ORIGINAL PAPER

CrossMark

Online solid-phase extraction liquid chromatography–mass spectrometry of hair cortisol using a surrogate analyte

Katarina Kostolanska[1] · Lucie Novotna[1] · Eva Taborska[1] · Ondrej Pes[1]

Abstract

Online SPE LC–MS with $^{13}C_3$-cortisol as a surrogate analyte was developed, validated and applied for obtaining hair cortisol levels of 114 healthy volunteers. The first 3 cm from a posterior vertex position were analyzed as three individual segments resulting in 310 hair cortisol levels, which were used, after log transformation, to predict a range of values observed in future. The median value of hair cortisol was 4.76 pg/mg. The employed method allowed simple processing, high throughput and may be readily expanded to analyze additional steroid compounds in hair.

Keywords Endogenous cortisol · Long-term hair concentration · Surrogate analyte · LC–MS · Online SPE

Introduction

Cortisol is a glucocorticoid hormone with major effect upon a range of physiological homeostatic mechanisms. It plays an important role in stressful situations when stressors activate the hypothalamic–pituitary–adrenocortical (HPA) system.

Cortisol has been routinely analyzed in the laboratory from saliva, serum/plasma, and/or urine. However, the HPA axis represents a highly dynamic system influenced by several factors and expose circadian rhythmicity. The most widely analyzed saliva or plasma reflect only acutely circulating cortisol levels, urine provides usually 24-h assessments of levels through the day. Measurements in the above-mentioned body fluids are limited in the temporal range of assessment. Cirimele et al. were first who detected cortisol and other glucocorticoids in hair (Cirimele et al. 2000). Cortisol is being constantly incorporated into a growing hair shaft and, accordingly, can allow the unique possibility to assess the long-term systemic levels and obtain a retrospective index of long-term hormone secretion. Based on an average hair growth rate of 1 cm per month (Wennig 2000), it is possible to distribute the hair along the length of individual segments and determine the concentration of cortisol reflecting secretion in the monitored period. But due to a rapid decrease of cortisol in hair, the measure may be limited to the past 6 months (Kirschbaum et al. 2009).

Concerning the diagnostic value, differences in cortisol levels can be used to evaluate disturbances of the HPA axis. It may serve in the diagnosis of Cushing's syndrome disease but it may also be useful as a marker of successful therapy. Changes in cortisol levels have been observed in mental diseases, e.g., depression, general anxiety disorder or post-traumatic stress disorder as well as physical diseases where it could serve as a marker for diagnosis or risk stratification (Russell et al. 2012).

Commonly used methods for a hair steroid analysis are immunoassays; principally enzyme-linked immunosorbent assays (ELISA). The major disadvantage of immunoassays is low specificity. Given to high structural similarity of steroid compounds, there is a risk of cross-reactions inside the immuno-complex that may overestimate the target concentration. In this context, cortisone especially has been observed as a major source of interference in immunoassays research of salivary cortisol (Miller et al. 2013). These approaches are often unsuitable for clinical applications that require a low detection limit (Taieb et al. 2002). Much higher specificity is provided by liquid chromatography–mass spectrometry (LC–MS). Compared to immunoassays with specificity to only a single analyte, LC–MS proves to measure a panel of multiple steroid hormones simultaneously, currently 28 steroids (Dong et al. 2017). LC–MS offers other advantages, such as high sensitivity,

✉ Ondrej Pes
ondramayl@gmail.com

1 Department of Biochemistry, Faculty of Medicine, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic

wide dynamic range, high throughput, and an option for automated sample preparation (Gao et al. 2016) Current and potential future applications of hair cortisol analysis have been summarized in a review by Wester and Rossum (Wester and van Rossum 2015), however; the exact physiological range of hair cortisol concentration has not yet been established.

In general, cortisol content in hair is very low. Raul et al. reported cortisol concentration ranged from 5.2 to 91 pg/mg with a mean value of 19 pg/mg. LC–MS/MS with electrospray ionization (ESI) in the positive mode was used for quantification; limit of detection (LOD) and limit of quantitation (LOQ) was 1 and 5 pg/mg, respectively (Raul et al. 2004). Another study reported a median cortisol level of 8.8 pg/mg for elementary school girls with a range of 5–1330.5 pg/mg (Vanaelst et al. 2013). Though, only 39 of 168 samples were higher than LOQ (5 pg/mg). Hair cortisol levels 12.7 ± 14 pg/mg in hair samples of healthy volunteers with an age range of 4–63 years were obtained by LC–MS/MS/MS (Quinete et al. 2015). The broad range of physiological values is likely affected by the fact that the mechanism of cortisol incorporation into the hair shaft has yet not been fully revealed.

