Original Article

MEASUREMENT OF CORTISOL AND CORTISONE IN HUMAN SALIVA BY UPLC-MS/MS

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ABSTRACT

Objective: To develop and validate a simple and rapid assay for simultaneous measurement of cortisol and cortisone in human saliva by ultra-performance liquid chromatography-tandem mass spectrometry.

Methods: Chromatographic analysis was performed on an Atlantis dC18 column (2.1 x 100 mm, 3 μm) using a mobile phase consisting of acetonitrile and 2 mmol ammonium-acetate (50:50, v; v) that was delivered at a flow rate of 0.3 ml/min. The eluents were monitored using electrospray ionization in the positive ion mode set at transition set of mass-to-charge (m/z): 363.11→ 121.00, 361.18 → 163.11, and 367.19 → 121.24 for cortisol, cortisone and internal standard (IS), respectively the method was validated for linearity, accuracy, precision, and recovery, according to international guidelines.

Results: The retention times of cortisol, cortisone and internal were about 1.38, 1.43 and 1.38 min, respectively. Cortisol level and cortisone level relationship to the ratio of their respective peak-area to IS's peak-area was linear (range of 0.5-100 ng/ml). Coefficients of variation and inaccuracy were, ≤9.9% and -0.3 to 6.9 for cortisol and≤8.4 and -1.5 to 4.8 for cortisone, respectively. Extraction recoveries for cortisol, cortisone, and the IS were 90%, 94%, and 98%, respectively. Cortisol and cortisone stability was evaluated in processed saliva samples (stored at room temperature for 24 h) and unprocessed saliva samples (stored at room temperature for 24 h or at-20 °C for 10 w) and after 3 freeze-thaw cycles was ≥ 86%.

Conclusion: The proposed method is simple, precise, and accurate for the rapid simultaneous measurement of cortisol and cortisone levels in saliva. The assay was successfully applied to determine levels of cortisol and cortisone in human saliva samples obtained from healthy volunteers.

Keywords: Cortisol, Cortisone, Cortisol-d4, Saliva, UPLC-MS/MS

INTRODUCTION

Cortisol, a steroid hormone secreted by the adrenal cortex, has an important function in metabolism, electrolyte balance, and the immune response, among others [1, 2]. Estimation of cortisol and cortisone in the saliva is used in evaluating the adrenal axis as it reflects that biologically active cortisol and sample assortment is not related to stress [3, 4]. Since cortisol is metabolized to cortisone (biologically inactive) by the salivary glands, via 11-ß-hydroxysteroid dehydrogenase type-2 (11ß-HSD2), an assay that can simultaneously measure cortisol and cortisone would allow better assessment of the adrenal axis and is essential for studying the salivary gland 11ß-HSD2 activity [5, 6].

Several assays for determining cortisol and cortisone levels in saliva and other biological fluids are reported [7-16]. Generally, commercially available kits are used for monitoring cortisol and cortisone levels by enzyme immunoassay and chemiluminescence individually [7, 8]. Whereas, high-performance-liquid-chromatography (HPLC) equipped with ultraviolet [9] or fluorescence [10] detector or tandem-mass-spectrometry (LC-MS/MS) [11-15] were used for simultaneous measurement of cortisol and cortisone levels in human plasma [11], serum [12] urine [13] and saliva [14, 15]. For Bioanalysis, often derivatizers [14] or expensive solid-phase extraction cartridges [13, 15] are utilized in sample preparation.

We report a simple, ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for simultaneous measurement of cortisol and cortisone levels in saliva, utilizing liquid-liquid extraction and cortisol-d4 as an internal standard (IS). The method was validated by following United States FDA guidelines [19] and it was utilized to monitor cortisol and cortisone levels in human saliva.

