interassay precision of the method were determined by analyzing replicate nail samples (n = 6), containing analytes of interest at concentrations of 50, 200 and 1000 pg/mg, at

the same day and on five consecutive days. The interassay values were based on five batches of n = 6 nails each. The results of these analyses are summarized in Table 3. Intra- and interassay precision calculated from repeated analyses was < 14% for all analytes. Accuracy expressed in terms of relative error ranged from -8.3% to 12.2% for the four compounds at all concentrations.

[Insert Table 3 around here]

Applications

UPLC-MS/MS was performed on fingernail samples of 10 adult women (age: median [range]: 32.5 [29-39] years). For the analyses, at least 1 mg of nail tissue was required. All participants provided more than 1 mg of nail sample (median [range]: 5.7 [2.4-10.6] mg).

All cortisol, cortisone, DHEA and DHEAS concentrations were above the LOQ (Table 4). Cortisol concentrations ranged from 36 to 158 pg/mg with a median value of 69.5 pg/mg; cortisone concentrations ranged from 32 to 133 pg/mg with a median value of 65 pg/mg; DHEA concentrations ranged from 50 to 1077 pg/mg with a median value of 211.5 pg/mg; and DHEAS concentrations ranged from 115 to 547 pg/mg with a median value of 245.5 pg/mg.

[Insert Table 4 around here]

Cortisol/cortisone ratios ranged from 0.98 to 1.26 with a median value of 1.13; cortisol/DHEA ratios ranged from 0.10 to 2.14 with a median value of 0.21; cortisol/DHEAS ratios ranged from 0.25 to 0.34 with a median value of 0.28; and DHEA/DHEAS ratios ranged from 0.14 to 2.55 with a median value of 1.31.

The concentrations of cortisol and cortisone, cortisol and DHEAS, and cortisone and DHEAS, respectively, were positively associated (all $P < 0.05$). The Kendall's τ correlation coefficients are depicted in Table 5.

[Insert Table 5 around here]

Discussion

We developed an analytical method to simultaneously measure cortisol, cortisone, DHEA and DHEAS in small quantities of human nails, using an LC-MS/MS assay, with good linearity, sensitivity, specificity and accuracy without interferences between the four molecules and a low limit of quantification. All hormones were quantifiable in nails of healthy adult women.

The cortisol LOQ of the here reported method was comparable to those of an enzyme immunoassay (EIA) method established for cortisol and DHEA determination in saliva that was previously used for the measurement of cortisol and DHEA in nails [29]. However, the here reported DHEA concentrations were 5 to 10 times higher than those previously measured with the EIA method for which data about accuracy were not reported [29]. In addition, we were able to quantify the four substances in quantities of nail substance as low as 1 mg. This is 50 times lower than the amount of nail substance used in previously reported analyses of cortisol and DHEA using EIA [29]. Therefore, UPLC-MS/MS allows easy and accurate concomitant assessment of cortisol, cortisone, DHEA and DHEAS in minimal amount of nail sample with high specificity and sensitivity and without interferences due to cross-reactivity with endogenous and exogenous steroids, as compared to immunoassays [22].

The cortisol concentrations detected in nails were higher than the previously reported cortisol concentrations in human hair measured with liquid chromatography mass spectrometry [11], while cortisone concentrations were virtually identical, ranging between 12 and 163 pg/mg in the previous study [11] and between 32 and 133 pg/mg in our data, which resulted in higher cortisol/cortisone ratios in nail as compared to hair. One possible explanation for this difference could be the wash-out of cortisol from hair [10], which may occur less in nails. Another explanation could be the differences in tissue incorporation. Alternatively, there may be higher hydroxysteroid (11-beta) dehydrogenase 2 (HSD11B2) activity in hair-associated tissues (e.g. sweat glands [11]), leading to lower cortisol/cortisone ratios in hair as compared to nail.

