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Research Article

MEASUREMENT OF CORTISOL IN HUMAN PLASMA AND URINE BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

Objective: The objective of this study is to develop and validate a simple, sensitive, specific, and rapid assay for quantification of clinically relevant cortisol level in human plasma and urine samples.

Methods: Ultra performance liquid chromatographic-tandem mass spectrometric (UPLC-MS/MS) analysis was performed on Atlantis dC18 column (2.1×100 mm, 3 µm) with a mobile phase consisting of acetonitrile and 2 mM ammonium acetate (50:50, v: v) that was delivered at a flow rate of 0.3 ml/min. Tolperisone (2 ng) was used as an internal standard (IS). Biological samples were extracted with a mixture of hexane and methyl tert-butyl ether (8:2, v: v). The eluents were monitored using electrospray ionization in the positive ion mode with transition mass to charge ratio set at 363.1 → 121.0 and 246.0 → 97.9 for cortisol and IS, respectively. The method was validated according to international guidelines.

Results: Retention times of cortisol and IS were about 1.4 and 2.3, respectively. Relationship between cortisol level and peak area ratio of cortisol to IS was linear ($R^2 \geq 0.987$) in the range of 2.5–400 ng/ml and 1.0–200 ng/ml in plasma and urine samples, respectively. Intra- and inter-day coefficient of variation and bias were ≤9.7% and ±11.1%, and ≤10.4% and ±11.5% for plasma and urine samples, respectively. Extraction recoveries were in 80–91% for cortisol plasma and 80–86% for cortisol in urine samples. Cortisol was ≥86% stable when stored at room temperature for 24 h or at −20°C for 24 weeks in plasma samples and ≥93% stable when stored at room temperature for 24 h or −20°C for 16 weeks in urine samples.

Conclusion: We report a validated, clinically relevant, simple, and rapid UPLC-MS/MS assay of cortisol level in human plasma and urine.

Keywords: Cortisol, Tolperisone, Human plasma, Urine, Ultra performance liquid chromatographic-tandem mass spectrometric.

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INTRODUCTION

Cortisol (Hydrocortisone, CAS: 50-23-7), the main glucocorticoid hormone in humans, plays several important physiological and pathological roles [1,2]. Measurement of its level in biological fluids is essential to establish a variety of related diagnoses [3,4].

Radioimmunoassay (RIA) is commonly used to determine cortisol level in biological fluids [5,6], whereas high-performance liquid chromatography (HPLC) with ultraviolet detection is mostly used for pharmaceutical preparations [7-9]. RIA in general has high sensitivity but often lack selectivity due to cross-reactivity with related substances [10,11]. To improve selectivity, Takao *et al.* [12] and Fenske [13] reported methods based on derivatization of cortisol with a fluorescent reagent, followed by HPLC or thin-layer chromatography, respectively. LC coupled with mass spectrometry (MS) or tandem MS/MS is a powerful and selective technique that has been used to determine steroid hormone levels [14-16]; however, most of the reported assays used d-4 cortisol as internal standard (IS), which may not be readily available [15,16].

In the present paper, we describe a simple, sensitive, specific, and rapid ultra performance liquid chromatographic-tandem mass spectrometric (UPLC-MS/MS) assay for determination of clinically related cortisol level in human plasma and urine samples, using tolperisone as an IS. The method involves simple liquid/liquid extraction and uses 0.5 ml plasma or 1.0 ml urine sample. The method was fully validated according to the international standards and used to determine cortisol stability under various clinical laboratory conditions.

METHODS

Chemicals and reagents

All chemicals were of analytical grade unless stated otherwise. Hydrocortisone and tolperisone were purchased from Acros Organics, NJ, USA, and Sigma-Aldrich MO, USA, respectively. Ammonium acetate, methyl tert-butyl ether, hexane, and acetonitrile (HPLC grade) were purchased from Fisher Scientific, NJ, USA. HPLC grade water was prepared by reverse osmosis and further purified by passing through a Milli-Q System (Millipore, Bedford, MA, USA). Human plasma was obtained from the blood bank of King Faisal Specialist Hospital and Research Center (KFSHRC), Riyadh, Saudi Arabia. The study was approved by the Research Ethics Committee of KFSHRC (RAC# 2160008).

