Mina Mirzaian*, Sofie K.M. van Zundert, Wim F. Schilleman, Mostafa Mohseni, Susanne Kuckuck, Elisabeth F.C. van Rossum, Ron H.N. van Schaik and Sjoerd A.A. van den Berg

Determination of cortisone and cortisol in human scalp hair using an improved LC-MS/MS-based method

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Abstract

Objectives: Human scalp hair is an easily available but complex matrix for determination of cortisone and cortisol, and has been shown to reflect long-term glucocorticoid exposure. Hair glucocorticoid analysis has been used to detect hypo- and hypercortisolism. In this study, we describe the development and validation of a LC-MS/MS method for quantification of cortisone and cortisol in human scalp hair, and provide a novel approach for analysis and interpretation of the results.
Methods: Improved sample preparation using pulverization and solid phase extraction allowed for low sample volumes (10 mg). Baseline chromatographic separation without matrix interference was achieved by reversed phase chromatography and MRM measurement in negative ion mode. Run-to-run time was 8 min. Mixed model analyses were performed to create individual patterns of cortisone and cortisol concentrations.
Results: Matrix matched calibration curves showed excellent linearity up to 100 pg (analyte)/mg (hair) for both cortisone and cortisol (R^2>0.995). LLOQ was 1.5 and 1.0 pg/mg for cortisone and cortisol, respectively. Matrix effect was negligible for hair color (recoveries 95–105 %). Cortisone and cortisol concentrations decreased from proximal to distal hair segments, following a predictable, but subject-specific pattern, with less individual variation for cortisone than for cortisol.
Conclusions: This improved LC-MS/MS method is able to accurately quantify cortisone and cortisol in human hair with minimum matrix interference. This new way of data analysis and interpretation including individual patterns of cortisone and cortisol will be of help with detection of pathological concentrations in both the high – and the low ranges of glucocorticoids.

Keywords: cortisol; cortisone; human hair; liquid chromatography; mass spectrometry

Introduction

Cortisol, also commonly known as the stress hormone, is a steroid hormone in the glucocorticoid class. Cortisol is involved in many vital processes, including mediation of the stress response, inflammatory response, and immune function [1, 2]. The hypothalamic-pituitary-adrenal axis (HPA) regulates both the production and secretion of cortisol. Upon release of the adrenocorticotropic hormone (ACTH), cortisol is released from the zona fasciculata of the adrenal cortex. The availability of cortisol is modulated by several enzymes. In kidney and colon 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which transforms cortisol to cortisone, and in the liver and adipose tissue 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which predominantly converts inactive cortisone to active cortisol by reverse reductive reaction [3]. An imbalance of cortisol levels can lead to various pathological conditions, e.g. Cushing's syndrome and Addison disease [4–6].

Cortisol is measurable in several body fluids, and shows a clean ultradian and circadian rhythm in blood, urine and saliva. Therefore, it represents a retrospective timespan of only a few minutes up to two days [5–12]. More than two decades ago, a novel approach was developed to analyze cortisone and cortisol using liquid chromatography-mass spectrometry

***Corresponding author: Mina Mirzaian**, PhD, Department of Clinical Chemistry, Erasmus MC, University Medical Center, P O Box 2040, Rotterdam 3000 CA, The Netherlands, Phone: +31 107043396, Fax: +31 107044895, E-mail: m.mirzaian@erasmusmc.nl
Sofie K.M. van Zundert, Department of Clinical Chemistry, Erasmus MC, University Medical Center, Rotterdam, The Netherlands; and Department of Obstetrics and Gynecology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
Wim F. Schilleman and Ron H.N. van Schaik, Department of Clinical Chemistry, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
Mostafa Mohseni, Susanne Kuckuck and Elisabeth F.C. van Rossum, Department of Internal Medicine, Division of Endocrinology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands; and Obesity Center CGG, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
Sjoerd A.A. van den Berg, Department of Clinical Chemistry, Erasmus MC, University Medical Center, Rotterdam, The Netherlands; and Department of Internal Medicine, Division of Endocrinology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands

(LC-MS) in human scalp hair, which was postulated to reflect long-term exposure to glucocorticoid hormones [13]. Scalp hair has the advantage over body fluids as the collection of hair is easy, non-invasive and independent of time of the day or fasting state, and it can be stored at room temperature. Determination of hair cortisol has been shown to be a promising diagnostic tool for Cushing's syndrome [14–17], although it is not included in clinical guidelines at this moment.