As cortisol is normally present in hair, it is difficult to achieve a high throughput methodology using an authentic matrix calibration curve. A method of the standard addition should be used instead (Tsakelidou et al. 2017). Some authors have considered a distal segment of hair as an analyte-free matrix even that ascertained cortisol concentration (1.2 pg/mg) was found in the 4 cm segment of the distal end of 20 cm long human hair (Binz et al. 2016). In such cases, the matrix varies from sample to sample and a lack of a reference material made all the validation studies ambiguous. Cortisol added to the solution of "blank hair" might behave differently and may significantly contribute to the high variation of hair cortisol, especially at low concentration levels. Hence, spiking with known amounts of reference standards should be avoided if the method of standard addition is not used for each sample. Alternatively, the results should be associated with the synthetic matrix only (Doroschuk et al. 2016).

The only approach using an authentic matrix and a surrogate analyte was demonstrated by Binz et al. (Binz et al. 2016) even as using a surrogate analyte might prove very advantageous in the analysis of endogenous compounds in challenging matrices and allows for facile method development when there is no analyte-free matrix available. The response of the isotopically labeled $^{13}C_3$-cortisol as a surrogate analyte was consistent and nearly identical with that of cortisol. Moreover, a method of a calibration curve in the authentic matrix could have been employed without the risk of deteriorating the results. The authors compared both approaches and validated the surrogate analyte method to

unambiguously determine the lowest cortisol levels in all hair samples.

In the present study, we aimed to assess a prediction interval based on data of hair cortisol in the local adult population using an approach based on online SPE LC–MS/MS with ^{13}C-labeled cortisol as a surrogate analyte.

Results and discussion

Online SPE LC–MS

An automated on-line SPE LC–MS provided high throughput of samples and minimized the retention time fluctuation as the autosampler loop (void volume > 100 μl) was excluded from the separation pathway. Moreover, we have, to our best knowledge, presented an inexpensive approach of utilizing a guard column cartridge (2 pcs) as a trapping column for the SPE purification/enrichment for the first time. The first 2 min of a chromatogram served for trapping and washing of the sample, which was done by a continuous flow of 2 mL of 10% MeOH from the secondary pump. When detection at 260 nm was used instead of MS the compounds originating from the washing step of SPE were eluted during the first milliliter of 10% MeOH (data not shown).

