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Simultaneous determination of hair cortisol, cortisone and DHEAS with liquid chromatography–electrospray ionization-tandem mass spectrometry in negative mode

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ABSTRACT

The present study aimed to develop a novel method for simultaneous assay of cortisol, cortisone and dehydroepiandrosterone sulfate (DHEAS) in human hair. The method was based on liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–MS/MS) in negative mode. Analytes were extracted from 20-mg hair incubated in 1 ml of methanol for 5 days. 100 μl non-SPE supernatant of the incubation solution was utilized in LC–MS/MS analysis. The liquid chromatography separation was performed on a reversed-phase C_{18} column with a mobile phase of 80:20 (v:v) methanol and deionized water containing 0.1% formic acid. The use of ESI in negative mode and the use of a small volume of the incubation solution significantly improved assay sensitivity. The linear range was 5–250 pg/mg for cortisol and cortisone, and 5–500 pg/mg for DHEAS. The limit of detection was 2 pg/mg for the three analytes in hair. The coefficients of variation for intra-day and inter-day assay were less than 10%. The method was applied to determine the three analytes mentioned above of hair samples from 103 participants. The results indicated that there was no significant effect of age and education level on the hair cortisol, cortisone and DHEAS levels. The simple treatment procedure developed in the present study may facilitate simultaneous measurement of more steroids in hair.

1. Introduction

Steroids play important physiological functions because of their anti-inflammatory, anti-allergic and immunosuppressive properties. The physiologically active cortisol in human metabolizes to inactive cortisone through the catalysis of 11β-hydroxysteroid dehydrogenase (11β-HSD) [1–3]. The activity of 11β-HSD influences the circulating level of cortisol and cortisone and its insufficiency results in mineralocorticoid excess syndrome, hypertension and hypokalemia [1]. Cortisol's function is directly antagonized by dehydroepiandrosterone (DHEA) which is circulated in a sulfate form (DHEAS) in human [4]. DHEA and DHEAS can decrease the glucocorticoid receptors, resulting in the impairment of cortisol functions. They can reduce the cortisol release through reducing the release of adrenocorticotropic hormone, and can inhibit the activity of 11β-HSD [4–6]. Additionally, cortisol is a biomarker of physiological and psychological stresses [7–12], anxiety [13] and depression [14,15]. DHEA and/or DHEAS are also related to chronic stress [6,16–18], anxiety [19] and depression [20–24]. Furthermore, endogenous cortisol in hair is considered as a novel biomarker of long-term response of hypothalamus–pituitary–adrenal axis (HPA) [25,26]. If hair grows regularly at a growth rate of 1 cm/month, cortisol in 1-cm hair segment will record the changes of HPA activity or stress exposure over one month [27]. Compared to serum, saliva and urine, hair is a better biological matrix because it has the characteristics of stress-free collection, easy transportation and storage, persistence, traceability and stability [28]. Therefore simultaneous determination of cortisol, cortisone and DHEAS in hair will be potentially useful for the diagnosis of clinical pathological syndromes and psychological status.

Due to their low levels in human hair, determination of cortisol, cortisone and DHEAS would require instruments with high sensitivity and selectivity, such as enzyme immunoassay (EIA) and radioimmunoassay (RIA) [29,30], gas chromatography–mass spectrometry (GC–MS) [31,32], liquid chromatography–mass spectrometry (LC–MS) [33–35] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [36–45]. Immunoassays were widely used for steroids determination because of their high sensitivity, simplicity and speed [29]. But they showed high cross-reactivity with other endogenous steroids and lack of specificity as a result of matrix interference. Immunoassays could quantify only one steroid at a time and their assay results were often inconsistent [46]. Additional disadvantage of RIA was radio-pollution from

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radioactive antibodies [30]. Although GC–MS was applied for multiple analytes [31,32], it was not suitable for steroids because of their poor volatility or easy denaturation with heat [34]. LC–MS/MS was much more accurate than single LC–MS due to its multiple reaction monitoring function in precursor ion/product ion [47]. It was considered to be more suitable for the assay of steroids.

Recently, main ionization methods used for LC–MS/MS determination of steroids are atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI) and electrospray ionization (ESI). In general, APCI and APPI are more apposite than ESI for ionization of steroids [44,48]. This is because most steroids are low polar and even non-polar. However, the analytical instrument with APPI was too expensive for most laboratories. Despite the fact that APCI was a soft ionization [49], it needed a high flow rate of LC based on its ionization principle, which interfered the detection of isomers and analogs [35,40]. Additionally, dehydration fragments, $[M-nH_2O]$, were more easily formed than $[M+H]^+$ or $[M-H]^-$ in APCI [48,50–52], resulting in a decrease in the analytes' response [35,40]. Compared to APCI and APPI, ESI showed wider application including non-polar materials, although non-polar molecules should be derived to increase their polarity ahead of determination [39,42]. Moreover, ESI was much more sensitive than APCI and APPI for polar compounds [41,44], such as steroids with 3-one-4-ene structure [48], aldosterone [53], estradiol [54] and testosterone [55]. Therefore, ESI might be a better ionization method for simultaneous determination of multiple analytes whose polarities are widely distributed.

