

Measurement of Hair Cortisol in Healthy Men by Ultra-performance Liquid Chromatography-tandem Mass Spectrometry

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ABSTRACT

Objectives: The goal of this study was to use a validated assay to measure hair cortisol levels in healthy persons. **Materials and Methods:** For quantitative detection of hair-cortisol, a high-performance liquid chromatography-tandem mass spectrometry approach was established and validated. Hair samples were incubated in methanol for 16 hr at 52°C, after which the clear supernatant was sonicated and transfer to a clean tube, where it was dried. The residual sample dissolved in 100 µl methanol containing cortisone (internal standard) and injected. The average extraction recovery was calculated (90.8%). A mobile phase of two mM ammonium acetate (pH 4.2) and acetonitrile (50:50, v:v) was supplied at a flow rate of 0.3 ml/min on an Atlantis dC₁₈ column (2.1 x 100 mm, 3 µm particle size). Multiple reaction monitoring was used in electrospray positive ion mode for mass spectrometry acquisition (m/z: 363.1 → 121.0 and 361.8 → 163.11 for cortisol and cortisone, respectively). **Results:** Cortisol and internal standard exhibited retention times of about 1.34 and 1.39 min, respectively. In the range of 2-100 ng/ml, the relationship between cortisol level and peak area ratio of cortisol to cortisone was linear, with a detection limit of 0.3 ng/ml. The

inter-day coefficient of variation and bias were 7.8% and 11.9%, respectively. 95% of the 88 men had cortisol detectable in their hair samples [mean (SD) length =1.3 (0.5) cm, weight = 33.9 (9.9) mg] and measurable in 49%, with a mean (median) 12.4 (8.7) pg/mg of hair. The method utilized for determining the stability and assessing the level of hair cortisol in samples taken from healthy adult men. **Conclusion:** For hair cortisol measurements in clinical labs, the described test is reliable, exact, and relevant.

Keywords: Cortisol, Cortisone, Hair, Healthy Men, UPLC-MS/MS.

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INTRODUCTION

Cortisol is a major glucocorticoid hormone that regulates a wide range of body systems. In routine practice, serum, plasma, or urine samples were used for cortisol measurements that indicate the current cortisol exposure that may likely influence by acute situational or individual stresses.¹⁻² Cortisol in plasma/serum has a short half-life and diurnal pattern with a 20%-50% increment, 30-45 min post-awakening, and declines.³⁻⁴ Whereas, measurement of cortisol in hair provides reliable information as it reflects cortisol level over the preceding period (3-4 months) that may not be influenced by psychological and physical stressors.⁵⁻⁷

It is reported that hair-cortisol is reliably measure using hair-sample length 3-4 cm from the scalp and then steady decline may occur due to leaching effects.⁸⁻⁹ Several assays were reported for hair-cortisol measurements that are based on either immunoassay¹⁰⁻¹² or liquid chromatography-tandem mass spectrometry assays (LC-MS/MS).¹³⁻¹⁴ In general, results obtained by immunoassays often reported a 20-30% higher levels of cortisol compared to LCMS/MS, because of cross-reactivity with related products.¹⁵

After validation, we have used ultra-high-resolution liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay in this investigation. Hair-cortisol levels were measured using this approach in samples collected from healthy subjects.

MATERIALS AND METHODS

Ethical Approval and Sample Collection

The study was carried out with the permission of the King Faisal Specialist Hospital and Research Centre (KFSHRC), Riyadh's

Research Ethics Committee under RAC# 2181261. Healthy volunteers (as screened by a clinical research coordinator using a standard questionnaire) provided informed consent and were compensated by having a free haircut. An assigned barber under the observation of the research coordinator hair collected. The length of the hair wasn't over three centimeters collected from the nape of the neck. Biohazard plastic bags were used for hair storage at room temperature until analyzed. All samples were analyzed within six months from date of collection.

