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Study on dissolution mechanism of cortisol and cortisone from hair matrix with liquid chromatography–tandem mass spectrometry

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ABSTRACT

Background: Hair cortisol has been used as a biomarker of chronic stress. The detected contents of hair cortisol might depend on the incubation duration in solvents for no-milled hair samples with 3-layer structure. However, there was no research on the dissolution mechanism of hair analytes.
Methods: After uniform mixture, no-milled hair samples were incubated in methanol and water for the 12 different durations and milled hair was done as comparison. Hair cortisol and cortisone were determined with high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS).
Results: The measured concentrations of hair cortisol and cortisone showed ≥2 maxima during the entire incubation in methanol and water from 5 min to 72 h for no-milled hair. Hair cortisol concentration measured by LC–MS/MS was increased with the incubation duration. Conversely, it was not held when hair was powdered prior to the incubation in methanol.
Conclusions: Hair cortisol and cortisone were dissolved from hair matrix through the 2-stage or multistage mechanism, which might depend on the hair 3-layer structure and its degree of damage.

1. Introduction

Hair cortisol was considered as a new retrospective biomarker to represent reliable basal activity of HPA system in cumulative exposure to chronic stress [1]. This was primarily because hair cortisol is biologically endogenous. Cortisol, together with other blood-borne substances, diffuses into the cells of hair follicle and is trapped and gradually deposited in the growing hair shaft with 3 concentric structures, cuticle, cortex and medulla [2,3]. Hair strands are also possibly incorporated by the cortisol from secreta of sebaceous gland and sweat gland and an exposure to cortisol atmosphere [2,3] and from the peripheral HPA system where hair follicle itself contains a function of cortisol synthesis [4]. The notion was supported by the significant correlation between hair cortisol and salivary cortisol [5–7] and urinary cortisol [8]. Moreover, it was consistently demonstrated by recent studies where there was the substantial increase of hair cortisol with the systemic exposure to a series of chronic stresses [5,9–17].

However, analysis methods for hair cortisol were varied with studies, such as traditional immunoassay methods [5,9–18], including enzyme immunoassay (EIA) and radioimmunoassay (RIA), high performance liquid chromatography with fluorescence detection (HPLC-FLU) [19], liquid chromatography–mass spectrometry (LC–MS) [20] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [7,12,21]. Because the performances and disadvantages varied with analytical methods, the treatment methods for hair samples and the extraction methods of hair cortisol varied with analytical methods. For instance, the treatment processes were relatively simple for immunoassay methods although they were usually impacted by higher cross reactivity with other steroids, resulting in higher deviation at 15–30% [9–18], and varied results with batches of expensive commercial kits. Although HPLC-FLU showed good performances matched immunoassay, this method consumed relatively more volumes of raw samples and required time-consuming extensive procedures, i.e., 2-step extractions including liquid–liquid extraction and solid phase extraction (SPE) [19]. For LC–MS/MS with high sensitivity and reproducibility, the simple pretreatment processes were performed for hair analysis [6,21]. In general, schematic treatment steps of analyzing cortisol from hair matrix include sampling and storage of hair, segmentation, decontamination by washing procedures, the pretreatment process (e.g. cutting to small pieces or grinding into fine powder), incubation in appropriate solvents, liquid–liquid extraction or solid phase extraction, and qualitative and quantitative detection with appropriate equipment as hair analysis of drugs was done [2]. Among the treatment steps, hair grinding, solid phase extraction and usage of solvents had important effects on the detection of hair cortisol detection.

In the previous studies [5–22], various kinds of the treatment methods were developed in order to extract more cortisol from hair matrix as quick as possible. The distinct differences in these treatment methods were whether hair samples were pulverized into powder in the ball mill prior to incubation in solvents, i.e. milled hair [12] and no-milled hair [18], and whether hair samples were washed prior

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to any of the pretreatment process, i.e. washing [12] and no washing [18]. These differences in the treatment methods might give rise to significant differences in hair cortisol contents because of complicated pathways of cortisol incorporation into hair shaft with 3-layered structure. For instance, washing procedures possibly removed the contaminations and non blood-borne cortisol coated on the outer surface of hair strands [2,19,20], resulting in lower cortisol content than no washing. It was regarded that less cortisol was dissolved out of no-milled hair with the small pieces [22] and more cortisol from milled hair [12] within the same incubation duration. Although so, some recent studies directly incubated no-milled hair samples in methanol for cortisol analysis because of simple pretreatment process [18]. Usually, it was better to incubate no-milled hair in solvents as long as possible in order to dissolve out completely all the cortisol from hair matrix. However, strong matrix effect existed and matrix effect varied with analytes when LC–MS/MS was applied in biological matrix with very low concentration of target compounds [23]. Matrix effect due to endogenous impurities suppressed the MS response of analytes in hair, such as cortisol [7,19] and cortisone [19]. Perhaps there was the optimum incubation duration to determine accurately the contents of hair cortisol and cortisone. Therefore the present study aimed to simultaneously investigate the dependence of hair cortisol and cortisone contents on the incubation duration for no-milled hair samples without any extraction and explore dissolution mechanism of cortisol and cortisone from hair matrix.