MATERIALS AND METHODS

Chemicals and solvents

We obtained cortisol (hydrocortisone) and cortisone from Acros organic NJ, USA, and cortisol-d4 from Sigma-Aldrich MO, USA. We purchased potassium phosphate (monobasic), sodium phosphate (dibasic), potassium chloride, sodium chloride, ammonium acetate, methyl-tert-butyl-ether, hexane (all AR grade) and acetonitrile (HPLC grade) from Fisher Scientific, NJ. USA. We prepared Milli-Q water by passing purified water through the Milli-Q System (Millipore, Bedford, MA, USA). The study was approved by the Research-Ethics-Committee, King Faisal Specialist Hospital and Research Centre, Riyadh (2191297).

Equipment and analytical conditions

Ultra-performance-liquid-chromatograph (UPLC) integrated solvent and sample manager (Acquity) equipped with tandem mass spectrometer (MS/MS) Xevo-TQD and an interface of Z-spray atmospheric pressure ionization (API) (Waters Corporation, Milford, MA, USA) were utilized for analysis. Analytes were estimated using an Atlantis dC18 steel column that was protected by an in-line filter. We used a mobile phase of 0.002 M ammonium-acetate and acetonitrile (50:50, v: v) that was passed through Supor membrane filter (Pall Gelman Laboratory, MI, USA) before delivery (0.3 ml/min). We operated the electrospray-ionization-source operated in the positive-ion-mode (capillary voltage 1.50 kV, cone voltage 35 volts). Nitrogen for nebulization/desolvation (1000 L/h) and argon for collision (3.6 x 10^{-3} mbar) were used. We found the optimum collision energy of 20 eV for cortisol, cortisone, and the IS. Ion source and desolvation temperatures were maintained at 150 °C and 500 °C, respectively. Cortisol, cortisone, and IS were detected in positive-ion-mode utilizing multiple-reaction-monitoring (MRM) at the transition of mass-to-charge (m/z) of: 363.11 → 121.00, 361.18→ 163.111, and 367.19 → 121.24 for cortisol, cortisone, and IS, respectively.

Standard and control samples

We prepared cortisol, cortisone, and IS solutions in 1.0 µg/ml methanol. We prepared 9 standards (0.5-100 ng/ml) and 4 quality-control solutions (0.5, 1.5, 50 and 90 ng/ml) in phosphate buffered saline (combination of 0.01 M sodium phosphate, 0.0018 M potassium phosphate, 0.137 M sodium chloride and 0.0027 M potassium chloride, pH=7.4, adjusted with HCl).

To validate the utilization of calibration standards in phosphate-buffered saline, about 230 ml human saliva was collected and levels of cortisol and cortisone were measured at baseline (blank). Four quality controls were prepared by spiking cortisol and cortisone in blank saliva at 0.5, 1.5, 50, and 90 ng/ml. Differences in levels of cortisol and cortisone between blank saliva and spiked saliva (as read against the calibration standards) were compared to the expected increase in level from the amount that were spiked. The differences were-0.03,-1.14, 1.77, and 5.28 ng/ml for cortisol and-0.01, 0.11,-0.31, 2.82 ng/ml for cortisone for four concentrations, respectively. We prepared an IS's solution (100 ng/ml) in methanol. All solutions were stored at 4 °C and used within four weeks of preparation.

Saliva sample collection

We collected unstimulated saliva by direct spitting in sterile tubes that were stored at-20 °C until analyzed.

Sample preparation

We added 200 µl IS's the solution (100 ng/ml) to 1.0 ml unknown, standard, or quality control samples that were then vortexed for 30 seconds. We added four ml combination of methyl-tert-butyl-ether and hexane (8:2, v: v), vortexed the mixture for 2 min, and then centrifuged it (4000 rpm) for 10 min at 20 °C. We collected the clear supernatant layer collected and dried it under a nitrogen stream (40 °C). We reconstituted the residue in methanol (100 µl) and analyzed 10 µl.

Recovery

We examined cortisol, cortisone, and IS extraction recovery by comparing peak-areas of extracted and un-extracted samples (five replicates, 0.5, 1.5, 50 and 90 ng/ml) for cortisol and cortisone. Similarly, IS recovery was determined at 100 ng/ml.