The cortisol/cortisone ratios detected in nails were approximately 2.5 to 10 times higher than those previously reported for saliva [20,21] and approximately 2 to 10 times lower than those previous reported in total and free plasma/serum [30,23] measured by different types of MS/MS. The cortisol/cortisone ratios detected in nails were in the same range as previously reported free urinary cortisol/cortisone ratios measured with different types of MS [22,31,17], and, interestingly, virtually identical to the ratio of their tetrahydro- and allo-tetrahydro-metabolites (THF + allo-THF)/(THE + allo-THE) (ranging between 1.01 to 1.23) recently determined by HPLC combined with fluorescence derivatization [32], indicating that the cortisol/cortisone ratio measured in nail may reflect the same physiological processes as the urinary ratio, above all the net balance of corticosteroid 11-beta-dehydrogenase isozyme 1 (HSD11B1) and corticosteroid 11-beta-dehydrogenase isozyme 2 (HSD11B2) activity [33] (see Figure 3). Indeed, it has been reported in human subjects that cortisol concentrations in keratinized tissue are not associated with those in saliva but with cortisol concentrations in urine [34].

[Insert Figure 3 around here]

Cortisol/DHEA ratios were lower than those previously determined in serum, saliva and nails assessed by immunoassays [29,28,35], while the here reported cortisol/DHEAS ratios were higher than those previously determined in serum and saliva by immunoassays [27,36,35]. Potential explanations may be differences in assay characteristics between UPLC-MS/MS and immunoassay methods, differences in age and gender of the participants, and differences in processes of incorporation of the substances from blood into saliva or nails, respectively, as described below.

DHEA/DHEAS ratios were substantially higher than those previously determined in serum by radioimmunoassay [37]. This could be explained by tissue-specific activity of steryl-sulfatase (STS) and bile salt sulfotransferase (SULT2A1), enzymes involved in the interconversion between DHEA and DHEAS [38,26] (see Figure 3). Indeed, sulfatase activity has previously been detected in nail tissue [39], where sulfatase deficiency is the basis defect of recessive X-linked ichthyosis [40].

Moreover, nail permeability to substances was shown to be negatively correlated to the molecular weight and the ionization of the molecule; it is significantly lower for ionic substances than for non-ionic substances and for substances with higher molecular weight, this in spite of the lipophilicity of the substances (40). As DHEA has a lower molecular weight (288.42 g/mol) than DHEAS (368.49 g/mol) cortisol (362.46 g/mol), and cortisone (360.44 g/mol), nails permeability coefficient for DHEA could be significantly higher than for DHEAS, cortisol, and cortisone which could in part explain the reversed hormone ratios compared to those found in the human biological fluids.

The nail concentrations of cortisol and cortisone, cortisol and DHEAS, and cortisone and DHEAS were mutually associated, suggesting that they partially reflect a shared underlying process, which may not be reflected by DHEA concentrations.

Evidence on potential routes of substance incorporation into the nail comes from a variety of *in vitro* and *in vivo* studies on nail permeability to drugs, suggesting that incorporation of drugs into the nail most likely occurs simultaneously via two ways, the nail matrix (located at the proximal ventral surface of the nail, responsible for cell proliferation and nail growth) and the nail bed filled with blood vessels [for details, see 14,41]. Nails grow along two different directions, length and thickness, with rates of 0.1 mm/day and 0.027 mm/day length growth, respectively [14]. Altogether, these data suggest that human nail matrix is likely to reflect cumulative endogenous hormone concentrations covering several weeks up to months rather than providing a snapshot of a short period of time [14]. This indicates the potential of the nail as matrix carrying biomarkers for chronic physiological processes. One advantage for the use of nail over hair, another keratin tissue, is the fact that the nail lacks pigments, such as different types of melanin, which may influence the affinity of the hair matrix for different substances [42].

Our study has several strengths. We developed a method with numerous advantageous characteristics, including non-invasive sample collection and convenient sample storage at room temperature, allowing ambulatory sample collection by study participants, low minimal amount of required sample, and a total analytical run time of 9 minutes for simultaneously assessing several hormones. Using

state-of-the-art approaches, we confirmed their high sensitivity and specificity, good accuracy and reproducibility. Furthermore, the UPLC-MS/MS method is free of interference in contrast to EIA. Finally, we provided proof-of-principle for using the method to determine hormone concentrations in human nails, opening the way for its further development for a wide range of potential applications in research and clinical settings, for example as a screening tool.

