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Cite this: *Analyst*, 2016, **141**, 2053

Quantification of cortisol in human eccrine sweat by liquid chromatography – tandem mass spectrometry†

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Received 18th November 2015,
Accepted 2nd February 2016

DOI: 10.1039/c5an02387d

www.rsc.org/analyst

Cortisol has long been recognized as the "stress biomarker" in evaluating stress related disorders. Plasma, urine or saliva are the current source for cortisol analysis. The sampling of these biofluids is either invasive or has reliability problems that could lead to inaccurate results. Sweat has drawn increasing attention as a promising source for non-invasive stress analysis. A sensitive HPLC-MS/MS method was developed for the quantitation of cortisol ((11β)-11,17,21-trihydroxypregn-4-ene-3,20-dione) in human eccrine sweat. At least one unknown isomer that has previously not been reported and could potentially interfere with quantification was separated from cortisol with mixed mode RP HPLC. Detection of cortisol was carried out using atmospheric pressure chemical ionization (APCI) and selected reaction monitoring (SRM) in positive ion mode, using cortisol-9,11,12,12-D4 as internal standard. LOD and LOQ were estimated to be 0.04 ng ml^{-1} and 0.1 ng ml^{-1}, respectively. Linear range of 0.10–25.00 ng ml^{-1} was obtained. Intraday precision (2.5%–9.7%) and accuracy (0.5%–2.1%), interday precision (12.3%–18.7%) and accuracy (7.1%–15.1%) were achieved. This method has been successfully applied to the cortisol analysis of human eccrine sweat samples. This is the first demonstration that HPLC-MS/MS can be used for the sensitive and highly specific determination of cortisol in human eccrine sweat in the presence of at least one isomer that has similar hydrophobicity as cortisol. This study demonstrated that human eccrine sweat could be used as a promising source for non-invasive assessment of stress biomarkers such as cortisol and other steroid hormones.

Introduction

Cortisol is a small steroid hormone (MW: 362.46) that plays many important roles in physiological and psychological processes. It is secreted from the adrenal glands, as a final product in the hypothalamic-pituitary-adrenal (HPA) axis activation cascade in response to stress or fear in the surrounding environment. Cortisol, the adrenal cortex's principle stress hormone, is crucial for homeostatic maintenance, by means of modulating, regulating or influencing vital systems including neural, immune, cardiovascular, metabolic, and endocrine systems. Abnormal circulating cortisol levels lead to illness. Prolonged elevation in cortisol levels can cause impaired cognitive performance, hyperglycemia, sleep disruption, elevated blood pressure, suppressed immune function, obesity, fatigue; and contribute to the development of Cushing's disease. Chronically lower levels (adrenal fatigue) have been associated with fatigue, low blood pressure and inflammation.[1,2]

A variety of analytical techniques have been employed for cortisol analysis in biofluids, including radio immunoassay (RIA),[3,4] enzyme-linked immunosorbent assay (ELISA),[5,6] chemiluminescence immunoassay (CLIA),[7] capillary electrophoresis-based immunoassay (CE-IA),[8,9] miniaturized immunosensors,[10,11] gas chromatography – mass spectrometry (GC-MS)[12,13] and high performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS).[14,15] Although widely used in biomedical and clinical studies, immunoassays of cortisol often lack specificity due to antibody cross-reaction with other unidentified endogenous steroids, administered drugs as well as their metabolites.[13,16] This leads to false positive results that could invalidate clinical test results. The high selectivity of SRM, combined with the separation power of HPLC, enables more reliable and specific quantification of

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cortisol. As compared to GC-MS, there is no need for cortisol derivatization and related complex sample preparation procedures, which is time consuming and labor intensive.[17]

Cortisol has been routinely measured in human blood, urine and saliva in clinical labs.[18,19] It has also been detected in interstitial fluid (ISF)[20] and hair.[21,22] Among these, blood and ISF sampling is invasive, and can thus trigger stress, thereby introducing artifacts and erroneous results. Although collection of urine and saliva is non-invasive, these have potential reliability problems as a source biofluid for cortisol detection, due to the cumbersome nature of collection and the potential for subject non-adherence.[23] Sweat, as a non-invasive source, can be collected continuously without human intervention *via* wearable devices, for subjects in physical exercise or heat conditions.