Reagents and Chemicals

Acros organic, NJ, USA, provided cortisol and cortisone (analytical reagent grade). Fisher Scientific, NJ, USA, provided acetonitrile, methanol (HPLC grade), and ammonium acetate. Purified water was sent through the Milli-Q System to create Milli-Q water (Millipore, Bedford, MA, USA).

Instrument and Analytical Conditions

Waters Corporation, Milford, MA, USA, provided the Acquity UPLC H-Class system, which was combined with a solvent and sample management, paired with a Xevo-TQD triple quadrupole mass spectrometer, and equipped with an API interface. A reversed phase Atlantis dC₁₈ column (2.1 x 100 mm, 3 µm particle size) was used for the analysis, which was carried out at room temperature and protected by a column guard in-line filter (2 mm, 0.2 m). The mobile phase was made up of two mM ammonium acetate (pH 4.2, adjusted with 1% formic acid) and acetonitrile (50:50, v:v), filtered through a 47 mm (0.2 µm pore size) Supor membrane disc filter (Pall Gelman Laboratory, MI, USA),

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and given at 0.3 ml/min. At a capillary voltage of 1.5 kV and a cone voltage of 36 V, an electrospray ionization (ESI) source was operated in the positive-ion mode. At a flow rate of 1000 L/hr, nitrogen was used as the nebulizing and desolvation gas. The collision gas was argon, and the cell pressure was kept at 3.6 E-3 mbar. For both cortisol and IS, an optimal collision energy of 20 eV was used. The temperatures of the ion source and desolvation were kept at 150°C and 500°C, respectively. In the positive ion mode, cortisol and IS were identified and quantified; in the multi reaction monitoring (MRM) mode, the product ion response was evaluated at specified transition mass to charge (m/z) of 363.1 → 121.0 and 361.18 → 163.11, respectively. The instrument parameters, data acquisition, peak integration, peak smoothing, and signal-to-noise ratio measurements were all controlled using Mass lynx Ver 4.1, Intellistart software (Waters Corporation, Milford, MA, USA) in a Microsoft Windows XP professional environment.

Preparation of Control and Standard Samples

Methanol was used to make cortisol and IS stock solutions (1.0 µg/ml). Nine calibration standards with concentrations ranging from 2-100 ng/ml and four quality control concentrations (2, 6, 50, and 90 ng/ml) were prepared in methanol. In methanol, an IS working solution (40 ng/ml) was prepared. Aliquots (5 ml) of the samples were transferred to 7 mL Pyrex glass culture tubes and stored at -20°C until needed.

Cortisol Extraction from Hair and Sample Processing

Hair cortisol extraction was allotted by adopting procedure reported by Brittany *et al.*¹⁰ Hair sample collected from a healthy volunteer dig into small segments using sharp scissor. 10 aliquots were weighed in range (11.5 - 51.8 mg) using (Mettler Toledo AG204 Analytical Balance), and placed into a 7 ml Pyrex glass culture tube. 2 ml methanol added to each tube, then incubated for 16 hr at 52°C and sonicated for 30 min, followed by vortexed for 10 min. The clear supernatant layer was transferred to a clean borosilicate culture tube and dried at 40 degrees Celsius under a moderate nitrogen steam. The residue was reconstituted in 100 µl methanol containing IS (40 ng/ml) and 10 µl clear solution was injected into the LC-MS/MS apparatus.

Bias, Precision, and Stability

At four QC concentrations (2, 6, 50, and 90 ng/ml), intra-day and inter-day precision and bias were assessed. Over the course of three days, inter-day accuracy and bias ($n=20$) were determined. Stability tests were conducted on hair samples taken from three subjects. Cortisol levels were tested before, and after storing processed hair samples at room temperature for twenty-four hours, and after storing unprocessed hair samples at room temperature for seven months.