Instrument and chromatographic conditions

The liquid chromatography-tandem mass spectrometer (LC-MS/MS) consists of Xevo-TQD detector equipped with Z-spray, an atmospheric pressure ionization interface, Acquity UPLC H-Class system, and integrated solvent and sample manager (Waters Corporation, Milford, MA, USA). Analysis was performed at room temperature using a reversed-phase Atlantis dC18 column (2.1×100 mm, 3 µm) steel column, protected by a column guard on-line filter (0.2 µm×2 mm). The mobile phase was composed of 2 mM ammonium acetate and acetonitrile (50:50, v: v) and was delivered at a flow rate of 0.30 ml/min. The electrospray ionization (ESI) source was operated in the positive ion mode at a capillary voltage of 1.5 kV and a cone voltage of 36 V. Nitrogen was used as the nebulizing and desolvation gas at a flow rate 1000 L/h. Argon was used as the collision gas, maintaining cell pressure at 3.5875 E^{-003} mbar. An optimum collision energy of 20 eV was applied for both cortisol and IS. The ion source and desolvation temperatures

were maintained at 150°C and 500°C, respectively. Cortisol and IS were detected and quantified in the positive ion mode; product ion response was measured at mass to charge (m/z) transition of 363.1 → 121.0 and 246.0 → 97.9, respectively. Mass lynx Ver 4.1 (Waters Corporation, Milford, MA, USA) software working under Microsoft Windows XP professional environment was used to control the instrument parameters, data acquisition, peak integration, peak smoothing, and signal-to-noise ratio measurements.

Preparation of standard and control samples

Cortisol and the IS stock solutions were prepared in methanol (1.0 µg/ml). Cortisol extracted human plasma, and urine samples were used as blanks for calibration standard and quality control samples' preparation. Nine calibration standards in the range of 2.5–400 ng/ml for plasma and 1–200 ng/ml for urine and four quality controls concentrations (2.5, 1.5, 200, and 380 ng/ml for plasma and 1.0, 3.0, 100, and 180 ng/ml for urine) were prepared in human plasma and urine, respectively. IS working solution was prepared in water (20 ng/ml). Standard and control solutions vortexed for 1 min. Aliquots of plasma (0.5 ml) and urine (1.0 ml) samples were transferred into 7 ml glass culture tubes and stored at -20°C until used. .

Preparation of samples

100 ml of the IS working solution was added to 0.5 ml unknown plasma or 1.0 ml urine samples, calibration standard samples, or quality control samples in a 7 mL glass culture tubes and vortexed for 30 s. 4.0 ml mixture of methyl tert-butyl ether and hexane (8:2,v: v) was added to each tube, vortexed for 2 min, and centrifuged at 4000 rpm for 10 min at 20°C. The clear supernatant layer was transferred to a clean borosilicate culture tube and dried under gentle steam of nitrogen at 40°C. The residue was reconstituted in 100 ml mobile phase, and 10 ml of the clear solution was injected into the LC-MS/MS system.

Effect of matrix

Matrix effect was evaluated by comparing peak area response obtained from spiked plasma and urine samples to peak area response obtained from direct injection of corresponding concentrations.

Stability studies

Two QC samples (7.5 and 360 ng/ml in plasma and 3.0 and 180 ng/ml in urine) were used for stability studies. Five aliquots of each sample were extracted and immediately analyzed (baseline). Five aliquots of each sample were allowed to stand on the benchtop for 24 h at room temperature before being processed and analyzed, and five aliquots were stored at −20°C for 16 or 24 weeks, before being processed and analyzed. Five aliquots were processed and stored at room temperature for 24 h or at −20°C for 48 h before analysis. Finally, fifteen aliquots of each sample were stored at −20°C for 24 h. After leaving them to completely thaw unassisted at room temperature, five aliquots were analyzed and the rest were stored at −20°C for another 24 h. The cycle was repeated 3 times.

RESULTS

Method development

MS/MS optimal analytical conditions were determined by infusing a standard mixture of cortisol and IS (1.0 µg/ml in methanol) using configured Intellistart software program. Precursor and product ion transitions for the measurement of cortisol and the IS response were set as m/z 363.1 → 121.0 and 246.0 → 97.9, respectively. MS/MS spectra and chemical structure of cortisol and tolperisone are shown in Fig. 1.