Sweat can be produced from three types of sweat glands: eccrine, apocrine and apoeccrine glands. Eccrine glands are distributed over almost the whole body surface, and open freely onto epidermal surface. Eccrine sweat contains over 99% of water, with less than 1% electrolytes, metabolites, proteins, peptides and hormones. Apocrine and apoeccrine sweat glands are restricted to hairy body areas such as axilla. Apocrine glands are open to and secrete into hair canal. Apocrine sweat is an oily fluid, containing proteins, lipids, steroids and sebum. Apoeccrine glands secrete an eccrine-like watery fluid. Currently sweat diagnostics mainly utilize eccrine sweat.[24] Although sweat has been a well-known source for diagnosing cystic fibrosis and drugs of abuse, there is little knowledge about cortisol in sweat. Our group has reported the quantification of neural and immune biomarkers in sweat using LC – LIF detection[25] and antibody microarray[26] platform. In these studies, we demonstrated the viability of using human eccrine sweat as a convenient and promising biomarker source for monitoring psychological and physiological disorders in ambulatory settings. Recently a study was published on cortisol quantitation in human sweat using ELISA to demonstrate the potential implications of sweat cortisol for hair cortisol accumulation.[27] As with all immunoassays, this method suffers potential interferences from other steroids, drugs and their metabolites in sweat.

A simple, sensitive, and specific analytical method for accurate cortisol quantification in human eccrine sweat is therefore needed to non-invasively measure this important stress hormone in settings where collection of blood is unfeasible or undesirable. Although the composition of eccrine sweat could be affected by hydration level, region of the body or exercise, in this work we focused on the establishment of a reliable analytical method for cortisol analysis, while leaving the clinical/biological correlation to follow-up studies.

In this paper we report the development and validation of a sensitive and specific HPLC-MS/MS method for detection of cortisol in human eccrine sweat. Baseline separation of cortisol from at least one unknown interfering isomer(s) was achieved for accurate evaluation of cortisol in sweat. Two sets of thermally induced sweat samples collected in a hot chamber at approximately 1 and 4 pm on the same day were analyzed. Sweat cortisol levels were found to be different at different times of day, corresponding to the diurnal effects in plasma cortisol levels. This work demonstrated the feasibility of employing HPLC-MS/MS for the accurate quantification of cortisol in human eccrine sweat.

Materials and methods

Materials

Cortisol ((11β)-11,17,21-trihydroxypregn-4-ene-3,20-dione) > 99% was purchased from ABIOX Company (Newberg, OR). Dihydrocortisone (5β-Pregnan-17,21-diol-3,11,20-trione) > 98% was purchased from Steraloids, Inc (Newport, RI). HPLC grade acetonitrile was bought from Fisher Scientific (Fair Lawn, NJ); HPLC grade water was bought from J. T. Baker, Inc. (Phillipsburg, NJ); formic acid (98%) was purchased from EMD Chemicals Inc. (Gibbstown, NJ). Artificial eccrine perspiration was bought from Pickering Laboratories, Inc. (Mountain View, CA); cortisol-9,11,12,12-D4 (97% atom %D) was obtained from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada).

Standard solution preparation

Stock cortisol solution was prepared to a concentration of 2 mg ml^{-1} in 50% acetonitrile and stored at −20 °C before use. This stock solution was serially diluted in 10% acetonitrile to make working solutions of 125.00, 62.50, 31.25, 6.25, 0.50 and 0.25 ng ml^{-1}. Sweat calibration standards were prepared fresh daily by spiking cortisol working standards to artificial eccrine perspiration to concentrations of 25.00, 12.50, 6.25, 1.25, 0.25, 0.10 and 0.05 ng ml^{-1}. Quality control standards were prepared by spiking cortisol working solutions into artificial eccrine sweat to concentrations of 0.63 and 20.0 ng ml^{-1}. Quality control standards were aliquoted and stored at −20 °C throughout the validation period.