Validation of the Method

The method was verified in accordance with the bioanalytical method validation guidelines published by the US Food and Drug Administration (FDA).¹⁶

RESULTS

LCMS/MS Condition Optimization

The ESI-MS/MS spectrum and chemical structures of cortisol and cortisone are shown in Figure 1. In the mass spectrometer, a standard mixture of cortisol and cortisone (1.0 µg/ml in methanol) was used to determine the product and precursor ions. A mobile phase of two mM ammonium acetate and acetonitrile (50:50, v:v) at a flow rate of 0.3 ml/min was used to optimize liquid chromatographic (LC) conditions. Low column backpressure and a shorter run time (around 2.0 min) were made possible by the relatively large amount of acetonitrile.

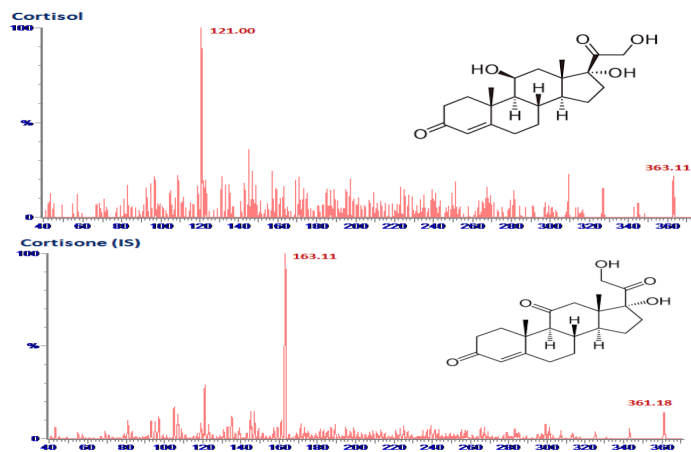


Figure 1: Mass spectrum and chemical structures of cortisol and cortisone (IS).

Extraction Recovery of Cortisol

Hair-sample (10 aliquots) were accustomed determine the extraction recovery. Samples were processed as described. However, to optimize and improve extraction efficiency, a further step of sonication half-hour followed by vortex 10 min was included in the procedure. Samples were re-processed after incubation for 32 and 48 hr of incubation at 52°C and analyzed on three different days. The mean (SD) measured level was 30.6 (2.4), 2.3 (0.4) and 0.8 (0.2) pg/ml, on day 1, 2 and 3 respectively. Data is summarized in (Table 1). Results indicate that ≥ 90% of cortisol is extracted from hair within the first extraction.

Specificity

Six separate hair samples were screened for six cortisol-related chemicals (progesterone, prednisone, 17-hydroxy progesterone, methyl prednisolone, prednisole, and testosterone) to determine the assay's specificity. 10 µl of 1.0 µg/ml methanol:water (1:1, v:v) solution was introduced into the system. Hair components/screened chemicals caused no influence with cortisol or IS peaks.

Linearity, Detection Limits, and Quantification

The assay's linearity was tested by assessing a series of cortisol standard solutions in methanol at nine different concentrations ranging from two to one hundred (ng/ml). Multivariate analysis was performed on the corresponding peak area ratio and concentration, $y = 0.0141(x) + (0.0012)$, with mean (SD) $R^2 = 0.9919$, was the average equation found from six calibration curves (0.0058). The detection and quantification limits were 0.3 ng/ml and 2.0 ng/ml, respectively.

Bias and Precision

Precision and bias within a day ranged from 2.6% to 8.5% and -11.3% to 7.0%, respectively. Precision and bias between days ranged from 6.7 percent to 7.8% and from -11.9% to 1.6%, respectively. The information presented in (Table 2).

Stability

Stability results are summarized in (Table 3). They indicate that cortisol is stable for at least 24 hr in processed hair samples (97%) and for at least seven months in unprocessed hair samples (99%).

Hair Cortisol level in Healthy Men

A typical MRM chromatogram of the hair sample with cortisol of 11.4 pg/mg is shown in Figure 2. Cortisol levels in hair samples collected from 88 men were determined using this method. Their mean (SD)

Table 1: Extraction of cortisol from hair.