The determination of cortisone and cortisol in hair is generally known to be complex due to sample heterogeneity, biometrics, damaging, and potentially influenced by contaminations by hair products [18]. In addition, genetics, ethnicity, sex and age can make hair analyses complex [16, 19–22]. In most studies, 2–3 cm of scalp hair was used [13, 23–26]. This approach, however, is hampered by the fact that both cortisone and cortisol are unstable in environmentally exposed hair, and that the rate of reduction in concentration is non-linear and differs per individual [24, 27]. Therefore, small or short perturbations in cortisol homeostasis may be masked when a subject's sample is tested against a reference cohort.

More recent studies reported measurements in 1 cm hair from scalp which allowed higher resolution of analysis [27–30]. To date, however, no studies have been performed that integrated individual trajectories of hair cortisone and cortisol concentrations in their analyses. In this study, we aimed to develop and validated an accurate, precise, sensitive, and especially specific LC-MS/MS method for determination of cortisone and cortisol in human scalp hair. We aimed to optimize sample preparation to allow low sample volume and high spatial resolution measurements. Furthermore, we described a statistical approach that takes into account within- and between-subject cortisone and cortisol concentrations in successive hair segments.

Materials and methods

The validation was performed according to Clinical and Laboratory Standards Institute (CLSI C50) and ISO15189:2012 guidelines.

Chemicals and reagents

Cortisone and cortisol standards were purchased from Cerilliant (Round Rock, USA), item numbers C-130 and C-106. $^{13}C_3$-Cortisol and $^{13}C_3$-Cortisone were obtained from IsoSciences (Ambler, USA), item numbers S14466-0.1 and S14465-0.1. LC-MS grade methanol (catalogue number (CN):13684101), acetonitrile (CN:12401), formic acid (CN:0006914131BS) were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium fluoride were obtained from Sigma-Aldrich (CN: 338869-25G) (St Louis, USA), ammonium hydroxide from Merck (CN: 5.33003.0050) (Darmstadt, Germany). Ultra-pure water was produced in house by Q-POD Ultrapure Remote Dispenser from Merck Millipore (Burlington, USA).

Equipment and supplies

Pulverizing of hair samples and first step extraction were performed by Lysera Bead Mill Homogenizer, Biotage (Uppsala, Sweden). A QUINTIX65-1S electronical scale was used for weighing the hair samples purchased from Sartorius (Göttingen, Germany). MAX µElution Oasis HLB solid phase extraction (SPE) and positive pressure manifold used for the second step of extraction/clean-up were both from Waters (Manchester, UK). Apricot designs^{MT} Evaporex EVX-192 Perkin Elmer (Waltham, USA) was used to dry the samples using nitrogen gas before and after the SPE step. Sample measurements were performed by reverse-phase liquid chromatography (UPLC) using UPLC-Xevo-TQS and an analytical column, Acquity UPLC HSS T3 (2.1 × 100 mm, 1.8 µm), both from Waters.

Hair samples

Hair samples used for quality control (QC) samples and calibration curves were obtained from anonymized volunteers. In total, 10 hair samples were separately pulverized (±300 mg). The samples were first extracted in triplicate in two runs for determination of their concentration and to choose QCs that approximate clinical decision limits (e.g. lower limit of normal (LLN), upper limit of normal (ULN)) as much as possible [31]. In addition, concentrations were specifically chosen near the expected lower limit of quantitation (LLOQ). The number of runs for within day and between day validation is 10. Samples were analyzed twice in triplicate and eight times in duplicate.

Calibration curves

The mixed analytes calibration curve was prepared for each experiment containing cortisone, cortisol, and their internal standards in neat solution as well as spiked in hair sample matrix, consisting of: 0–3.9–7.2–15.6–31.3–62.5–125–250 and 500 pg/xmg. The concentration of $^{13}C_3$-Cortisol and $^{13}C_3$-Cortisone in the calibration curve is 200 pg/xmg. X represents the exact weight of hair, which approximated ~10 mg in all experiments. A blank was added containing no exogenous analyte, no internal standards for each calibration curve. The calibration curves for matrix interferences were extended to 1,000 pg/xmg.