Human eccrine sweat collection and preparation

All eccrine sweat samples were handled in accordance with approved institutional review board (IRB) protocols at University of Arizona School of Medicine. Sweat collection was done in a heat environment at 41 °C and ~55% humidity. To minimize epidermal and skin surface contamination, skin of the left leg of a volunteer was cleansed with alcohol pads followed by distilled water, and dried before the thermal sweat induction. Sweat was collected off the skin directly into 1.5 ml Eppendorf LoBind microcentrifuge tubes. Two sampling sessions were carried out at 1 pm and 4 pm of the same day, controlling physical activity, food intake and body hydration levels. Collected sweat samples were transferred to dry ice immediately, and subsequently to −80 °C freezer until use.

Sample extraction procedure

For standards and controls: 50 μl of the calibration standards or controls, 10 μl of the IS working solution (250 ng ml^{-1} in 10% acetonitrile), 200 μl artificial sweat and 200 μl 0.1 M ammonium acetate pH 5.5 were mixed in a 2 ml microcentri-

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fuge tube. The mixture was vortexed and 1 ml of ethyl acetate was added, followed by vortex and centrifuge at 12 600 rcf at 4 °C for 10 minutes. The upper organic layer was transferred to glass test tubes. The ethyl acetate extraction was repeated twice and organic layer was combined in test tube. The organic solvent was then evaporated to dryness in a centrifugal evaporator (SpeedVac, Thermo Fisher, Milford, MA). The dry residues were reconstituted with 100 μl 10% acetonitrile and transferred to autosampler vials, where 50 μl were injected into HPLC-MS system for analysis.

For human sweat samples: 200 μl sweat samples were mixed with 50 μl 10% acetonitrile, 200 μl 0.1 M ammonium acetate pH 5.5 and 10 μl internal standard working solution in a 2 ml microcentrifuge tube, followed by the same extraction procedure as the standard solutions above.

Liquid chromatography – mass spectrometry

The HPLC-MS/MS system consisted of a Finnigan Surveyor LC system and a TSQ Quantum triple quadrupole mass spectrometer with Finnigan Surveyor MS Pump Plus and Finnigan Surveyor AS Plus autosampler (Thermo Electron, San Jose, CA). Initially a Zorbax 300 SB-C18 column (0.5 ID × 150 mm L, 5 μm, Agilent Technologies, Germany) was used for separation, with 0.1% formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B) as mobile phases. Eventually chromatographic separation was achieved on a SIELC Primesep 100A column (2.10 ID × 250 mm L, 5 μm, SIELC Technologies, Prospect Heights, IL) with a Primesep 100A guard column (2.10 ID × 10 mm L), and binary gradient elution. Mobile phase A consisted 0.1% formic acid in water, and mobile phase B consisted 0.1% formic acid in acetonitrile. Flow rate was 250 μl min^{-1} with the following gradient: 20%B (0–2.0 min), from 20 to 40%B (2.0–12 min), 40%B (12–16 min), from 40 to 20%B (16–16.1 min), 20%B (16.1–25 min). Sample vials were maintained at 5 °C in the autosampler tray.

Ionization of the mass spectrometry analysis was atmospheric pressure chemical ionization (APCI) operated in positive ion mode, with a discharge voltage of 3300 V and a vaporizer temperature of 421 °C. The capillary offset was 35 V, and capillary temperature was 229 °C. Sheath gas (nitrogen) flow was 32 arbitrary units (AU), and the flow rate of auxiliary gas was 9 AU. Both Q1 and Q3 were operated in unit mass resolution with a scan time of 0.1 seconds. Argon was used as the Q2 collision gas and maintained at a constant pressure of 0.80 mTorr. Quantification of cortisol and IS was in selected reaction monitoring (SRM) mode monitoring the most abundant ion transitions: cortisol 363→121 and cortisol-d4 367→121. Transition 363→327 was also monitored for cortisol identification confirmation during early stages of method development and human sweat sample measurement. The collision energy used was 37 eV. The mass spectrometry conditions were optimized for the best detection sensitivity. HPLC-TSQ Quantum system control, data acquisition and processing were done with Xcalibur (version 2.0) software.

Stability of cortisol in human eccrine sweat

Aliquots of the same sweat samples were left at room temperature for a duration of 3 hours, with each aliquot analyzed every 30 minutes in first hour and every hour afterwards.

Method validation

The calibration standards were prepared on each analysis day from a single batch of cortisol working solutions. The linearity of the detection was evaluated with single measurement of spiked artificial sweat sample, zero (artificial sweat spiked with IS) and each of the 6 standards with concentration from 0.05 to 25.00 ng ml^{-1}.