Hair weight (mg)	Measured level of cortisol (pg/mg)		
	Day-1	Day-2	Day-3
27.1	33.49	2.51	0.96
21.8	34.79	2.32	0.88
21.2	31.90	2.12	0.78
24.2	29.62	2.29	0.53
43.2	27.71	1.59	0.68
21.3	28.11	2.11	0.65
29.8	28.32	2.69	0.47
32.4	32.05	2.93	0.97
32.6	29.54	2.62	0.79
29.4	30.25	1.98	0.91
Mean	30.6	2.3	0.8
SD	2.4	0.4	0.2
Recovery (%)	90.8	6.9	2.3

Recovery (%) = Mean measured cortisol level on each day ($n=10$) / Mean measured cortisol level in three days ($n=30$) \times 100. SD= Standard Deviation.

Table 2: Intra- and inter-day precision and accuracy of hair cortisol assay.

Nominal level (ng/ml)	Intra-day (n=10)			Inter-day (n=20)		
	Mean (SD) measured level	CV (%)	Bias (%)	Mean (SD) measured level	CV (%)	Bias (%)
2.0	1.8 (0.1)	6.4	-11.3	1.8 (0.1)	6.7	-11.9
6.0	6.1 (0.5)	8.5	2.2	6.1 (0.5)	7.8	1.6
50	52.7 (1.4)	2.6	5.4	49.4 (3.9)	7.8	-1.2
90	96.3 (3.2)	3.3	7.0	90.6 (6.7)	7.4	0.6

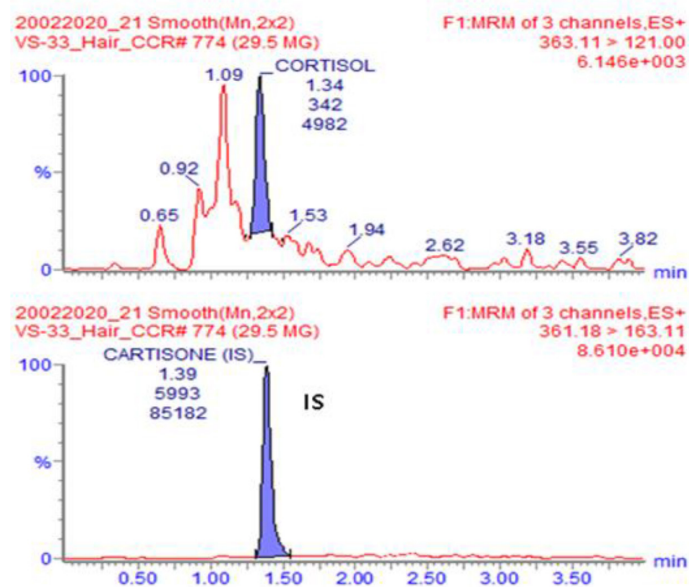
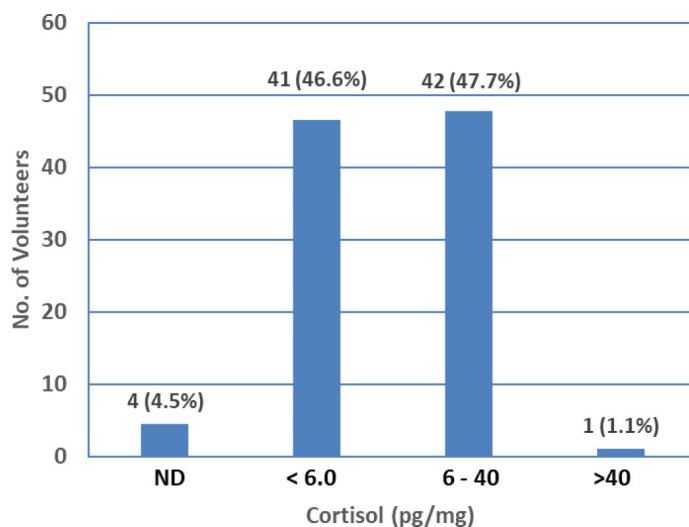
SD, standard deviation. CV, coefficient of variation = standard deviation divided by mean measured concentration \times 100. Bias = measured level – nominal level divided by nominal level \times 100.