Representative LC/MS chromatograms are shown in Fig. 1. Figure 1a shows a hair sample from the hair pool; Fig. 1b, c show a hair pool sample spiked with $^{13}C_3$-cortisol and D_8-cortisone, and Fig. 1d shows a representative hair sample of a participant with spiked D_8-cortisone. In most cases, the endogenous cortisol levels in hair pool were below limits of detection, however; as the sample matrix was not homogenous there were cases when the quantitation would have been ruined if the surrogate cortisol calibration had not been used. The response factor for $^{13}C_3$-cortisol/cortisol was 0.94. Peaks of cortisol and cortisone were chromatographically resolved to eliminate errors resulting from very similar mass-to-charge ratios of target compounds. This was achieved by employing > 50% MeOH in the mobile phase during a single gradient run. On the other hand, the system backpressure was relatively high (~ 300 bar), which may shorten the analytical column lifetime. The separation conditions were chosen to allow the simple introduction of additional steroid compounds into the method in future. Due to the high MeOH content in the mobile phase, it might be advantageous to employ a heating gradient inside the ESI chamber (Şener et al. 2017).
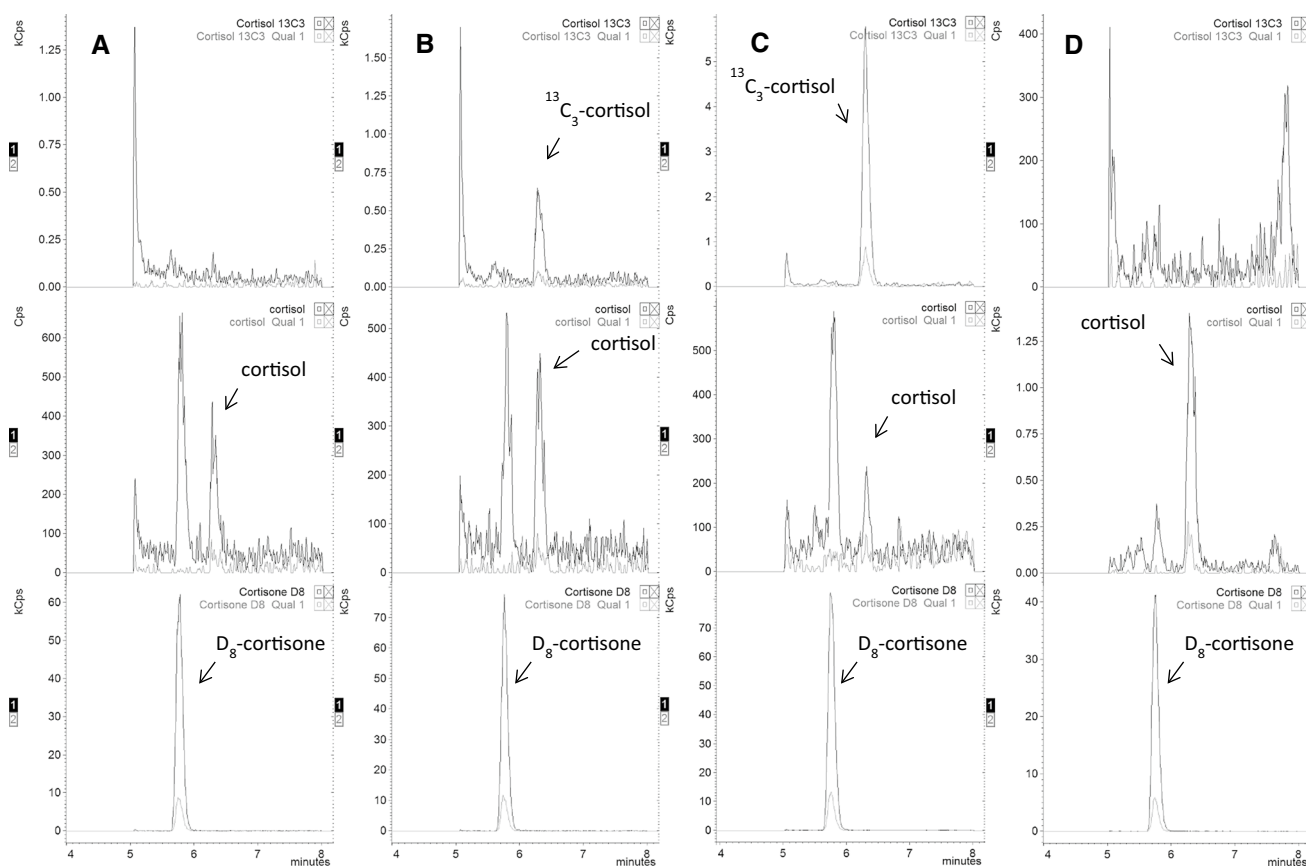
Fig. 1 Online SPE LC–MS chromatograms of **a** a hair pool sample, **b** a hair pool sample spiked with $^{13}C_3$-labeled cortisol (1.25 pg/mg), **c** a hair pool sample spiked with $^{13}C_3$-labeled cortisol (12.5 pg/mg), and **d** a real hair sample (8 mg). Two SRM transitions for $^{13}C_3$-cortisol ($t_R = 6.3$ min), cortisol ($t_R = 6.3$ min), and IS D_8-cortisone ($t_R = 5.8$ min) are represented by the upper, middle, and lower frames, respectively. Please notice, that the endogenous cortisol was found in the hair pool sample at rather low, however, detectable levels

Linearity, LOD, and LOQ

Calibration was carried out using a $^{13}C_3$-labeled cortisol. The calibration curve showed linearity in the entire range 1–100 pg/mg. The square of the correlation coefficient (R^2) of the calibration curve was 0.9995. The intercept was tested by regression analysis and found insignificant (p value 0.076, $\alpha = 0.05$). The LOD and LOQ were found 0.3 and 0.8 pg/mg for $^{13}C_3$-cortisol with a signal–noise ratio of 3:1 (LOD) and 10:1 (LOQ), respectively. The LOD and LOQ were in a similar range than previously published methods with LC–MS. A calibration curve from 1 to 100 pg/mg was used to determine cortisol levels in authentic hair samples as the cortisol concentrations in hair normally exceed 1 pg/mg.