The within-day and between-day variations were also evaluated *via* spiking 0.63 and 20.00 ng ml^{-1} of cortisol into aliquots of artificial sweat samples. 5 measurements for each concentration were performed within a validation batch for within-day variation assessment. This analysis was repeated over a 3-day period for between-day variation assessment. The concentrations of the QC standards were determined from the calibration curve prepared for each day. The RSD of the concentration determined within a run (5 replicates) and among 3 runs were used to show the within-day and between-day precision of the method, respectively.

The percent difference between measured and theoretical concentrations within a run and among 3 runs were used to determine within-day and between-day accuracy of the method, respectively.

The extraction recovery of cortisol was determined by comparing the peak areas for the quality control standards spiked before extraction to peak areas for standards spiked after extraction. Matrix effect was evaluated by comparing the peak areas for the quality control standards spiked after extraction to peak areas for standards in 10% acetonitrile. Matrix spike recovery of cortisol in human eccrine sweat was also examined by calculating the ratios of the peak areas for sweat samples to peak areas for spiked sweat samples using the below equation:

$$\%R = 100 \times \frac{M_{sp} - M_o}{M_t} \quad (1)$$

where $\%R$ represents percent recovery, M_{sp} represents the total amount of cortisol detected in spiked sweat sample aliquot; M_o represents the amount of cortisol in original sweat sample aliquot, and M_t represents the theoretical amount spiked.

Results and discussion

Sweat pretreatment

Sweat is a relatively new source biofluid for biomarker analysis using HPLC-MS/MS. Highly selective and efficient sample preparation techniques that are suitable for automation, such as solid phase extraction (SPE), have been reported for cortisol analysis.[28,29] In our work, since sweat is a new source of biomarkers, we intended to use a less selective method that still features high recovery efficiency, in an effort to explore other steroids hormones in future studies. Liquid–liquid extraction

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(LLE), which is traditionally employed for general unknowns, or categories of analytes, was therefore adopted for the early stage exploratory sweat research. In this study, a liquid–liquid extraction procedure using ethyl acetate was developed to reduce potential matrix effects. The extraction recovery averaged at 107% was obtained (Table S1†).

Stability test of cortisol in human eccrine sweat

Sweat collection normally takes time to complete, especially at low sweat rates. A key question is whether cortisol is stable in sweat, and if not, how to develop sweat collection and preprocessing methods to minimize cortisol loss in sweat. Spiked human eccrine sweat samples were tested over a period of 3 hours at room temperature. Data are shown in Table S2.†

Method validation

Sweat cortisol calibration curves were constructed using the ratios of the peak area of cortisol to cortisol-d4 *versus* cortisol concentrations in artificial sweat samples. Linear least-squared regression with a weighting factor of $1/y$ was used for curve fitting. Linear range was found to be from 0.1 ng ml^{-1} to 25 ng ml^{-1}. The lower limit of quantitation (LLOQ) is defined as the concentration corresponding to 10 times of the blank signal, with a RSD of less than 20% and an accuracy of 80–120% of nominal concentration. The lower limit of detection (LLOD) is defined as the lowest concentration that showed a peak response 3 times compared to bank signal. Based on these definitions, the LLOD and LLOQ were estimated to be ~0.04 ng ml^{-1} and ~0.1 ng ml^{-1}.

Table 2 below lists the method accuracy, precision and extraction recovery. Within-day analysis evaluated accuracy and precision during a single analytical run of 5 replicates for each QC sample. The concentrations of these QC samples were determined by using a calibration curve prepared for each batch. The between-day accuracy and precision were determined from 3 analytical runs of 5 replicates for each QC sample over three days. The concentrations of these QC samples were determined by a calibration prepared each day. The within-day %RSD and % difference were from 2.5 to 9.7 and from 0.5 to 2.1, respectively. The between-day %RSD and % difference were from 12.3 to 18.7 and from 7.1 to 15.1, respectively. Extraction recoveries for cortisol and for the IS were between 90% and 110%.