2. Materials and methods

2.1. Chemicals and solution preparation

Analytical grade cortisol and cortisone were purchased from National Institutes for Food and Drug Control, China, and deuterated cortisol (cortisol-9, 11, 12, 12-d4) as internal standard was from Isotec, Sigma Aldrich, St. Louis. MO. Water used throughout the experiments was triple-distilled deionized water.

Stock solutions of cortisol and cortisone were prepared in methanol at 100 μg/ml. Cortisol-d4 was prepared in methanol at stock solution of 50 μg/ml and at final concentration of 10 ng/ml. The mobile phase was 8:2 (v:v) methanol and deionized water containing 0.1% formic acid which was filtered through micro porous membrane (0.22 μm) prior to use.

2.2. Hair collection

Hair samples were collected from 19 healthy participants (S01–S19) recruited in the present study (age range: 15–45 y, mean age: 23.2 ± 12.5 y) to determine the dissolution mechanism. Participants with dyed or perm or bleached or hairs of <1 cm were excluded. All participants provided their basic information and the written consent form. Hair samples with >1 cm in the posterior vertex were cut with iron scissors as close as possible to the scalp. As-collected hair samples were stored in dry tubes at room temperature for LC–MS/MS analysis. The 1-cm hair segments closest to the scalp were utilized and were mixed uniformly for the following treatment procedures.

2.3. Treatment of hair samples

In the case of milling, hair samples from 5 participants (S15–19) were pulverized with a ball mill (MM400 type, Retsch, Germany) at 30 Hz for 5 min and divided into 12 portions with 30 mg/portion, and then incubated in 1.5 ml methanol at 25 °C for 12 different desired durations, 5, 30, 90, 240, 480, 720, 1440, 1800, 2160, 2880, 3600 and 4320 min. The incubation medium was separated by centrifugation at 12,000 rpm for 10 min. The 1-ml supernatant was transferred to a dry tube and 100 μl 10 ng/ml cortisol-d4 was added, and then was evaporated to dryness and the dry residue was redissolved in 100 μl methanol for LC–MS/MS analysis.

For no-milled hair, the 1-cm hair segments from fourteen participants (S01–S14) were mixed uniformly and divided into 12 portions with 30 mg/portion, and then incubated at 25 °C for the 12 different desired durations in methanol for S01–S10 and in water for S11–S14. The subsequential procedures were the same as the corresponding ones for the milled hair samples described above.

After hair samples from S09 and S10 were incubated in methanol and from S11 and S12 in water, the incubated hair samples were dried with pure nitrogen and cut into pieces with iron scissors and re-incubated at 25 °C again for another 48 h in 1 ml methanol to extract the remaining hair cortisol and cortisone. The re-incubation medium was separated by vortex and 600 μl supernatant was transferred to a dry tube and 50 μl 10 ng/ml cortisol-d4 was added in the supernatant, then was evaporated to dryness and the dry residue was redissolved in 50 μl methanol for LC–MS/MS analysis.

Additionally, in order to verify the matrix effect on concentration determination of cortisol-d4 and hair cortisol and cortisone, 600 mg hair was incubated in 30 ml methanol for 48 h and the incubated solution was used as hair matrix. The desired volume of the incubated solution in addition of 100 μl 10 ng/ml cortisol-d_4 and 100 μl solution of standard cortisol and cortisone with definite concentration was evaporated to dryness by pure N_2, and then the dry residue was resuspended in 100 μl methanol for LC–MS/MS analysis.

2.4. Hair analysis of cortisol and cortisone

The chromatographic separation was performed on Agilent 1200 HPLC system (Agilent Technologies, Waldbronn Germany). A 5 μl volume of the extract 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/deionized water (80/20, v/v) with 0.1% formic acid which was filtered through micro porous membrane (0.22 μm) prior to use. The flow rate was 200 μl/min and the column temperature was 30 °C.

Detection was performed in a 3200 QTRAP liquid chromatography tandem mass spectrometer (ABI, Foster City CA) equipped with an electrospray ionization source (ESI). Nitrogen (99.999%) was used as desolvation and cone gas. The ionization mode was maintained in negative mode with ion-spray voltage at −4500 V, symmetrical heaters at 400 °C, curtain gas at 10.0 psi, gas 1 (ion source gas) at 40.0 psi, gas 2 at 40.0 psi and collision gas at medium. Ions were detected in multiple reaction-monitoring mode. The optimum conditions for ionization and fragmentation selectivity were listed in Table 1. Three analytes were monitored with a dwell time of 200 ms.

2.5. Validation method

The calibrated curves of standards was obtained at final concentration of 5 to 500 pg/mg in the presence of 2 ng IS. The limit of detection (LOD) was determined as the signal to noise ratio (S/N) equaled 3. Recovery (n = 5), intra-day and inter-day precisions (n = 5) were determined at 5, 25, 250 pg/mg for cortisol and cortisone (n = 5 for each concentration).

Table 1
Optimum ionization and fragmentation conditions for cortisol, cortisone and cortisol-d4.

	Precursor ion (Da)	Product ion (Da)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Cortisol	407.10	331.30	−32.78	−4.71	−26.72	−27.18	−2.15
Cortisone	405.20	329.20	−35.93	−4.02	−26.65	−26.05	−3.20
Cortisol-d4	411.20	335.20	−39.26	−4.90	−21.33	−25.02	−3.54

DP: declustering potential; EP: entrance potential; CEP: collision cell entrance potential; CE: collision energy and CXP: collision cell exit potential.