Recovery and matrix effect

The data for recovery and matrix effects are summarized in Table 1. Recovery was 91.4, 100.2 and 90.1% for the low,

Table 1 Recovery and matrix effect, ($N=5$)

	$^{13}C_3$-cortisol		
	Low (5 pg/mg)	Medium (20 pg/mg)	High (100 pg/mg)
Recovery (%)	91.4	100	90.1
RSD (%)	11	8.8	9.0
Matrix effect (%)	116	97.4	106
RSD (%)	6.2	9.6	5.8

medium and high concentration, respectively, with the relative standard deviation (RSD) below 12%. Matrix effects were not observed for $^{13}C_3$-cortisol (≥ 75 and $\leq 125\%$).

Precision and accuracy

The results for accuracy and precision for $^{13}C_3$-cortisol as an analyte are summarized in Table 2. In this case, all the data met the required criteria (RSD $< 15\%$).

Table 2 Accuracy and precision, on 5 consecutive days

$^{13}C_3$-cortisol	Low (5 pg/mg)	Medium (20 pg/mg)	High (100 pg/mg)
Characteristics ($N = 25$)			
Mean (pg/mg)	5.09	20.2	102
SD (pg/mg)	0.307	1.46	6.01
Accuracy			
Variance (pg/mg)	0.090	0.20	2.0
Bias (%)	1.7	1.0	2.0
Intraday precision ($N = 5$)			
RSD (%)	3.5	6.2	4.9
Interday precision ($N = 25$)			
RSD (%)	6.0	7.2	5.9

Clinical application

To indicate a range of hair cortisol values, which might be applicable as a reference range, a series of 114 volunteers (40 men, 74 women, age 19–27 years) has been analyzed for hair cortisol levels, see Table 3. Such effort was already made by other groups, however; a range of expected values should be determined among the population it is going to be applied to. As we plan to correlate hair cortisol levels of people with disorders associated with high levels of cortisol in the organism, we had to compare the range obtained with local population with that assessed by other groups. Although we attempted to minimize within individual variation in hair growth by taking hair from the vertex posterior region of the head, hair growth rates may vary among individuals. Furthermore, the participants in this cohort at this phase have a mean age of 20.5 years; therefore, it may not be possible to generalize the findings of this study to older adults, even as the age-related variability appears mostly in the range 0.5–18 years (Binz et al. 2018).

The original data were log-transformed to obtain normal distribution, which was used to obtain re-transformed mean (4.76 pg/mg), standard deviation (1.88 pg/mg), and a prediction interval ($\alpha = 0.05$; 1.22–18.7 pg/mg) in the first three centimeters of hair (Table 3). The prediction interval could be defined as an estimate of an interval in which future observations will fall, with a certain probability and it was very similar to the 2.5–97.5 quantile interval.

To reveal the relationship of the hair cortisol concentration with the physical state of the organism, we asked the participant with the value of hair cortisol, which was marked as an outlier, to be sampled again after a 3-month period. In this case, the hair sample was obtained from three posterior vertex positions approximately 2 cm from each other (left, middle, and right) in two replicates; each of the hair strands was cut into six 1 cm segments. Essentially, it could have represented hair cortisol concentrations approximately over the past 6 months (Table 4). Despite the high variability between individual sampling locations, it may be clearly stated that hair cortisol had been increased in the time of the first sampling (4–6 cm) and was probably lowered by a washing-out effect. The values from the 1–3 cm may suggest that the overall cortisol balance in the organism has been settled and that the former values had been momentarily increased. However, the participant was not able to provide any additional information on the cause of the elevated values. Careful interpretation has to be made as, according to another study, the participant could have been identified as a person with Cushing's syndrome (cut-off = 31.1 pg/mg) (Wester et al. 2017).

Materials and methods

Chemicals and calibration standards

Reagents, stock solutions of $^{13}C_3$-cortisol (2,3,4-$^{13}C_3$) (100 µg/mL), deuterated D_8-cortisone (2,2,4,6,6,9,12,12-D_8), formic acid (HCOOH), and methanol (MeOH, LC–MS) were obtained from Sigma Aldrich (Switzerland). All water used was of an ultra-pure grade supplied by an in-house Milli-Q system (Millipore, MA, USA).