Application to human eccrine sweat cortisol analysis

The optimized method was successfully applied to the sensitive analysis of cortisol in human eccrine sweat. Sweat samples were collected from a volunteer in a hot chamber during two sessions in the same day: 1 pm to 1:30 pm and 4 pm to 4:25 pm. Plasma cortisol levels are known to undergo diurnal effect with highest concentrations at wakeup and lowest at late night. Data in Table 3 showing lower sweat cortisol levels at 4 pm than those at 1 pm. This trend is in accordance with plasma cortisol circadian cycle. Multiple factors other than stress can affect sweat cortisol concentrations: sweat rate, body location of sweat collection, food intake, physical exercise and

Table 1 Ratios of two common cortisol MRM transitions in eccrine sweat samples

Sample ID	T1: 363.18→120.83	T1: 363.18→327.10	Ratio T1/T2	Average ratio
A1	29 222	61 338	0.48	
A2	27 342	59 038	0.46	0.47
Standard_01	41 921	39 005	1.07	
Standard_02	49 420	47 804	1.03	1.05
A3	23 879	43 809	0.55	
A4	34 152	72 476	0.47	
A5	29 563	58 464	0.51	0.51
B1	20 704	15 311	1.35	
B2	19 865	14 992	1.33	1.34
C1	26 080	21 841	1.19	
C2	29 333	21 230	1.38	1.29
D1	22 935	33 370	0.69	
D2	20 271	32 806	0.62	0.65

Table 2 Accuracy and precision of the method

	Theoretical cortisol concentrations (ng ml^{-1})	
	20.000	0.625
Within day		
Mean measured concentration (n = 5)	20.422	0.628
Standard deviation	0.504	0.061
Precision (%RSD)	2.5%	9.7%
Accuracy (%difference)	2.1%	0.5%
Between day		
Mean measured concentration (n = 15)	21.420	0.531
Standard deviation	2.631	0.099
Precision (%RSD)	12.3%	18.7%
Accuracy (%difference)	7.1%	−15.1%
Extraction recovery (%, n = 5)	97	103
Matrix spike recovery (%, n = 3)	92	

Precision is expressed as %RSD: (standard deviation)/(mean measured concentration) × 100. Accuracy is expressed as % difference: [(measured − theoretical conc)/theoretical conc] × 100.

Table 3 Cortisol levels in human eccrine sweat collected at different times of the day

Collection time	1–1:30 pm	4–4:30 pm
Concentration (ng ml^{-1})	1.832	0.686
	1.829	0.736
	1.746	0.342
	1.626	0.240
	1.953	0.340
	1.068	0.507
	1.215	0.670
	2.802	
	1.470	
	1.307	
Average concentration (ng ml^{-1})	1.68 ± 0.49	0.50 ± 0.20

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hydration levels, therefore caution was used to minimize the possible cortisol variations caused by these factors.

Separation and quantification of cortisol in human sweat

Although HPLC-MS/MS is well known for its selectivity/specificity for small molecule such as steroid analysis, it is also documented that interfering species (isomer metabolites and drugs) are likely present in human biofluids, due to the similarity in structure and overlapping fragmentation patterns of these molecules. Therefore, during the early stages of method development and sweat cortisol analysis, two of the well-known cortisol SRM transitions, 363→121 and 363→327 were both monitored. With the Zorbax column, a single peak at the same retention time as standard cortisol was observed, without separation or peak split when a few different gradient elution programs were tried. As a measure of caution, we calculated and compared the ratio of the peak areas of the 2 transitions (363→121 and 363→327) as a "confirmatory parameter" for sweat samples collected from different subjects. This ratio varies from ~0.5 to ~1.5 (Table 1) for cortisol in sweat samples collected from different volunteers, indicating that there could be isomer(s) co-eluted with cortisol under the chromatographic separation conditions, and that this isomer(s) contributes to at least one of the transitions we were monitoring.

A SIELC Primesep A 100A mixed mode column was therefore employed to replace the Zorbax in order to achieve separation of cortisol from its isomer(s) in human eccrine sweat samples. This column is a RP column with embedded cation-exchange functional groups. Fig. 1 shows a typical extracted ion chromatograph of the sweat cortisol and its isomers (363→121 transition). As can be seen, two isomers were separated from cortisol. The retention times of the three compounds were 11.4 min and 11.9 min for the other cortisol isomers; and 12.4 min for cortisol (11β)-11,17,21-trihydroxy-pregn-4-ene-3,20-dione. This distinction of the retention times of the two compounds confirmed the presence of isomers of cortisol in human eccrine sweat. No peaks were observed in the above retention times when extracted artificial sweat samples were analyzed. To further illustrate the difference of cortisol and its isomers, we show in Fig. 2 the APCI CID mass spectra of cortisol and one of its isomers under optimal separation conditions. Clear distinction in the fragmentation patterns were observed, which further confirmed the presence of at least one isomer in human eccrine sweat.