LC conditions

LC conditions were optimized using mobile phase composed of 2 mM ammonium acetate and acetonitrile (50:50, v: v) at a flow rate 0.3 ml/min. The high proportion of acetonitrile facilitated lower back pressure of the column and shorter analysis time (<3.0 min).

Extraction recovery

The absolute recovery of cortisol was assessed by comparing absolute peak area of spiked human plasma and urine samples to mobile phase samples, using five replicates for each of four concentrations (2.5, 7.5, 200, and 360 ng/ml for plasma and 1.0, 3.0, 100, and 180 ng/ml for urine). Similarly, the recovery of the IS was determined by comparing the peak area of the IS in 5 aliquots of human plasma and urine spiked with 20 ng/ml IS to the peak area of equivalent aliquots prepared in mobile phase.

Effect of matrix

Atmospheric pressure ESI (APESI) is a widely used technique in bioanalytical applications. Although it is expected that analyte response may supress more in APESI compared to atmospheric chemical ionization mode [17], in the present study, in the present study, mean matrix effect on cortisol and IS response were low when measured using APESI (−11.1% and −5.9%), respectively.

Method validation

The method was validated according to the international guidelines. The validation parameter included selectivity, recovery, linearity, accuracy, precision, and stability [18,19].

Selectivity

The selectivity/specificity of the assay was determined by screening six different batches of blank human plasma and urine; none showed interference. Further, the potential interference of seven structurally similar compounds, i.e., cortisone, progesterone, 17 α-hydroxyprogesterone,

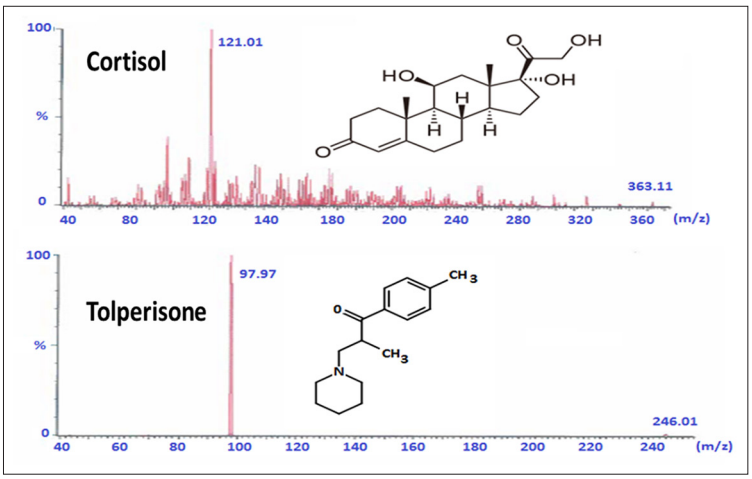

Fig. 1: Mass spectrometric (MS)/MS spectra and chemical structures of cortisol and tolperisone (internal standard), solution concentration 10 µg/ml each in methanol

prednisone, prednisolone, methyl prednisolone, and testosterone was also examined. 1.0 µg/ml solutions were prepared in methanol:water (1:1, v: v) and 10 µl were injected into the system. No compound showed interference with the peaks of cortisol or the IS. Fig. 2 depicts representative chromatograms of human plasma and urine that was used in the preparation of calibration curve and quality control samples.

Recovery

Recovery of cortisol and IS was assessed by comparing analyte peak areas obtained from spiked plasma, urine, and mobile phase samples, using five replicates of four cortisol concentrations (2.5, 7.5, 200, and 360 ng/ml for plasma and 1.0, 3.0, 100, and 180 ng/ml for urine) and 20 ng/ml for IS. Extraction recoveries were in 80–91% for cortisol in plasma, 80–86% for cortisol in urine, and ≥84% for the IS in plasma and urine. The results are presented in Table 1.

Linearity and limit of quantification

Linearity of the assay was evaluated by analyzing a series of cortisol standards at nine different concentrations over the range of 2.5–400 ng/ml in plasma and 1.0–200 ng/ml in urine. Corresponding peak area ratios and concentrations were subjected to regression analysis. The mean equation obtained from eight standard curves was y=0.0031 (x)+0.0010, with R^2 (SD)=0.9971 (0.0028), and y=0.0093 (x)+0.0030, with R^2 (SD)=0.9949 (0.0058), for plasma and urine samples, respectively. The detection and quantification limits were as 1.0 ng/ml and 2.5 ng/ml for plasma and 0.5 ng/ml and 1.0 ng/ml for urine samples, respectively.