Sample preparation and extraction

The hair samples were pulverized by Lysera. For each QC hair-pool ±300 mg, hair was put in a 30 mL tube with metal beads. The following parameters were used on the Lysera system for preparation of QC samples: Speed (S), Time (T), Cycli (C), Dwell (D) and temperature (temp). The device setting was on S=4.00 m/s, T=1:00 min, C=04, D=0:30 min, temp=22 °C (room temperature). For pulverizing of each control and patient sample ±10 mg hair was transferred in a 2 mL tube with metal beads. Device setting was on S=6.00 m/s, T=0:45 min, C=03, D=0:20 min, temp=22 °C. For the Lysera extraction step ±10 mg pulverized hair 100 µL internal standard mixture and 1.1 mL methanol were added and extracted on S=5.30 m/s, T=1 min, C=03, D=0:20 min, temp=22 °C. Subsequently, the tubes were transferred in the Hettich Mikro centrifuge for 5 min at 15 °C and 21,250 *g*. The supernatant (1 mL) was transferred to Micronic tube of 1.40 mL, and the sample was dried under a stream of nitrogen gas ±40 L/min per plate at 50 °C. The residue was dissolved in 5 % methanol (SPE loading solvent) as follows: 25 µL methanol was

added, stirred and put on the plate shaker for 5 min, 475 µL water was added, stirred and put on the plate shaker for 5 min. The sample was centrifuged for 5 min at 15 °C and 21,250 *g*.

Solid phase extraction by MAX µElution Oasis HLB

The SPE column was pre-conditioned first by adding 150 µL methanol at ±3 psi, next 150 µL water was added at ±3 psi. Sample was transferred on the column at ±5 psi. The wash steps were performed with 100 µL 0.1 % formic acid and 10 % acetonitrile in water and 100 µL 0.1 % ammonia and 10 % acetonitrile in water, respectively. The sample was eluted with 100 µL 60 % acetonitrile in water. The sample was then dried under a stream of nitrogen gas (±40 L/min) at 50 °C. The residue was dissolved well in 75 µL methanol by stirring and shaking for 5 min. Next 75 µL water was added and the sample was put on the shaker for 10 min and centrifuged for 2 min at 935 *g* and at 15 °C. For LC-MS/MS measurement 35 µL was injected.

LC-MS/MS optimization

The MS tuning was performed with different approaches. By direct infusion and/or combined 1 µM analyte in methanol: water (1:1) either containing 2 mM ammonium fluoride (direct infusion) and eluent at 0.1 mL/min flow containing 0.2 mM ammonium fluoride (combined). The analyte was also tuned using IntelliStart software. Once the LC condition was optimized the analyte was tuned using LC-MS setting. All analytes and their internal standards were tuned in both positive and negative ion modes. The LC gradient was optimized using Acquity UPLC HSS T3 (2.1 × 100 mm, 1.8 µm) analytical column at 50 °C, and autosampler temperature at 15 °C. The weak eluent was 0.2 mM ammonium fluoride in water (Eluent A) and strong eluent was methanol (Eluent B). Weak needle wash and strong needle wash were methanol: water (1:1) and methanol, respectively. The seal wash was 10 % methanol in water. LC flow set to 0.4 mL/min, run time 8 min. Finally, a LC-MS/MS measurement program was developed based on multiple reaction monitoring mode (MRM) method in electrospray negative ion mode (ESI^-) and positive ion mode (ESI^+). The next parameters were taken into account to choose most suitable transitions for MRM measurements: maximum intensity of signal of each analyte and internal standard, minimal interferences and adequate quantifier/qualifier ion ratio.

Linearity and limit of determination

For determination of linearity, three approaches were used. First, cortisone and cortisol were spiked in hair matrix (0, 3.9, 7.2, 15.6, 31.3, 62.5, 125, 250 and 500 pg/xmg, n=7). Second, samples with high concentrations were processed in duplicate using different amounts of starting mass of hair (5–10–15–20 mg). Third, samples with high and medium concentration of analytes were mixed with hair with low concentration using different amount of starting mass (3–5–10 mg, final amount of hair ±10 mg), and were extracted in duplicate.

Stability test

The hair samples were tested for three different conditions. First, stability of cortisone and cortisol in pulverized hair exposed to light and air. Three different pools were chosen for the light and air stability test. The samples were exposed for one month to air and daylight on a shelf in the laboratory. For cortisone, the selected samples had concentrations of ±2.5–5–10 pg/mg, for cortisol this was ±2–5–11 pg/mg. Samples were extracted in triplicate and the results were compared with the same samples, which were protected from air and light, and were also extracted in triplicate. Second, we tested stability of the extracted samples at 4 °C for 48 h. For 4 °C stability, 13 samples were extracted in triplet. The samples were once measured at day of extraction and for the second time after 48 h. Third, we tested stability of the extracted samples at −20 °C for one week. For stability of the samples in the freezer, 11 samples were extracted in duplicate and measured for the first time at day of extraction. The samples were then placed in the freezer for one week and measured again.