A stock solution of deuterated internal standard (IS) D_8-cortisone was prepared at the final concentration of 100 ng/mL and was also used as a working solution. A stock solution of a stable isotope-labeled form of the analyte, $^{13}C_3$-cortisol, was prepared in methanol to a concentration of 50 ng/mL. Working standard solutions were prepared by diluting the stock solution $^{13}C_3$-cortisol to a

Table 3 Hair cortisol concentration (pg/mg) of the study participants ($N = 114$) containing mean (X), median (M), re-transformed mean (X_R), re-transformed standard deviation (SD_R), prediction interval (PI), and a 2.5–97.5 quantile interval (Q_{95})

Hair segment	N	%	X	M	X_R	SD_R	PI	Q_{95}
1st cm	114	100	6.02	4.86	4.85	1.84	1.44–16.3	1.45–16.5
2nd cm	101	88.6	7.13	4.90	5.00	2.03	1.22–20.6	1.35–17.8
3rd cm	95	83.3	6.63	4.20	4.59	2.09	1.05–20.0	1.13–23.1
3 cm length	310	–	6.57	4.76	4.76	1.88	1.22–18.7	1.32–19.4

Table 4 Hair cortisol concentrations (pg/mg) of a single sample cut into 6 segments in two replicates

Position	Hair segment					
	1 cm	2 cm	3 cm	4 cm	5 cm	6 cm
Left						
A	7.82	8.45	8.13	13.7	27.6	35.2
B	7.58	10.2	9.62	13.1	20.2	22.7
X	7.70	9.33	8.87	13.4	23.9	29.0
SD	0.168	1.24	1.05	0.44	5.23	8.82
RSD (%)	2.18	13.3	11.8	3.26	21.9	30.5
Middle						
A	8.82	10.2	16.0	41.0	60.2	52.4
B	11.0	28.2	23.5	51.8	73.1	61.1
X	9.90	19.2	19.7	46.4	66.7	56.8
SD	1.53	12.7	5.29	7.63	9.13	6.11
RSD (%)	15.4	66.4	26.8	16.4	13.7	10.8
Right						
A	11.4	11.1	11.4	12.6	18.9	19.0
B	10.7	12.3	13.5	14.5	21.3	19.9
X	11.1	11.7	12.5	13.6	20.1	19.5
SD	0.506	0.810	1.50	1.37	1.63	0.616
RSD (%)	4.57	6.92	12.0	10.1	8.13	3.17

Values of the mean (X), standard deviation (SD), and relative standard deviation (RSD) were calculated

concentration of 0.08, 0.4, 0.8, 1.6, and 8 ng/mL resulting in the final concentration levels in the calibration curve of 1–100 pg/mg.

All standard and stock solutions were individually diluted with methanol and stored at − 20 °C until use.

Hair sample collection and preparation

The hair samples were taken from healthy volunteers following the basic criteria for collection defined by the Society of Hair Testing (Cooper et al. 2012). The protocol was approved by the Masaryk University Ethical Committee, and all participants gave written informed consent. The hair strands with a thickness of several millimeters in diameter were cut with fine scissors from a posterior vertex position as closely as possible to the scalp. Subsequently, they were tagged on the proximal side and stored individually in a folded piece of paper in labeled foiled bags. Until the day of analysis, the hair strands were stored at room temperature in a dry environment away from direct sunlight. For the analysis, the first 3 cm of the proximal segment was cut into 1 cm segments. A minimum of 8 mg of each segment was weighted to evaluate and determine the level of cortisol.

Four centimeters of the distal end of 20 cm long human hair was used as authentic matrix. The hair was cut into snippets, homogenized and used as a hair pool during the method development.

Hair sample extraction

Prior to analysis, the hair samples were manually washed in vials by shaking them two times with 2 ml of isopropanol for 1 min at room temperature. Subsequently, they were dried on a piece of filter paper and uniformly mixed with a laboratory tweezer to ensure maximum homogeneity. Approximately 8 mg of washed and dry hair was carefully transferred into a glass tube. Sequentially, 1800 μl of methanol and 10 μl of IS from the working solution (100 ng/mL) were added.

Calibration curve standards were prepared following the same procedure using the hair pool matrix. The 8-mg hair sample was spiked with $^{13}C_3$-cortisol (100 μL from the working standard solutions resulting in final concentrations of 1–100 pg/mg) and 10 μl of IS from the working solution (100 ng/mL) was added prior to incubation. The hair was incubated for 18 h at room temperature, in darkness and without shaking. Afterward, 1600 μl of the extract was transferred into a new tube and fully evaporated at 40 °C under a constant stream of nitrogen. The residue was reconstituted in 500 μl of 20% MeOH, vortexed for 1 min, and transferred into the LC–MS vials. A volume of 50 μl was directly injected into the online SPE LC–MS/MS system for analysis.