LC–ESI-MS/MS was mostly performed in positive mode when it was applied to determine steroids in urine [40], saliva [42] and serum [39,44,56]. However, such assay methods could not satisfy the requirement of hair analysis because their limits of detection (LOD) were usually higher than analytes' contents in hair. Interestingly, Labadie et al. and Salste et al. found that ESI in negative mode showed low background noises [57,58]. Henriksen et al. proved that ESI in negative mode was more sensitive than in positive mode for low and non-polar materials [59]. Bevalot et al. and Higashi et al. ever attempted to detect cortisol and cortisone in hair [34] and DHEAS in saliva [41] in ESI negative mode. Unfortunately, these efforts failed to detect DHEAS in hair samples. The present study aimed to develop a sensitive LC–MS/MS method for simultaneous analysis of hair cortisol, cortisone and DHEAS with wide assay window of ESI and high sensitivity in its negative mode. The present study also explored the optimum treatment process to reduce the effect of hair matrix on the sensitivity of ESI. Finally, the validated method was applied to hair samples of 103 participants from two cohorts.

2. Materials and methods

2.1. Chemicals and solutions

HPLC grade methanol was purchased from Dikma Lake Forest, CA. HPLC grade formic acid (HCOOH) was from Tedia, Fairfield, OH. Analytical grade cortisol and cortisone were from National Institutes for Food and Drug Control, China. Analytical grade dehydroepiandrosterone sulfate (DHEAS) and deuterated cortisol (cortisol-9, 11, 12, 12-d4) were from Isotec, Sigma Aldrich, St. Louis, MO.

Calibrator stock solutions were prepared in methanol to a concentration of 100 μg/ml for cortisol, cortisone and DHEAS. Working calibrators were then prepared by diluting stock solutions in methanol to concentration of 0.05, 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/ml. A 50 μg/ml stock solution of cortisol-d4 was prepared in methanol as internal standard (IS). This was diluted in methanol to a working concentration of 20 ng/ml.

2.2. Hair collection and incubation

Hair strands longer than 1 cm in the posterior vertex were cut with scissors as close as possible to the scalp from 103 volunteers who provided written informed consent. Because the surface structure of some hair samples was possibly heavily damaged, the hair strands were stored in sealed container at −50 °C until analysis in order to prevent the loss of the analytes. The hair strands were cut into 1-cm segments prior to use. The 1-cm hair segments closest to the scalp were used as hair samples in the subsequent incubation. The 1-cm distal segments 12 cm away from the scalp of a female adult were used as blank hair matrix where concentrations of cortisol, cortisone and DHEAS were beyond the limits of detection of our mass spectrometry because of wash-out effect [25] or solarizing effect [60].

Before incubation in methanol, 20-mg hair samples were washed with 2 ml of methanol for 2 min and dried at room temperature (25 °C). The hair samples were finely cut into pieces and transferred to a dry tube. Sequentially, 900 μl of methanol and 100 μl of IS working solution were added. The resulting samples were incubated for 5 days at 25 °C in the dark. After centrifuged at 12,000 rpm for 5 min, 100 μl of the supernatant was utilized for LC–MS/MS analysis. The 5-day incubation was selected based on our preliminary results that 3-day incubation in methanol could dissolve most of cortisol and cortisone from the hair samples and 5-day incubation could dissolve most of DHEAS.

2.3. Optimum treatment process of hair samples for the reduction of matrix effect

The 20-mg blank hair matrix was washed with 2 ml of methanol for 2 min, dried at room temperature, and incubated in 1 ml of methanol for 5 days. After the 5-day incubation period, the solution was centrifuged at 12,000 rpm for 5 min. A 100 μl and a 1 ml of supernatant were utilized as blank matrix, respectively. The two different volumes of the supernatant in combination with solid phase extraction (SPE) were applied to investigate the matrix effect on the sensitivity of our LC–MS/MS method.

In order to determine the calibration curve with blank hair matrix, the contents of standards (i.e. cortisol, cortisone and DHEAS) ranged from 0.01 to 50 ng and 2.0 ng IS were added in the supernatant from the methanol incubation of blank hair matrix. In the case of SPE, the desired volume of supernatant was transferred to Pro Elut SPE C_{18} column (Dikma) which was activated with 3 ml of methanol and washed with 3 ml of deionized water before use. After the supernatant was loaded, the deposit on the SPE C_{18} column was rinsed with 3 ml of deionized water to remove impurity. Afterwards, a vacuum pump was used to vacuum the column. The sample was eluted with 1 ml of methanol. The eluate was transferred to another dry tube and dried in N_2 at 50 °C. The dried sample was redissolved in 100 μl of methanol under 2-min vibration with a votexer for LC–MS/MS analysis. In the case of no SPE, the desired volume of supernatants was directly dried in N_2 at 50 °C and redissolved in 100 μl of methanol for LC–MS/MS analysis.

2.4. Simultaneous analysis of cortisol, cortisone and DHEAS

Chromatographic separation was performed on Agilent 1200 HPLC system (Agilent Technologies, Waldbronn Germany). 5 μl of the redissolved solution was injected onto a 150 mm × 4.6 mm 5 μm C18 analytical column (Zorbaxtm Eclipse XDB-C18, Agilent) which was protected by a 10 mm × 4.6 mm 5 μm C18 guard cartridge (Agilent). The mobile phase was methanol and deionized water (80:20, v/v) with 0.1% formic acid in water which was filtered through micro porous membrane (0.22 μm) before use. The flow rate was 200 μl/min. The column temperature was 30 °C.