Patterns of cortisone and cortisol concentrations over time

From nine subjects (all female) 12 cm of hair from scalp was taken to determine per cm the stability of the concentration of cortisone and cortisol along successive hair segments of each individual. The absolute and relative concentrations of cortisone and cortisol per cm hair from scalp of each subject was plotted separately. To calculate the relative concentrations, concentrations of the first cm were set to 100 %. Mixed model analysis was performed as it takes into account within-subject variations. For model selection the Akaike Information Criterion (AIC), Bayesian information criterion (BIC), and marginal and conditional R^2 were used. Using the final model both marginal (average) and subject-specific predictions of cortisone and cortisol were plotted per cm hair.

Matrix interferences

For determination of matrix interferences in hair, the formulas defined by Matuszewski et al. were used [32] (Supplementary Figure 1). Different hair types were used for matrix effect. Blond (1 pool), gray (2 pools), brown (1 pool), and dyed hair (1 pool). Three calibration curves were performed: neat standard solution, for each hair pool pre-extraction, and post-extraction spike. The concentration range of calibration curves was 0.0–15.6–31.3–62.5–125–500 and 1,000 pg/xmg hair and the slope of the curves was calculated.

Data generation and analysis

Waters MassLynx version 4.2 software was used during the whole process. Data generated by the MRM methods were further analyzed by the data processing program TargetLynx XS according to the following criteria generated by two high concentration points from neat standard calibration curves: retention time analytes, retention time internal standards, relative retention time (RRT≤2.5 %), ion ratio (≤20 %), and signal to noise (S/N) set on peak-to-peak (S/N≥10). The current LC-MS/MS method was compared to our previously published method [24] by Passing-Bablok regression.

For the statistical analyses R version 3.5 (R for Windows) and Analyse-it (for Excel) were used [33].

Results

LC-MS/MS optimization

The major precursor (>90 %) in ESI^+ mode was $[M+H]^+$ and in ESI^- $[M-H-30]^-$ for both cortisone and cortisol. Although ESI^+

had a higher response for all analytes, ESI^- was clearly more specific, especially in dyed hair matrix (Figure 1). We therefore opted to validate in ESI^- (Table 1). The tune file parameters were for capillary voltage 3 (kV), desolvation temperature 600 °C, desolvation gas 1,000 L/h, cone gas 200 L/h and source temperature at 150 °C. For ESI^+ see Supplementary Table 1.