Online SPE LC–MS

The online SPE LC–MS method for analysis of cortisol was developed using a Dionex Ultimate 3000RS (ThermoScientific, CA, USA) module, equipped with a binary

high-pressure gradient pump (HPG), a quaternary low-pressure gradient pump (LPG) connected to an auto-sampler (AS), and a column oven with a 6-port, two-way valve. The trapping column (SPE column) consisted of a mounted guard column holder (Phenomenex, CA, USA) with two pieces of a SecurityGuard^{TM} cartridge (2×4 mm, C18, Phenomenex, CA, USA). With the switching valve in the sample loading position (A), the LPG pump was used to load within 2 min 50 μl of the samples on the SPE column operating at room temperature using 10% MeOH in water at 1 ml/min. From 2 to 2.5 min (Fig. 2), the valve automatically switched to its alternate position (B) and then back to the loading position (A), allowing the analytes to be transferred (in a backflush mode) from the SPE column onto the analytical column (2.1 mm×100 mm, 2.7 μm C18 Ascentis, Phenomenex, CA, USA), shielded by a guard column (C18, 2×2 mm, Phenomenex, CA, USA), both operated at 20±0.1 °C. The HPG binary mobile phase system consisted of water with 0.1% formic acid (v/v) and MeOH. The flow-rate was set

to 0.2 ml/min and the following 12-min gradient program was employed. The initial 50% of MeOH was kept constant for 2 min and then linearly increased from 50 to 85% over the next 7.5 min. It was held at 85% for 1 min, followed by equilibration under the initial conditions for another 1.5 min. From 4.0 to 8.5 min, the SPE column was regenerated using 100% MeOH and then re-equilibrated using 10% MeOH.

The outlet of the analytical column was connected to a Bruker EVOQ Qube triple quadrupole mass spectrometer (Germany) operated in the negative heated-electrospray ionization mode with the following parameters: Spray voltage 3500 V, cone temperature 350 °C, cone gas flow 20 psi, heated probe temperature 350 °C, probe gas flow 40 psi (nitrogen), and nebulizer gas flow 50 psi (nitrogen). Argon was used as collision gas. All other parameters were set at default values provided by the manufacturer. The flow from LC was diverted to waste in the range of 0–5 and 8.5–12 min. Target compounds were scanned in the selected reaction monitoring mode (SRM) as fragments of ionic adducts with
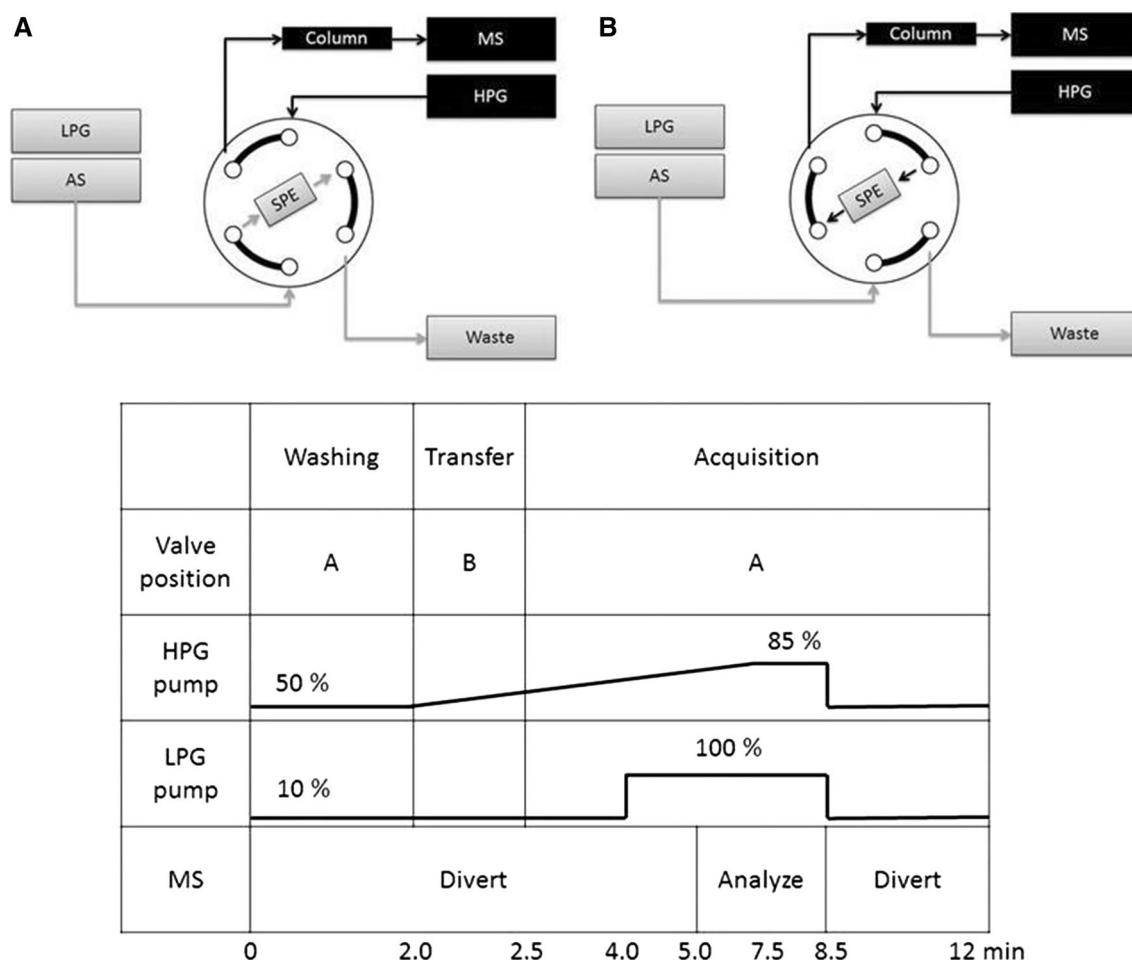