Table 3: Stability of cortisol in hair samples.

Sample	Cortisol level (pg/mg)		Stability (%)	Cortisol level (pg/mg)		Stability (%)
	Processed hair sample			Unprocessed hair sample		
	Baseline	24 hrs	Baseline	7 months		
1	27.0	26.9	100	28.6	27.0	94
2	13.6	13.5	99	12.8	13.6	106
3	6.0	5.6	93	6.2	6.0	97
Mean			97			99

Processed and unprocessed hair samples were stored at room temperature for the indicated times. Stability = level determined after storage/ level determined before storage \times 100.

age and BMI were 35.1 (9.2) years and 25.7 (3.7) kg/m², respectively. None reported a history of psychiatric disorders, had clinical evidence for cortisol excess was treated with systemic or local glucocorticoids, or used hair bleach. Of the 88 men, 73.9% were of East Asian, 21.6% of geographic region, and 4.5% of African ethnicity; 54.5% were

**Figure 2: Representative MRM chromatogram of hair sample from a healthy man with a hair cortisol level of 11.4 pg/mg hair.****Figure 3: Measured level of hair-cortisol in eighty-eight healthy volunteers.**

self-classified as athletes; and 31.8%, 8%, and 5.7%, respectively, reported perceived psychological stress, shift work, and chronic pain within the previous three months. Cortisol was detectable in hair samples [mean (SD) length = 1.3 (0.5) cm, weight = 33.9 (9.9) mg] of 95% of the 88 men and quantifiable in 49%. The measured level of hair-cortisol in eighty-eight healthy volunteers is shown in Figure 3.

DISCUSSION

The main advantage of hair cortisol measurement is providing information about the integrated exposure of internal cortisol over the past 2-4 months (similar to hemoglobin A1C for glycemic exposure). Hair-cortisol is usually measured by utilizing immunoassay or LC-MS/MS techniques followed by the procedure as reported.¹⁰⁻¹⁴ The procedure involved incubation of hair in methanol at 52°C for 16 hr that results from an extraction recovery range of 87.4-88.9%.^{8,10} In order to improve extraction recovery, we added a step of sonication for half-hour,

followed by vortex of 10 min that yields an average recovery (90.8%). However, after the primary extraction, the same samples were used for re-extraction on day-2 and day-3. The results of the analysis indicate that a rise in recovery of 6.9% and 2.3%, respectively. The stability of hair-cortisol was determined in processed (after extraction) and unprocessed (before extraction) samples. It absolutely was observed that hair-cortisol is stable a minimum 24 hr at room temperature in (processed samples) and seven months at room temperature in unprocessed samples.

The results of the current analysis indicate that no cortisol was detected in 4 (4.5%) of 88 hair samples. Whereas it was detectable in 84 (95.4%) and quantifiable in 43 (48.8%) with a mean (median) of 12.4 (8.7) pg/mg of cortisol in hair.

CONCLUSION

This ultra-high-performance liquid chromatography tandem-mass spectrometry assay is simple and accurate. The assay was used to determine hair cortisol levels in healthy volunteers' samples. Furthermore, the assay was utilized to assess cortisol stability in settings that are commonly encountered in clinical laboratories.

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CONFLICT OF INTREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

UPLC-MS/MS: Ultra-performance Liquid Chromatography-Tandem Mass Spectrometry; **IS:** Internal Standard; **MRM:** Multi Reaction Monitoring; **ESI-MS/MS:** Electro Spray Ionization Tandem Mass Spectrometry; **SD:** Standard Deviation; **CV:** Coefficient of variation.