One limitation of our method is that we cannot finally exclude that decontamination of the samples extracted parts of the endogenous hormones, thereby reducing the detectable hormone levels. We dealt with this limitation by additionally calculating hormone/metabolite ratios, which are more robust against underestimation, as aberrant decontamination would relate to both hormone and metabolite. Finally, it may be speculated that cosmetic products influence incorporation of endogenous hormones into the nail. However, 4 out of the 10 women reported use of nail polish and nail polish remover, but concentrations of all four hormones were virtually identical in those using and not using nail polish (data not shown).

Future studies should further address the role of cosmetics and hygiene. Moreover, future studies should aim at further reducing the minimum amount of required sample by increasing the sensitivity of the method, for example using gas-chromatography instead of LC. Analytical methods to measure DHEA in hair with a LOQ at 0.5 pg/mg have recently been described [43]. Finally, further studies should test the functional significance of the method and provide data on its validity. Indeed, as proof of principle, we have recently shown that DHEA concentrations in nails of infants are associated with intrauterine exposure to maternal stress [44].

The method may have the potential for future applications in different fields of endocrinology and beyond, offering a non-invasive tool to assess cumulative steroid concentrations in pathological conditions characterized by disturbances of steroid hormones and related enzymatic activity.

To the best of our knowledge, this is the first study demonstrating that cortisol and DHEA, and their metabolites cortisone and DHEAS, are simultaneously detectable and quantifiable in small amounts of human fingernail, using a simple and reproducible LC-MS/MS assay with good linearity, sensitivity,

specificity and accuracy without interferences for the four molecules and a low LOQ. It may be a useful easily accessible biomarker providing cumulative information on HPA hormone concentrations and related enzymatic activity. This non-invasive method may be a promising tool for a wide range of clinical and research applications in the field of endocrinology and beyond.

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Table 1: Multiple reactions monitoring parameters for cortisol, cortisone, DHEA, DHEAS, and internal standard detection

Compound	Precursor/parent ion (m/z)	Product/daughter ion (m/z)	Cone voltage (V)	Collision energy (eV)
Cortisol	363.1	120.9*	35	20
	363.1	309.0	35	10
Cortisone	361.1	163.0*	40	25
	361.1	120.9	40	20
DHEA	289.1	253.1*	20	10
	289.1	213.1	20	15
DHEAS	367.1	96.8*	50	30
Cortisol-d3	366.1	120.9	35	20

* quantification ion

DHEA = dehydroepiandrosterone, DHEAS = dehydroepiandrosterone sulfate.

Table 2: Extraction recovery and matrix effect

		Recovery (n = 6)		Matrix Effect (n = 6)	
Compound	**(pg/mg)**	**%**	**CV (%)**	**%**	**CV (%)**
Cortisol	50	87.8	7.6	90.0	11.6
	200	89.5	6.5	91.5	9.5
	1000	94.1	5.0	95.3	9.1
Cortisone	50	91.4	8.3	98.9	10.8
	200	93.7	5.1	96.6	10.2
	1000	97.2	4.7	99.1	8.9
DHEA	50	82.9	10.9	93.9	12.9
	200	84.6	9.8	94.7	12.2
	1000	87.3	9.1	96.1	10.4
DHEAS	50	93.4	6.4	105.4	11.4
	200	95.8	6.1	103.7	9.8
	1000	98.6	4.3	98.5	9.2

CV = coefficient of variation, DHEA = dehydroepiandrosterone, DHEAS = dehydroepiandrosterone sulfate.

Table 3: Precision and accuracy

		Intra-assay (n = 6)		Inter-assay (n = 6)	
Compound	**(pg/mg)**	**Precision (CV, %)**	**Accuracy (RE, %)**	**Precision (CV, %)**	**Accuracy (RE, %)**
Cortisol	50	9.2	4.9	11.7	12.2
	200	7.8	3.8	10.9	9.4
	1000	6.3	-0.6	8.9	5.9
Cortisone	50	8.6	-0.1	12.4	6.4
	200	8.2	-2.2	10.1	4.9
	1000	5.9	-4.9	9.7	0.7
DHEA	50	10.4	-1.4	13.5	-8.3
	200	9.9	-2.7	12.1	-6.4
	1000	8.1	2.6	11.7	-5.9
DHEAS	50	8.8	-2.7	11.2	1.5
	200	7.5	2.8	10.6	3.1
	1000	5.4	3.7	9.9	5.9

CV = coefficient of variation, RE = relative error, DHEA = dehydroepiandrosterone, DHEAS = dehydroepiandrosterone sulfate.