Fig. 2 Switching valves positions. **a** Loading and washing. **b** Sample transfer. The table specifies individual times of events during a single sample run

formic acid $[M+HCOO]^{-1}$: cortisol 407.1–331.1 (12 eV) and 407.1–297.1 (29 eV); cortisol $^{13}C_3$ 410.1–334.1 (12 eV) and 410.1–300.1 (29 eV); D_8-cortisone 413.3–337.1 (10 eV) and 413.1–139.1 (36 eV). Dwell time for each transition was 50 ms.

Method validation

As the validation for using the $^{13}C_3$-cortisol calibration instead of endogenous cortisol calibration was done by Binz et al. 2016, we have validated only the parameters related to the $^{13}C_3$-cortisol calibration. Statistica (v13, TIBCO Software Inc.) was used for data processing.

LOD and LOQ

Five calibration levels (1–100 pg/mg) were used. Three replicates for each calibration level (1, 5, 10, 20, 100 pg/mg) were analyzed. The calibration curve was estimated by the least-squares regression procedure. LOD and LOQ were determined for $^{13}C_3$-cortisol by analyzing the lowest calibration point of calibration and calculating a theoretical signal-to-noise ratio of 3:1 for LOD and 10:1 for LOQ. The values were verified by measuring $^{13}C_3$-cortisol samples using the calculated concentrations.

Accuracy, precision, matrix effects, and recovery

Accuracy and precision of the method were evaluated by measuring five replicates of a QC sample at three different concentration levels (low 5 pg/mg, medium 20 pg/mg, high 100 pg/mg) on 5 consecutive days. Matrix effect was measured by comparing the calculated concentration of $^{13}C_3$-cortisol spiked hair after extraction (A) to the spiked solvent at the same concentration (B) (Matrix effect $= A/B \times 100$). Recovery was measured by comparing the calculated concentration of $^{13}C_3$-cortisol from spiked hair before extraction (C) to spiked hair after extraction (D) (recovery $= C/D \times 100$). Five replicates at low (5 pg/mg), medium (20 pg/mg) and high (100 pg/mg) concentration levels were used for both parameters.

Conclusions

An online SPE LC–MS with a surrogate analyte was developed and validated to analyze the hair cortisol concentration. A range and median concentration levels of physiological values correspond to those previously published in the literature. Cortisol in hair represents a long-term value of cortisol in the organism even that the incorporation mechanism has not been fully uncovered yet. We have obtained a prediction interval for hair cortisol to help differentiate between short-term physical stress-related issues and long-term non-physiological conditions, such as Cushing's syndrome. Hence, when hair cortisol values are being determined we recommend analyzing a longer segment of a hair strand to obtain a decline curve, which might negate the enormous individual and sampling variability. Careful interpretation of hair cortisol levels within the prediction interval should be considered as well.