Stability

Five aliquots of 2 QC concentrations (1.5 and 90 ng/ml) were analyzed without storage (baseline), 5 aliquots were kept at room temperature 24 h before analysis, 5 were kept in-20 °C for 10 w before analysis, and 5 were kept at room temperature for 24 h after processing and before analysis. Finally, 15 aliquots were kept at −20 °C for 24 h before thawing at room temperature, 5 aliquots were analyzed and 10 returned to-20 °C for 24 h. This was repeated 3 times.

Matrix-effect

We studied the matrix-effect by contrasting peak-areas of cortisol and cortisone (0.5, 1.5, 50, and 90 ng/ml) and IS (100 ng/ml) in spiked-after-extraction samples with the corresponding peak-areas of standards in methanol.

Assay-validation

We validated the assay according to the guidelines of the United States FDA [16].

Collection of saliva from healthy volunteers

We collected 2-3 ml saliva samples by direct spitting in the morning (7-9 AM) and kept on-20 °C.

RESULTS

Separation and quantification

We determined the product and precursor ions of cortisol, cortisone, and cortisone-d4 (IS) by infusing a standard mixture containing cortisol, cortisone, and cortisol-d4 in the mass-spectrometer. We used a configured software program (Intellistart, Waters Corporation, Milford, MA, USA). Fig. **1** depicts the MS/MS spectrum and chemical structures of these compounds. The best liquid-chromatographic conditions were obtained with a mobile phase of 2 mmol ammonium-acetate and acetonitrile (50:50, v: v) with a flow rate 0.3 ml/min. The mass-spectrometry acquisition was achieved with multiple-reaction-monitoring in positive-ion mode. Cortisol, cortisone, and IS retention times were around 1.38, 1.43, and 1.38 min, respectively.
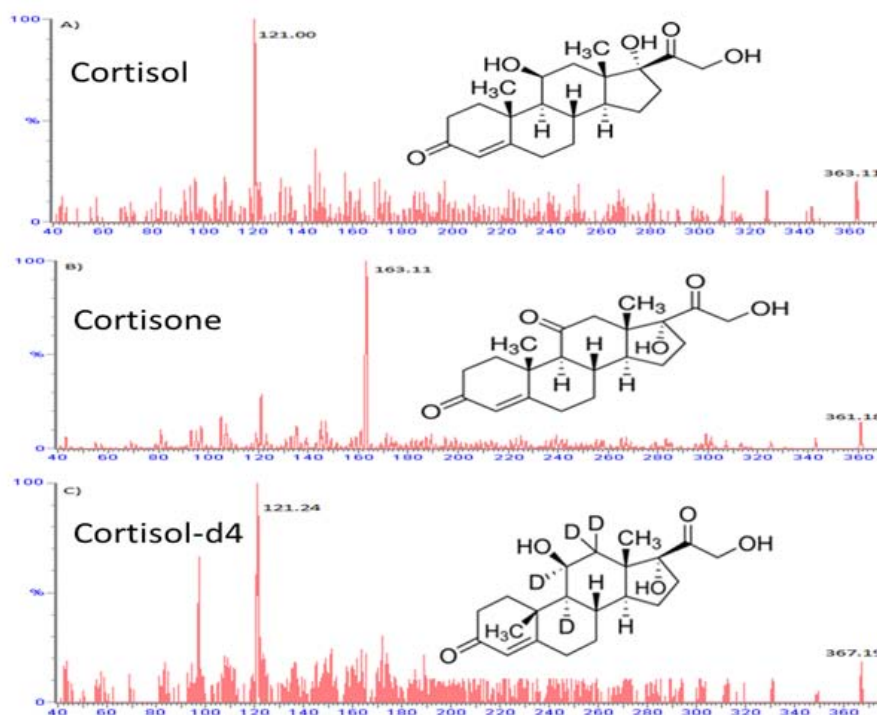

Fig. 1: MS/MS spectrum and chemical structures of cortisol, cortisone, and cortisol-d4 (internal standard)

Matrix-effect

Mean-matrix effect (at 0.5, 1.5, 50 and 90 ng/ml) was measured as ion suppression,-4.5%,-2.6%, and-3.6% for cortisol, cortisone and IS, respectively.