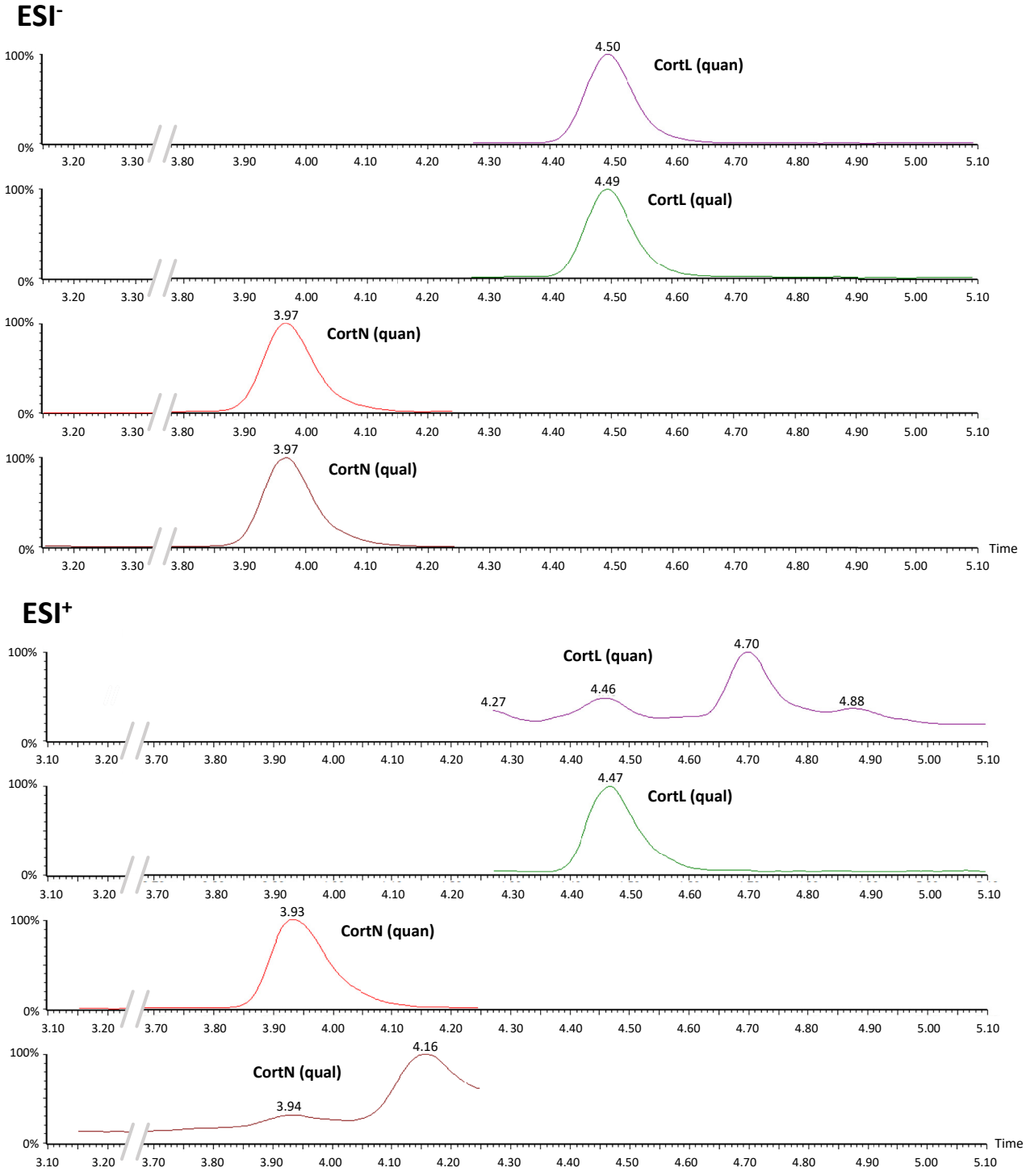

Figure 1: Example of chromatogram of cortisone (8.0 pg/mg) and cortisol (4.3 pg/mg) in dyed hair. No interfering peaks are observed in the negative ion mode (ESI^-, upper panel) in contrast to in the positive ion mode (ESI^+, lower panel).

Table 1: Electrospray ionization negative ion mode, multiple reaction monitoring method for cortisone (CortN) and cortisol (CortL) and their internal standards.

Compound	*m/z* Parent	*m/z* Daughter	Cone, V	CE, V	Dwell, s	Quan/ qual
CortN	329	137	40	30	0.08	Quan
CortN	329	301	40	10	0.08	Qual
$^{13}C_3$-CortN	332	137	40	30	0.08	Quan
$^{13}C_3$-CortN	332	304	40	10	0.08	Qual
CortL	331	297	40	20	0.08	Quan
CortL	331	282	40	30	0.08	Qual
$^{13}C_3$-CortL	334	300	40	20	0.08	Quan
$^{13}C_3$-CortL	334	285	40	30	0.08	Qual

The tune file parameters are for capillary voltage 3 (kV), desolvation temperature 600 °C, desolvation gas 1,000 L/h, cone gas 200 L/h and source temperature at 150 °C.

LC-gradient

A mobile-phase gradient was used during the 8 min run. To protect the source from contaminants, at start of the run the LC flow was guided to the waste and switched to detector at 2.70 min and diverted to waste at 5.30 min. The data was collected between 3.15 and 4.25 min for cortisone and $^{13}C_3$-Cortisone, and between 4.27 and 5.1 min for cortisol and $^{13}C_3$-Cortisol (Table 2). The peaks of cortisone (3.97 min) and cortisol (4.50 min) and their internal standards were fully separated (Figure 1, upper panel).

Calibration curves

Calibration curves for cortisone and cortisol were prepared in hair matrix with a concentration range between ±1–100 pg/mg, and were linear (R^2 of 0.996 and 0.997, respectively).

Precision

Pooled hair samples with concentrations near the expected LOQ as well as previously published clinical relevant concentrations (LLN, mean and ULN) were analyzed to assess reproducibility of the method (Table 3) [31]. LLOQ for cortisol was set to 1.0 pg/mg (average pools 5 and 9) and for cortisone to 1.5 pg/mg (average pools 5 and 9).

Table 2: Liquid chromatography gradient for cortisone and cortisol and their internal standards.

Compound	Time	Flow	%A	%B	Curve
1	Initial	0.400	55.0	45.0	Initial
2	2.00	0.400	55.0	45.0	6
3	7.00	0.400	35.0	65.0	6
4	7.02	0.400	2.0	98.0	11
5	8.00	0.400	55.0	45.0	11

Table 3: Cortisone and cortisol pg/mg hair within run and between run (number of runs=10).