REFERENCES

1. Bauer ME. Chronic stress and immunosenescence: A review. *Neuroimmunomodulation*. 2008;15(4-6):241-50. doi: 10.1159/000156467, PMID 19047801.
2. McKay LI, Cidlowski JA. Pharmacokinetics of corticosteroids. In: Kufe DW, Pollock RE, Weichselbaum RR, *et al.*, editors *Holland-Frei medicine*. 6th ed Hamilton (ON): BC Decker; 2003.
3. Chahal HS, Drake WM. The endocrine system and ageing. *J Pathol*. 2007;211(2):173-80. doi: 10.1002/path.2110, PMID 17200939.
4. Fries E, Dettenborn L, Kirschbaum C. The cortisol awakening response (CAR): Facts and future directions. *Int J Psychophysiol*. 2009;72(1):67-73. doi: 10.1016/j.ijpsycho.2008.03.014, PMID 18854200.
5. Wright KD, Hickman R, Laudenslager ML. Hair cortisol analysis: A promising biomarker of HPA activation in older adults. *Gerontologist*. 2015;55;Suppl 1: S140-5. doi: 10.1093/geront/gnu174, PMID 26055775.
6. Karlén J, Ludvigsson J, Frostell A, Theodorsson E, Faresjö T. Cortisol in hair measured in young adults - a biomarker of major life stressors? *BMC Clin Pathol*. 2011;11:12. doi: 10.1186/1472-6890-11-12, PMID 22026917.
7. Rana D, Kristin H, Stanvan U, Michael G, Catherine PB. Hair cortisol concentrations in war-affected adolescents: A prospective intervention trial *Psychoneuroendocrinology*. 2018;89:138-46.
8. Gow R, Thomson S, Rieder M, Van Uum S, Koren G. An assessment of cortisol analysis in hair and its clinical applications. *Forensic Sci Int*. 2010;196(1-3):32-7. doi: 10.1016/j.forsciint.2009.12.040, PMID 20096513.
9. Kirschbaum C, Strasburger CJ, Jammers W, Hellhammer DH. Cortisol and behavior: 1. Adaptation of a radioimmunoassay kit for reliable and inexpensive salivary cortisol determination. *Pharmacol Biochem Behav*. 1989;34(4):747-51. doi: 10.1016/0091-3057(89)90269-4, PMID 2623029.
10. Sauvé B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med*. 2007;30(5):E183-91. doi: 10.25011/cim.v30i5.2894, PMID 17892760.
11. Laura M, Jan WK, Steven WJL, Elisabeth FCVR. Evaluation of a method to measure long term cortisol levels. *Steroids*. 2001;76(10-11):1032-6.
12. Noppe G, Van Rossum EF, Koper JW, Manenschijn L, Bruining GJ, de Rijke YB, *et al.* Validation and reference ranges of hair cortisol measurement in healthy children. *Horm Res Paediatr*. 2014;82(2):97-102. doi: 10.1159/000362519, PMID 25115629.
13. Papafotiou C, Christaki E, Van den Akker EL, Wester VL, Apostolakou F, Papassotiropoulos I, *et al.* Hair cortisol concentrations exhibit a positive association with salivary cortisol profiles and are increased in obese prepubertal girls. *Stress*. 2017;20(2):217-22. doi: 10.1080/10253890.2017.1303830, PMID 28270027.
14. Tina MB, Ueli B, Markus RB, Thomas K. Development of an LC-MS/MS method for the determination of endogenous cortisol in hair using (13) C3-based-labeled cortisol as surrogate. *J Chromatogr B*. 2016;1033:65-72.
15. Santos-Montes A, Gonzalo-Lumbreras R, Izquierdo-Hornillos R. Simultaneous determination of cortisol and cortisone in urine by reversed-phase high-performance liquid chromatography. Clinical and doping control applications. *J Chromatogr B Biomed Appl*. 1995;673(1):27-33. doi: 10.1016/0378-4347(95)00253-f, PMID 8925071.
16. Bioanalytical method validation, guidance for industry US department of health services, Food and Drug Administration, CDER, CVM; 2018.

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