Specificity

We evaluated specificity by analyzing 6 batches of blank human saliva and 7 cortisol-related compounds, namely, cortisone, progesterone, 17 α-hydroxyprogesterone, prednisone, prednisolone,

methylprednisone and testosterone. The solutions were prepared as 1.0 μg/ml in methanol: water (1:1, v: v). 10 μl were injected. No interference with analytes' peaks was observed. Fig. 2 shows chromatograms of human saliva samples used to prepare quality controls.

Recovery

Mean extraction recoveries of cortisol and cortisone (0.5, 1.5, 50 and 90 ng/ml, 5 aliquots each) were 90%, and 94%, respectively. Recovery of IS (100 ng/ml) was 98%.
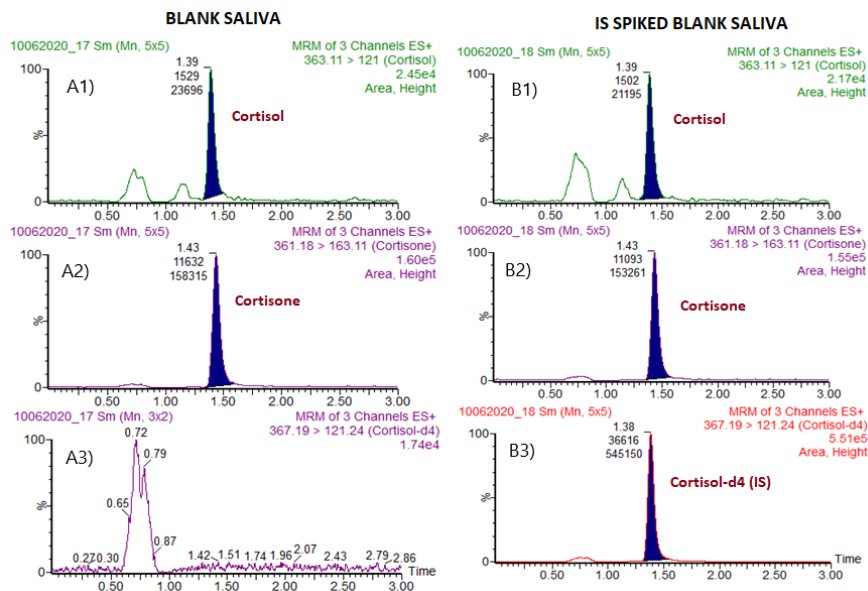

Fig. 2: Chromatogram of multiple-reaction-monitoring of human saliva used to prepare quality controls. Blank saliva (A1-A3). Blank saliva spiked with internal standard (B1-B3)

Linearity, detection limit, and quantification limit

We evaluated assay linearity by analyzing standard mixtures containing cortisol and cortisone in phosphate buffered saline at 9 different concentrations (0.5–100 ng/ml). Corresponding peak-area ratios and concentrations were analyzed by regression analysis. Mean (n=8) equations were y= 0.0531 x-0.0218, r^2 = 0.9991 for cortisol and y= 0.0454 x–0.0163, r^2 = 0.9989 for cortisone. Limits of detection and quantification (for both cortisol and cortisone) were 0.3 ng/ml and 0.5 ng/ml, respectively.

Precision and inaccuracy

Intra-day and inter-day precision and inaccuracy were assessed for four quality control samples (0.5, 1.5, 50, and 90 ng/ml). Intra-day (n=10) coefficients of variation (CV) and inaccuracy ranged from 2.8% to 9.8% and-1.0% to 9.7%, for cortisol and from 4.0% to 7.9% and-1.0% to 6.2% for cortisone, respectively. Inter-day (n=20) CV and inaccuracy ranged from 5.5% to 9.9% and-0.3% to 6.9% for cortisol, and from 5.2% to 8.4% and-1.5% to 4.8% for cortisone, respectively (table 1).