Hair pool	Mean[a]	SD	%CV	Mean[b]	SD	%CV
Cortisone						
1	5.7	0.2	4	5.3	0.4	7
2	1.7	0.2	12	1.8	0.1	7
3	9.8	0.9	9	10.8	0.4	4
4	4.7	0.4	9	4.8	0.3	6
5	2.0	0.2	9	1.8	0.2	12
6	2.8	0.2	6	2.9	0.2	5
7	7.2	0.6	8	7.1	0.4	6
8	18.5	0.7	4	18.2	0.9	5
9	1.2	0.05	4	1.3	0.1	9
10	22.2	1.8	8	21.8	1.4	6
Cortisol						
1	15.8	1.4	9	13.4	1.6	12
2	2.0	0.4	18	2.2	0.3	11
3	4.8	0.3	7	4.8	0.3	5
4	1.6	0.1	6	1.5	0.1	8
5	1.2	0.05	4	0.9	0.1	14
6	3.0	0.3	10	2.8	0.3	12
7	2.2	0.1	6	2.1	0.05	2
8	5.0	0.3	7	4.9	0.3	5
9	1.1	0.1	12	1.1	0.2	14
10	7.1	0.5	7	7.0	0.4	6

[a]Within run; [b]between run. CV, coefficient variation.

Linearity and limit of determination

For all three approaches for determination of linearity the R^2>0.995 for both cortisone and cortisol. The calibration curves for both cortisone and cortisol in hair matrix with a concentration ranged between ±1–100 pg/mg has R^2 of >0.995.

Trueness

When comparing our previously published method [24] to the currently described method, one sample was excluded because of matrix interference measured by the previous method. No absolute difference, but a significant relative difference was found between methods for both analytes (slope 1.20 (95 % CI 1.06–1.34) and 1.21 (95 % CI 1.07–1.39) for cortisone and cortisol, respectively).

Stability test

Stability of cortisone and cortisol in hair was tested for four different conditions. First, the stability was tested in pulverized

Table 4: Cortisone (CortN) and cortisol (CortL) protected and exposed (pg/mg) and matrix interferences.

A. Protected[a] and exposed[b]						
Hair pool	**Mean[a]**	**SD**	**%CV**	**Mean[b]**	**SD**	**%CV**
CortN (6)	2.6	0.2	7	2.6	0.2	7
CortN (1)	5.1	0.3	6	4.8	0.03	0.7
CortN (3)	9.8	0.3	3	9.4	0.7	8
CortL (6)	2.2	0.4	17	2.6	0.5	17
CortL (3)	4.5	0.04	1	4.2	0.3	7
CortL (1)	11.1	0.4	3	12	1.9	15

B. Matrix interferences cortisone[a] and cortisol[b]						
Hair pool	**%ME[a]**	**%RE[a]**	**%PE[a]**	**%ME[b]**	**%RE[b]**	**%PE[b]**
Blond	105	96	101	104	96	100
Gray-1	103	98	101	104	100	104
Gray-2	99	98	97	100	98	98
Brown	101	102	103	99	102	101
Dyed	101	98	99	102	96	98

ME, matrix effect; RE, extraction recovery; PE, process efficiency; CV, coefficient variation. In Table 4A, [a]referes to protected, [b]referes to exposed.

hair exposed to light and air. The slope of cortisone and cortisol were 0.91 ($p<0.01$), and 0.98 ($p<0.01$), respectively (Table 4A). Second, stability of the extracted samples was tested at 4 °C for 48 h. The slope for cortisone and cortisol were 0.99 ($p<0.01$), and 0.97 ($p<0.01$), respectively. Third, stability of the extracted samples was tested at −20 °C for one week. The slope for cortisone and cortisol were 1.01 ($p<0.01$) and 1.01 ($p<0.01$), respectively.

Patterns of cortisone and cortisol concentrations over time

From nine subjects (all female), 12 consecutive cm of hair were analyzed separately per cm hair. Individual, but predictable, trajectories were apparent for both cortisone and cortisol and a strong non-linear relationship among consecutive concentrations within the hair strand was found for all subjects studied (Figure 2A). A second order polynomial fit resulted in a conditional R^2 of 0.947 and 0.925 for cortisone and cortisol, respectively (Supplementary Table 2). Increasing the degree of fit to a third order, or using a spline model, did not improve the model substantially. The marginal (average) and subject-specific predictions are included in Figure 2A and B, respectively. Furthermore, analyte concentrations showed a negative decline even within the first 2 cm in almost all subjects. Consistent with the observation over 12 cm, the degree of decline was subject-specific (Supplementary Figure 2). Finally, as expected from the assays performance characteristics, cortisone concentrations showed less individual variation and better predictability when compared to cortisol.