Although theoretically the matrix effect should be largely minimized with the use of stable isotope labeled internal standard, possible ion suppression was also tested using post column infusion method. In this experiment, due to the lack of genuine "blank human eccrine sweat", we used simulated sweat in place of blank sweat samples. No ion suppression was observed at around the retention times of cortisol as well as its isomers. The matrix spike recovery of sweat cortisol analysis may also be used as one quick and easy way to detect the presence or lack of matrix effects.[30,31] As shown in Table 2,

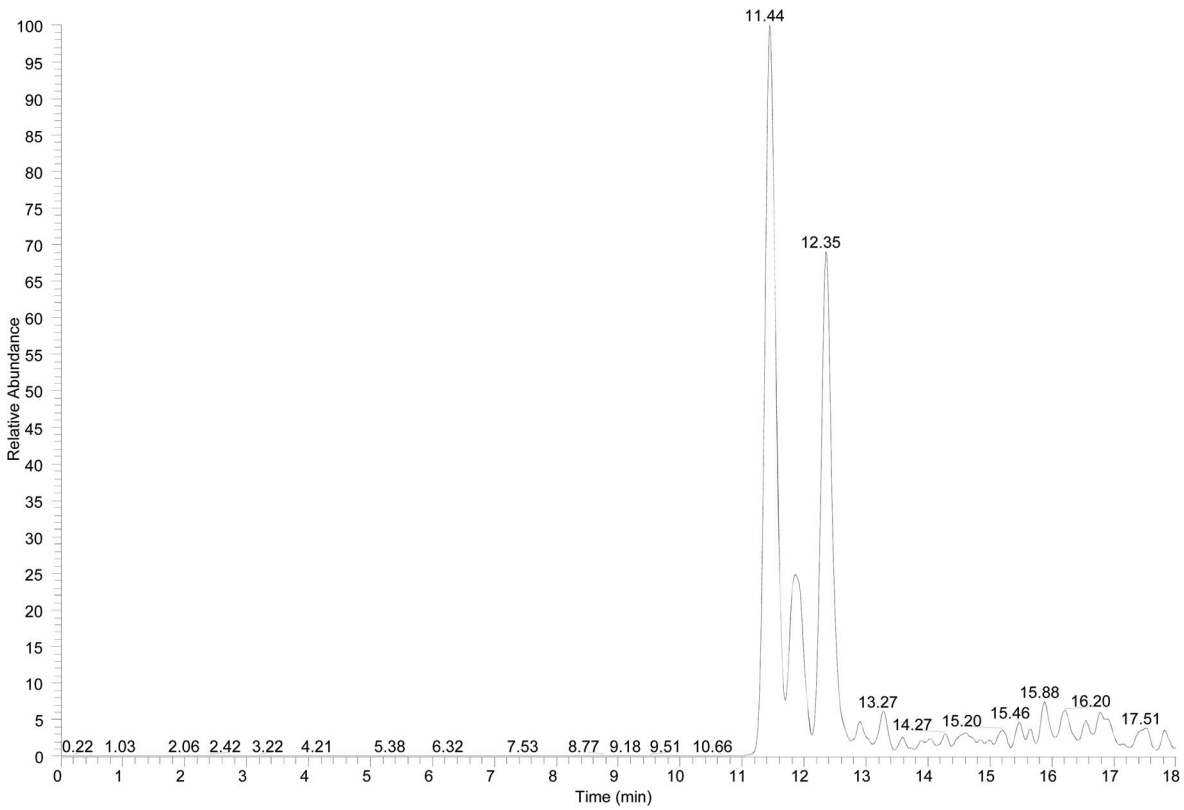

Fig. 1 Typical extracted ion chromatogram of cortisol and its isomers in human eccrine sweat (363→121 transition).