Table 1: Intra-day and inter-day precision and inaccuracy of cortisol and cortisone assays

Nominal level (ng/ml)	Intra-day (n=10)			Inter-day (n=20)		
	Mean (SD) measured level	CV (%)	Bias (%)	Mean (SD) measured level	CV (%)	Bias (%)
Cortisol						
0.5	0.49 (0.05)	9.8	-1.0	0.50 (0.05)	9.9	-0.3
1.5	1.65 (0.05)	2.8	9.7	1.60 (0.09)	5.5	6.9
50	52.19 (2.30)	4.4	4.4	50.31 92.88)	5.7	0.6
90	93.24 (3.98)	4.3	3.6	89.83 (5.61)	6.3	-0.2
Cortisone						
0.5	0.50 (0.04)	7.9	-1.0	0.49 (0.05)	8.4	-1.5
1.5	1.59 (0.10)	6.5	6.2	1.56 (0.11)	7.2	3.9
50	49.93 (3.53)	7.1	-0.1	52.13 (3.86)	7.4	4.3
90	92.27 (3.73)	4.0	2.5	94.34 (4.86)	5.2	4.8

SD is the standard deviation. CV is the coefficient of variation (SD/mean measured level multiplied by 100). Inaccuracy= measured level-nominal level/nominal level multiplied by 100.

Stability

We assessed the stability of cortisol and cortisone under common laboratory conditions. Little or no change in concentration was

observed. (Table 2) In processed saliva samples, cortisol and cortisone were stable at room temperature for 24 h (≥ 91%). In unprocessed saliva samples, they were stable for 24 h at room temperature (≥ 86%), 10 w at −20 °C (≥ 88%), and after 3 freeze-thaw cycles (≥ 89%).

Table 2: Stability of cortisol and cortisone in various laboratory conditions

	Processed		Unprocessed		Freeze and thaw		
	Zero time	24 h (RT)	24 h (RT)	10 wks (-20 °C)	Cycle-1	Cycle-2	Cycle-3
Cortisol	1.55	1.41	1.33	1.36	1.29	1.38	1.46
Level ng/ml, (SD)	(0.09)	(0.07)	(0.14)	(0.04)	(0.06)	(0.09)	(0.07)
Stability (%)		(91)	(86)	(88)	(83)	(89)	(94)
	95.27	91.44	89.50	85.51	86.93	92.57	83.85
	(1.98)	(1.98)	(1.13)	(2.09)	(1.83)	(1.54)	(0.78)
	(96)	(94)	(90)	(112)	(97)	(88)	
Cortisone	1.42	1.53	1.55	1.36	1.59 (0.06)	1.33 (0.11)	1.27 (0.13)
Concentration	(0.12)	(0.09)	(0.08)	(0.04)	(112)	(94)	(89)
ng/ml, (SD)	(108)	(109)	(96)				
Stability (%)	90.94	94.67	99.67	85.51	93.60	82.99	84.87
	(2.45)	(4.62)	(2.89)	(2.09)	(5.00)	(3.06)	(1.00)
	(104)	(110)	(94)	(103)	(91)	(93)	