Matrix interferences

Results of all matrix experiments are presented in Table 4B. The R^2 of both cortisone and cortisol were always >0.999. The result showed a matrix effect (%ME) of maximal 5 %, extraction recovery (%RE) of ≥98 % and a process efficiency (%PE) ≥97 %. The absolute %ME, %RE and %PE were also calculated by slope of area of analyte and the extraction lost was taken into account. For cortisone this was 97, 89 and 84 %, respectively. For cortisol this was 97, 90 and 89 % respectively. The R^2 on average was >0.995, only in case of pre-extraction spike this was on average 0.99.

Discussion

We developed an accurate, sensitive and robust LC-MS/MS method for quantification of cortisone and cortisol in human scalp hair. This method is novel in several aspects. It is sensitive and only requires 10 mg of hair. Several optimized conditions in combination with the MS/MS measurement in ESI^- mode ensure selectivity, sensitivity and minimize the risk of incorrect quantification of analytes within the hair matrix and/or misinterpretation. In addition, a good separation of the analytes using a RP-column with suitable LC-gradient combined with use of $^{13}C_3$-labelled internal standards ensures the robustness of the newly developed method for determination of cortisone and cortisol in hair. Furthermore, the LC-MS method was sufficiently sensitive and precise to detect

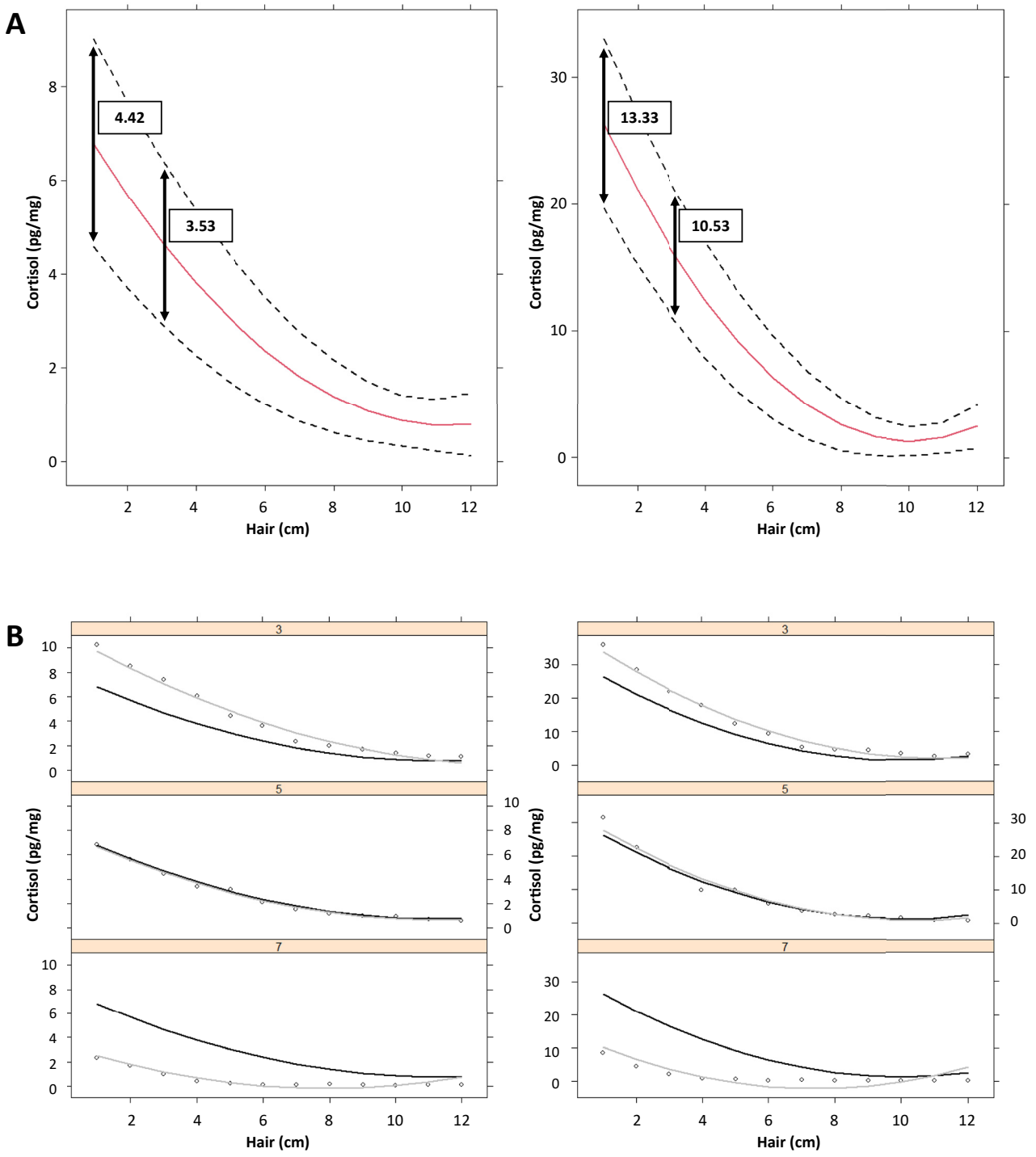

Figure 2: Marginal predictions (A) and subject-specific predictions (B) of cortisone and cortisol concentrations per centimeter hair. The dotted lines represent the 95% confidence interval (95%CI). The differences between the upper and the lower bound of the 95%CI (delta) at 1 cm and 3 cm are presented in the white boxes with black outline (A). Comparison of the marginal and subject-specific predictions of the subjects 3, 5 and 7 (B). The black lines denote the marginal predictions and the grey lines the subject-specific predictions. The black circles present the observed data.

variations within-subjects in cortisone and cortisol concentrations between successive hair segments of 1 cm. Using mixed model analysis, a statistical approach for within-subject data, strongly conserved individual patterns of cortisone and cortisol concentrations could be calculated.

New way of data interpretation

Our statistical approach takes into account within-subject cortisone and cortisol concentrations in successive hair segments, which opens the door for more reliable ways to detect pathological concentrations. Most previous studies determined cortisone and cortisol concentrations in hair by averaging absolute concentrations in the first 2 or 3 cm (overview of LC-MS studies in Supplementary Table 3). A possible pitfall of this single measurement approach is that short term perturbations may be missed as the associated elevation in cortisol is averaged out [34]. As an illustration, a hypothetical patient has an average hair cortisol concentration in the first 3 cm of 3 pg/mg. Whether the hair cortisol concentrations per 1 cm were 4–3–2 pg/mg or 2–5–2 pg/mg cannot be deduced. This may have clinical implications as the first case might reflect a physiological reduction of hair cortisol concentrations, while the second case might present a patient with an episode of cortisol excess (e.g. cyclic Cushing's syndrome). To determine the exact link between perturbations in the HPA-axis and cortisone and cortisol trajectories, our findings need to be validated in a large prospective cohort study.