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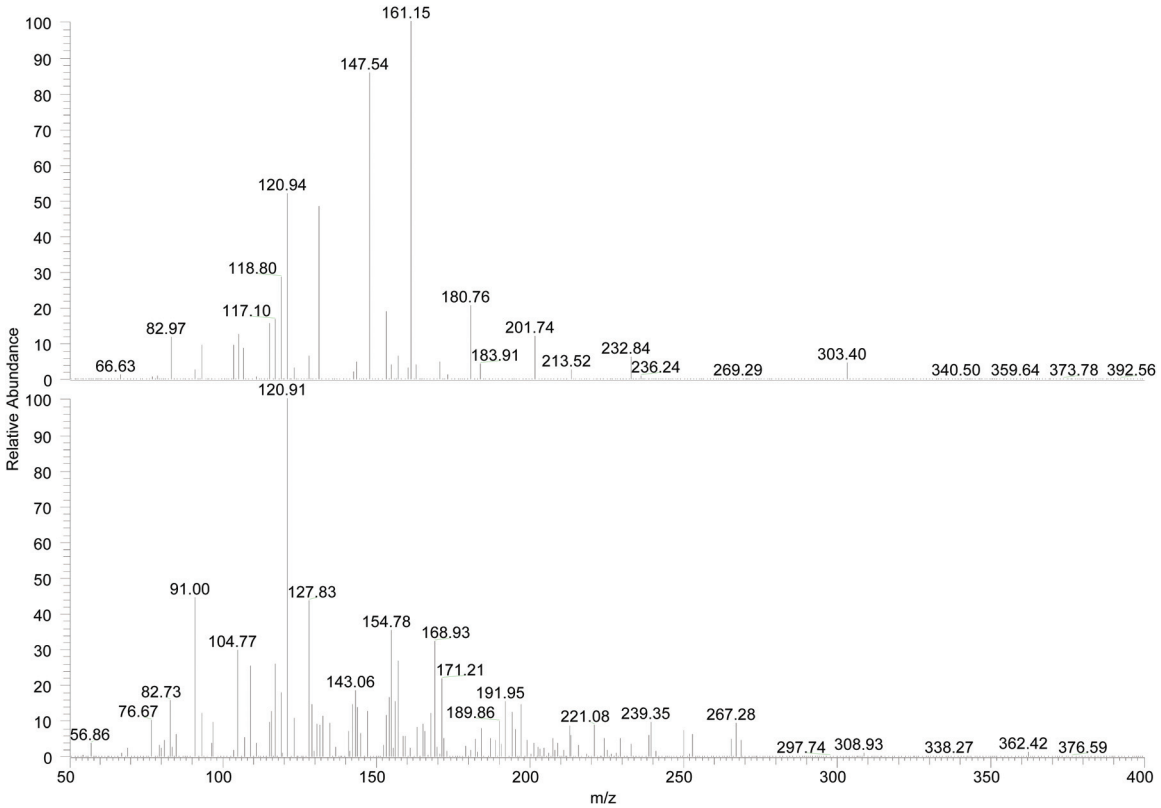

Fig. 2 APCI CID mass spectra for cortisol (retention time 12.4 min, bottom panel) and one of its isomer (retention time 11.4 min, top panel).

close to 100% matrix spike recovery of cortisol in eccrine sweat was obtained using the liquid–liquid extraction method, which confirms the lack of matrix effects in the developed method.

The cortisol concentrations we obtained using the developed HPLC-MS/MS method were much lower than the results from a previous ELISA analysis of human eccrine sweat.[27] In this ELISA study, an average of 74.62 ± 41.51 ng mL^{-1} sweat cortisol was obtained, with concentration ranged from 8.16–141.7 ng mL^{-1}. The time course of cortisol levels agrees with our findings using HPLC-MS/MS: lower in evening and higher in morning, during collection period 7:40 am to 22:00 pm. These results are much higher as compared to our results of 2.80–0.24 ng ml^{-1} using HPLC-SRM from 1 pm to 4 pm. There are several possible reasons for this discrepancy. First, EIA of cortisol and other steroids are known to suffer significant interferences with polar metabolites with known or unknown structures (such as cortisol ring-A metabolites), which leads to false positives.[16] We believe such interferences/cross-reactions likely account for the high cortisol concentrations in study,[27] especially considering that sweat is a novel biofluid for cortisol analysis and its composition of steroid metabolites are largely unknown. Second, in ref. 27, the sweat samples were collected soon after the subjects underwent intense exercise. It was reported that medium to high intensity exercise provokes increasing circulating cortisol levels.[32] The percent change of post exercise cortisol increase in plasma was reported to be less than 90%, therefore, the high cortisol concentrations observed in the previous study may not be only attributed to the effects of physical activity. Rather, it is more likely that other matrix components, including other steroids, metabolites, drugs or as we shown in this study, structurally similar isomers may be responsible for the high sweat cortisol concentration levels obtained using ELISA.