References

Binz TM, Braun U, Baumgartner MR, Kraemer T (2016) Development of an LC–MS/MS method for the determination of endogenous cortisol in hair using C-13(3)-labeled cortisol as surrogate analyte. J Chromatogr B Anal Technol Biomed Life Sci 1033:65–72. https://doi.org/10.1016/j.jchromb.2016.07.041

Binz TM, Rietschel L, Streit F, Hofmann M, Gehrke J, Herdener M, Quednow BB, Martin NG, Rietschel M, Kraemer T, Baumgartner MR (2018) Endogenous cortisol in keratinized matrices: systematic determination of baseline cortisol levels in hair and the influence of sex, age and hair color. Forensic Sci Int 284:33–38. https://doi.org/10.1016/j.forsciint.2017.12.032

Cirimele V, Kintz P, Dumestre V, Goulle JP, Ludes B (2000) Identification of ten corticosteroids in human hair by liquid chromatography–ionspray mass spectrometry. Forensic Sci Int 107:381–388. https://doi.org/10.1016/S0379-0738(99)00180-2

Cooper GAA, Kronstrand R, Kintz P (2012) Society of hair testing guidelines for drug testing in hair. Forensic Sci Int 218:20–24. https://doi.org/10.1016/j.forsciint.2011.10.024

Dong Z, Wang CH, Zhang JL, Wang Z (2017) A UHPLC-MS/MS method for profiling multifunctional steroids in human hair. Anal Bioanal Chem 409:4751–4769. https://doi.org/10.1007/s00216-017-0419-2

Doroschuk VO, Grogul AB, Mandzyuk YS, Makukha OG, Grytsyk NO (2016) Cloud point extraction of disulfiram for its HPLC–MS/MS determination in synthetic urine. Chem Pap 70:1316–1321. https://doi.org/10.1515/chempap-2016-0074

Gao W, Kirschbaum C, Grass J, Stalder T (2016) LC–MS based analysis of endogenous steroid hormones in human hair. J Steroid Biochem Mol Biol 162:92–99. https://doi.org/10.1016/j.jsbmb.2015.12.022

Kirschbaum C, Tietze A, Skoluda N, Dettenborn L (2009) Hair as a retrospective calendar of cortisol production-increased cortisol incorporation into hair in the third trimester of pregnancy. Psychoneuroendocrinology 34:32–37. https://doi.org/10.1016/j.psyneuen.2008.08.024

Miller R, Plessow F, Rauh M, Groschl M, Kirschbaum C (2013) Comparison of salivary cortisol as measured by different immunoassays and tandem mass spectrometry. Psychoneuroendocrinology 38:50–57. https://doi.org/10.1016/j.psyneuen.2012.04.019

Quinete N, Bertram J, Reska M, Lang J, Kraus T (2015) Highly selective and automated online SPE LC–MS3 method for determination of cortisol and cortisone in human hair as biomarker for stress related diseases. Talanta 134:310–316. https://doi.org/10.1016/j.talanta.2014.11.034

Raul JS, Cirimele V, Ludes B, Kintz P (2004) Detection of physiological concentrations of cortisol and cortisone in human hair.

Clin Biochem 37:1105–1111. https://doi.org/10.1016/j.clinbiochem.2004.02.010

Russell E, Koren G, Rieder M, Van Uum S (2012) Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. Psychoneuroendocrinology 37:589–601. https://doi.org/10.1016/j.psyneuen.2011.09.009

Şener H, Anilanmert B, Cengiz S (2017) A fast method for monitoring of organic explosives in soil: a gas temperature gradient approach in LC–APCI/MS/MS. Chem Pap 71:971–979. https://doi.org/10.1007/s11696-016-0042-2

Taieb J, Benattar C, Birr AS, Lindenbaum A (2002) Limitations of steroid determination by direct immunoassay. Clin Chem 48:583–585

Tsakelidou E, Virgiliou C, Valianou L, Gika HG, Raikos N, Theodoridis G (2017) Sample preparation strategies for the effective quantitation of hydrophilic metabolites in serum by multi-targeted HILIC–MS/MS. Metabolites. https://doi.org/10.3390/metabo7020013

Vanaelst B, Rivet N, Huybrechts I, Ludes B, De Henauw S, Raul JS (2013) Measurement of cortisol and cortisone in children's hair using ultra performance liquid chromatography and tandem mass spectrometry. Anal Methods 5:2074–2082. https://doi.org/10.1039/c3ay26570f

Wennig R (2000) Potential problems with the interpretation of hair analysis results. Forensic Sci Int 107:5–12. https://doi.org/10.1016/S0379-0738(99)00146-2

Wester VL, van Rossum EFC (2015) Clinical applications of cortisol measurements in hair. Eur J Endocrinol 173:M1–M10. https://doi.org/10.1530/Eje-15-0313

Wester VL, Reincke M, Koper JW, van den Akker ELT, Manenschijn L, Berr CM, Fazel J, de Rijke YB, Feelders RA, van Rossum EFC (2017) Scalp hair cortisol for diagnosis of Cushing's syndrome. Eur J Endocrinol 176:695–703. https://doi.org/10.1530/Eje-16-0873