Comparison with previous studies

In accordance with previously published data from our and other groups, hair cortisone and cortisol concentrations were lower in more distal fragments [24, 27]. In our study, analyte concentrations showed a negative decline even within the first 3 cm in all subjects. Therefore, averaging absolute concentrations in the first 2 or 3 cm may under- or overestimate the concentration. Furthermore, our findings showed that the variation between individuals is the largest at 1 cm from scalp, and the smallest in most distal hair segments. This may hamper drawing conclusions from concentrations of cortisone and cortisol in distal hair segments. This is in contrast with a recent study which did not find such a decline in all patients, and stated that cortisol concentrations can be measured accurately two years back in time (up to 24 cm hair) [30].

The potential of cortisone

Hair cortisol is predominantly used to measure long-term stress, whereas its inactive metabolite cortisone is often not assessed, although both are measures of long-term exposure to glucocorticoid hormones [23, 24, 35–37]. However, our results demonstrated that cortisone might be a more suitable measure for exposure to glucocorticoid hormones than the conventional cortisol, since cortisone concentrations showed less individual variation than cortisol concentrations. The possible validity of cortisone has been described in the field of anthropometry [38] and metabolic syndrome [39].

Recommendations for future studies

Based on our findings, cortisone and cortisol concentrations should be assessed in consecutive hair segments of 1 cm, and subject-specific concentration patterns should be determined rather than assessing one single sample to identify perturbed cortisol metabolism. To determine reference trajectories and altered trajectories, large prospective cohort studies including more data of healthy patients and of patients with perturbations in the HPA-axis are required. This is not only clinically important for diseases such as Cushing's and Addison's disease, but also since elevations in hair cortisone and cortisol levels have been associated with the metabolic syndrome [39–42], obesity [35, 43, 44] and cardiovascular diseases [45–48]. In addition, expanding the LC-MS/MS method by including other steroids, such as 21-deoxycortisol and 11-deoxycortisol, holds the potential to provide valuable insights into the overall steroid profile [24, 49]. However, it is important to consider the challenges associated with these measurements. Lower concentrations of certain steroids compared to cortisone and cortisol pose a challenge in accurate quantification [50]. Furthermore, variations in the metabolism and distribution of different steroids within hair samples introduce further complexities. However, it is important to note that measuring these additional steroids falls beyond the scope of the present study and remains a recommendation for future research.

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