RT indicates room temperature, hr indicates hours, wks indicates weeks, FT indicates Freeze-Thaw. Stability (%) equals average measured level (n=5) at specified time/average measured level (n=5) at baseline x 100.
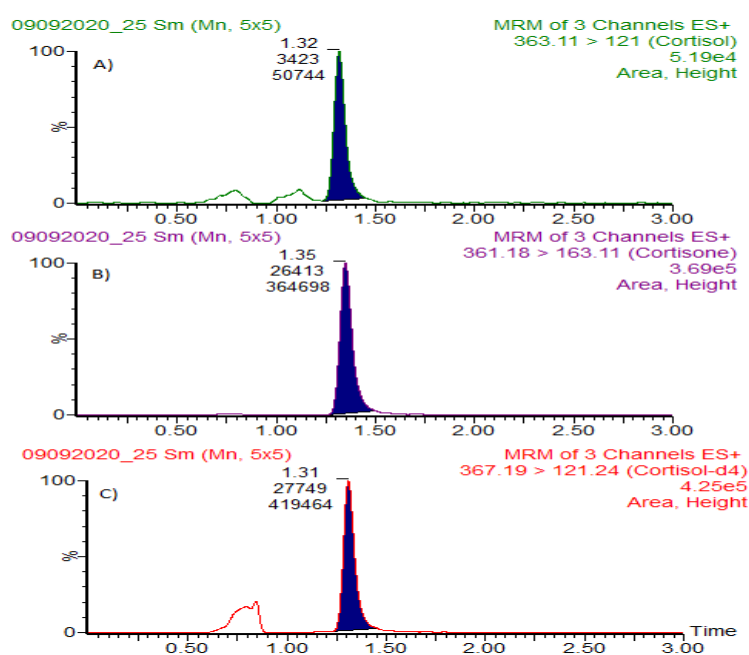

Fig. 3: Multiple-reaction-monitoring chromatogram of saliva sample collected from healthy volunteer spiked with IS (20 ng). Measured level of A) Cortisol (2.4 ng/ml) B) Cortisone (26.7 ng/ml) and C) Cortisol-d4 (IS)

Application of the method

The validated assay was used to determine the level of cortisol and cortisone in saliva samples collected from healthy volunteers. Fig. 3 depicts a typical chromatogram of saliva sample that represents measured levels of cortisol and cortisone were 2.4 ng/ml and 26.7 ng/ml, respectively.

DISCUSSION

Analysis of human saliva is a non-invasive and inexpensive test that can serve as a source of valuable information for diagnosis of several hormonal diseases including cushing's and nephrotic syndrome in adult and children [17, 18]. The measurement of cortisol in saliva reflects the free and biologically active fraction of cortisol in plasma/serum. Due to the auto-conversion process, cortisol metabolized to cortisone (biologically inactive) by the salivary glands, via 11-ß-hydroxysteroid dehydrogenase type-2 (11ß-HSD2) [5-6]. Therefore, presences of cortisol and cortisone in saliva are always expected. The measurement of cortisol and cortisone levels in saliva provides a more comprehensive picture of the adrenal axis function than the measurement of cortisol alone. Further, the measurement also essential for studying 11-ß-hydroxysteroid dehydrogenase type-2 activity in salivary glands. Usually, immunoassay techniques were used for measurements cortisol and cortisone in biological fluid. However, the technique often suffer the issue of interferences due to cross-reactivity with structurally related substances. In order to avoid the cross-reactive, Vieira *et al.* reported a liquid chromatography-tandem mass spectrometry method for simultaneous measurement of cortisol and cortisone in saliva by adopting the procedure that involves derivatization with hydroxylamine [14]. Whereas, Allende F, *et al.* and Jones RL, *et al.*, used solid-phase extraction procedure utilizing commercially available disposable cartridges in sample preparation [13, 15]. In comparison to these reported methods, we described here a simple and rapid method for simultaneous measurement of cortisol and cortisone, using a liquid-liquid extraction procedure that does not require expensive reagents or disposable cartridges. The recovery of all analytes was measured more than 90%, precision and inaccuracy of the method were than 10%, respectively.

CONCLUSION

A validated UPLC-MS/MS assay for simultaneous measurement of cortisol and cortisone levels in human saliva is reported. The assay is based on liquid-liquid extraction and use of cortisol-d4 as an

internal standard. The assay was used to determine the stability of cortisol and cortisone under common laboratory conditions and was applied in determining cortisol and cortisone levels in the saliva of healthy volunteers.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

Dr. Syed N Alvi: Performed experiments, analyzed data and drafted manuscript.

Dr. Muhammad M Hammami: Critically reviewed data and revised manuscript.

CONFLICTS OF INTERESTS

Declared none

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