Conclusions

We developed and validated a sensitive, selective HPLC-MS/MS method for quantitation of cortisol ((11β)-11,17,21-trihydroxypregn-4-ene-3,20-dione) in human sweat samples. A LLOD of 0.04 ng ml^{-1} and LLOQ of 0.1 ng ml^{-1} were achieved. The linear range of this method is 0.1–25 ng ml^{-1}. We found that at least one isomer existed in human eccrine sweat, which has very similar retention time and fragmentation patterns to cortisol ((11β)-11,17,21-trihydroxypregn-4-ene-3,20-dione). We demonstrated for the first time that caution must be taken when analyzing cortisol in human eccrine sweat, and possibly other steroids using HPLC-MS/MS, although HPLC-MS/MS is well known for its specificity in small molecule analysis as compared to immunoassays. Therefore, careful scrutiny of potential co-eluting isomer(s) may be critical for the accurate determination of cortisol and in certain human biofluids.

It was previously reported that other endogenous or exogenous steroid/drug metabolites such as fenofibrate could

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co-elute with cortisol when a regular C-18 RP column was used, and interfere with cortisol analysis.[33] However, we have not seen publications detailing the potential interferences by isomers during HPLC-MS/MS quantitation of cortisol. Considering the similar polarity of the observed isomers to that of cortisol, we found that these isomers could easily co-elute with cortisol with a regular C-18 RP column. With the same molecular mass as cortisol, these isomers also produce transition(s) overlapping the major SMR transitions of cortisol (363→121 or 363→327) that is frequently used in cortisol quantitation. Due to these characteristics of these isomers, and the fact that the C-18 RP column has been the most commonly used stationary phase for cortisol analysis in the literature, we believe these isomers could represent potential risks and pitfalls of interferences in cortisol quantitation by HPLC-MS/MS. We propose an initial check using the ratio of two transitions to examine the possible presence of cortisol isomers, as a standard analytical procedure during the early stage of method development for cortisol analysis in human biofluids.

The structure elucidation of the isomer(s) in human eccrine sweat will be researched in our future work. Future work will also involve more extensive application of this method for dynamic assessment of cortisol in human sweat and other human biofluids, such as saliva and urine, under different stress and other psychological conditions. We believe with this sensitive and highly selective HPLC-SRM methods, the potential of use of human eccrine sweat as a source for non-invasive stress analysis with cortisol will hold great promise.

Acknowledgements

This material is based on research developed through a contract awarded to the authors by the NBMC (Nano-Bio Manufacturing Consortium), a consortium sponsored by the Air Force Research Laboratory under agreement number FA8650-13-2-7311. We would like to thank NBMC and its members for all their advice and support. The U.S. Government is authorized to reproduce and distribute reprints for Government purposes notwithstanding any copyright notation thereon. The views and conclusions contained herein are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of Air Force Research Laboratory or the U.S. Government. The preliminary mass spectrometry data were acquired at the Arizona Proteomics Consortium supported by NIEHS grant ES06694 to the SWEHSC, NIH/NCI grant CA023074 to the UA Cancer Center and by the BIO5 Institute of the University of Arizona. The ABI/SCIEX 4000 QTRAP mass spectrometer was provided by grant 1S10RR022384-01 from NIH/NCRR. We would like to thank Drs George Tsaprailis and Linda Breci for the helpful discussions regarding the preliminary mass spectrometry results; Ms Andria Pizzato for helping with sweat sample collection; and Ms Paula Cook for administrative assistance. We wish to thank Dr. Charles Raison and Mr. Clemens Janssen for providing access to their hyperthermia facility in the Department of Psychiatry, University of Arizona College of Medicine.

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