Table 4: Concentrations of cortisol, cortisone, DHEA and DHEAS in nail samples of participants.

Subject No	Cortisol (pg/mg)	Cortisone (pg/mg)	DHEA (pg/mg)	DHEAS (pg/mg)
1	70	66	447	252
2	36	32	196	130
3	69	64	199	239
4	107	85	50	354
5	158	133	97	547
6	53	54	224	202
7	115	102	128	345
8	105	92	1077	422
9	65	63	393	232
10	39	32	287	115

DHEA = dehydroepiandrosterone, DHEAS = dehydroepiandrosterone sulfate.

Table 5: Kendall's τ coefficients (*P*-values) of the associations between cortisol, cortisone, DHEA, and DHEAS concentrations in nail samples of participants.

	Cortisol	Cortisone	DHEA	DHEAS
Cortisol	– –	0.944* (< 0.001)	–0.156 (0.531)	0.822* (0.001)
Cortisone	– –	– –	–0.135 (0.590)	0.899* (< 0.001)
DHEA	– –	– –	– –	–0.156 (0.531)

* $P < 0.05$

DHEA = dehydroepiandrosterone, DHEAS = dehydroepiandrosterone sulfate.

Figure legends

Figure 1: Chromatogram of a nail extract. Concentrations measured were 66 pg/mg, 70 pg/mg, 447 pg/mg, and 252 pg/mg, respectively, for cortisone, cortisol, dehydroepiandrosterone, and dehydroepiandrosterone sulfate. The y-axis represents relative signal intensity.

Figure 2: Chromatogram of blank nails spiked with cortisone, cortisol, dehydroepiandrosterone, and dehydroepiandrosterone sulfate at limits of quantification (10, 10, 50 and 10 pg/mg respectively). Retention times were 4.59, 4.51, 6.49, and 5.35 minutes, respectively. The y-axis represents relative signal intensity.

Figure 3: Chemical structure and metabolic dependency of cortisol, cortisone, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS).

Figure 1:

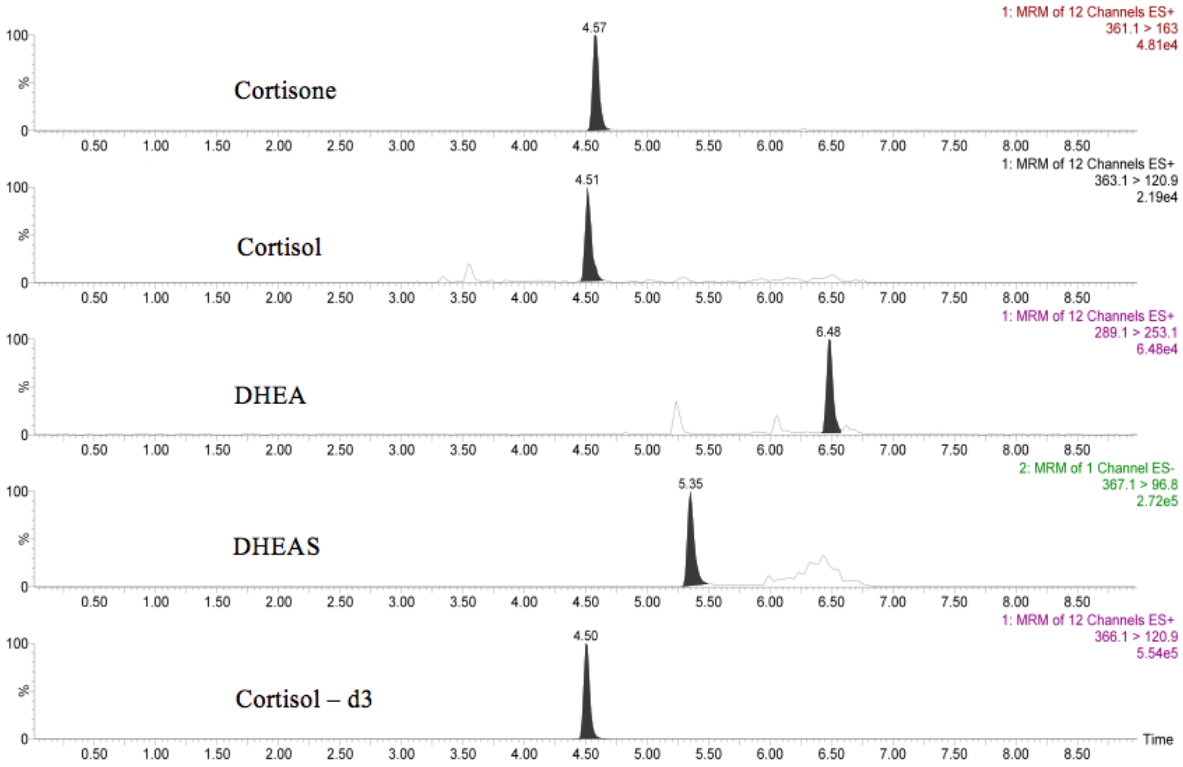

1: MRM of 12 Channels ES+
361.1 > 163
4.81e4
4.57
Cortisone
1: MRM of 12 Channels ES+
363.1 > 120.9
2.19e4
4.51
Cortisol
1: MRM of 12 Channels ES+
289.1 > 253.1
6.48e4
6.48
DHEA
2: MRM of 1 Channel ES-
367.1 > 96.8
2.72e5
5.35
DHEAS
1: MRM of 12 Channels ES+
366.1 > 120.9
5.54e5
4.50
Cortisol – d3
100
%
0
0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50 7.00 7.50 8.00 8.50
Time

Figure 2:

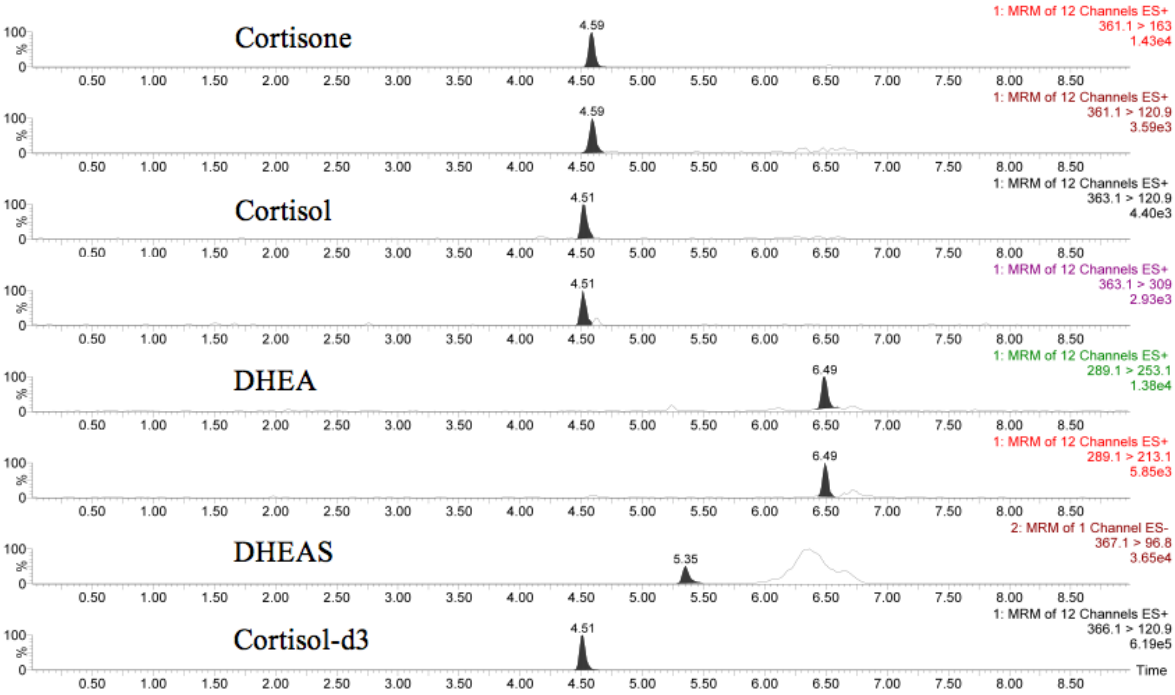
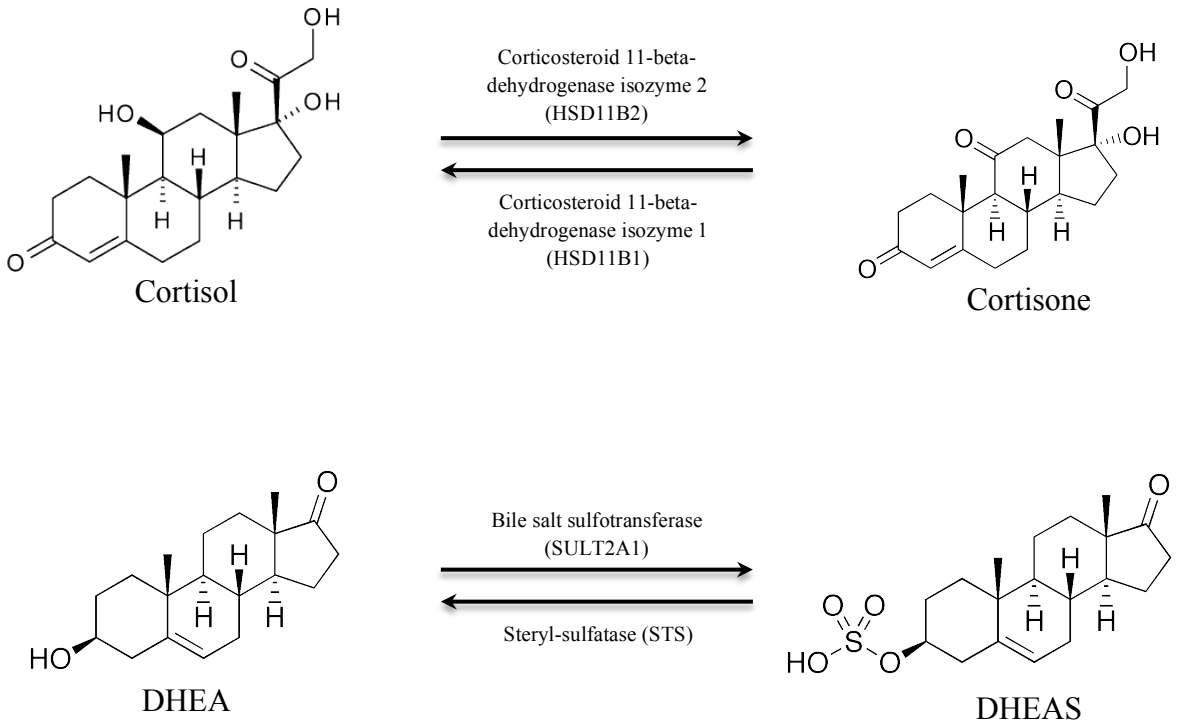

Cortisone
4.59
1: MRM of 12 Channels ES+
361.1 > 163
1.43e4
4.59
1: MRM of 12 Channels ES+
361.1 > 120.9
3.59e3
Cortisol
4.51
1: MRM of 12 Channels ES+
363.1 > 120.9
4.40e3
4.51
1: MRM of 12 Channels ES+
363.1 > 309
2.93e3
DHEA
6.49
1: MRM of 12 Channels ES+
289.1 > 253.1
1.38e4
6.49
1: MRM of 12 Channels ES+
289.1 > 213.1
5.85e3
DHEAS
5.35
2: MRM of 1 Channel ES-
367.1 > 96.8
3.65e4
Cortisol-d3
4.51
1: MRM of 12 Channels ES+
366.1 > 120.9
6.19e5
100
%
0
0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50 7.00 7.50 8.00 8.50
Time

Figure 3:


OH
O
HO
OH
H
H
H
O
Cortisol
Corticosteroid 11-beta-dehydrogenase isozyme 2 (HSD11B2)
Corticosteroid 11-beta-dehydrogenase isozyme 1 (HSD11B1)
OH
O
O
OH
H
H
H
O
Cortisone
O
H
H
H
HO
DHEA
Bile salt sulfotransferase (SULT2A1)
Steryl-sulfatase (STS)
O
H
H
H
O O
HO S O
DHEAS