Accuracy and precision

Accuracy and precision were determined by measuring cortisol levels at four concentrations (2.5, 7.5, 200, and 360 ng/ml for plasma and 1.0, 3.0, 100, and 180 ng/ml for urine). Intraday (n=10) imprecision and bias were ≤9.7% and ±10.8% and ≤10.0% and ±11.5%, for plasma and urine samples, respectively. Interday (n=20) imprecision and bias, determined over 3 days, were ≤9.6% and ±11.1% and ≤10.4% and ±6.5%, for plasma and urine samples, respectively. Data are summarized in Table 2. Fig. 3a and b depicts LCMS/MS chromatograms of cortisol in human plasma and urine samples spiked with 100 µl of IS (20 ng/ml).

Stability

It is necessary to perform stability studies of the analyte and IS to determine the range of appropriate conditions and times of storage. The availability of long-term stability studies is essential for sample collection planning and results in interpretation as described by Syed *et al*.[20].

In the present study, cortisol and IS stability in processed and unprocessed plasma and urine samples was investigated. Cortisol in processed plasma (7.5 and 360 ng/ml) and urine (3.0 and 180 ng/ml) samples was stable for 24 h at room temperature (≥ 90%) and 48 h at ×20°C (≥90%). Cortisol in unprocessed plasma samples was stable for at least 24 h at room temperature (≥86%), 24 weeks at −20°C (≥90%), and after three freeze-and thaw cycles (≥83%). Similar stability was seen for cortisol in urine samples. The data are summarized in Table 3. Further, no significant change in chromatographic behavior of cortisol or the IS was observed under any of the above conditions.

DISCUSSION

In general, cortisol levels in serum/plasma or urine were measured by radioimmunoassay (RIA) utilizing commercially available kits. Most of the reported methods indicated that the performance of RIA for urinary free cortisol is relatively less satisfactory than plasma/serum cortisol [10]. Wood *et al.* [11] reported that urine-free cortisol levels measured by RIA are 1.6 and 1.9 times overestimated compared to gas chromatography (GC)-MS and LCMS/MS, respectively. On the other hand, results obtained by GC-MS and LC-MS/MS have good agreement, indicating that chromatographic assays are more reliable than RIA for cortisol measurements. Several LCMS/MS methods have been described for quantitative measurement of cortisol in serum and urine [15,16]. However, most of them are based on solid-phase extraction and deuterated cortisol as IS, which may not be readily available. We described a simple and convenient LCMS/MS method that is based on liquid-liquid extraction and that uses a commercially available IS, tolperisone. Intra- and inter-day coefficient of variation and bias were ≤9.7% and ±11.1% and ≤10.4% and ±11.5% for plasma and urine samples, respectively, and extraction recoveries were 80–91% for plasma cortisol and 80–86% for urine cortisol. The assay was successfully used to determine the stability of cortisol in biological

Table 1: Extraction recovery of cortisol and tolpirisone (IS) from human plasma and urine

Cortisol (ng/ml)	*Plasma	*Mobile phase	†Recovery (%)	Cortisol (ng/ml)	*Urine	*Mobile phase	†Recovery (%)
2.5	3699	4317	86	1.0	1089	1304	84
7.5	12635	13931	91	3.0	3070	3562	86
200	217809	270711	80	100	74837	92971	80
360	529223	584303	91	180	125576	152344	82
IS (20)	199045	236899	84	IS (20)	64951	76209	85

*Mean peak area of 5 replicates. †Mean peak area of cortisol in human plasma or urine divided by mean peak area in mobile phase×100. IS: Internal standard

Table 2: Intra- and inter-day precision and bias of cortisol assay

Nominal	Intraday (n=10)			Interday (n=20)		
Level (ng/ml)	Measured level			Measured level		
	Mean (SD)	CV (%)	Bias (%)	Mean (SD)	CV (%)	Bias (%)
Plasma sample						
2.5	2.7 (0.3)	9.7	6.0	2.7 (0.2)	9.2	6.3
7.5	7.1 (0.6)	8.7	-5.2	7.3 (0.7)	9.6	-2.2
200	180.8 (15.3)	8.5	-9.6	182.0 (13.9)	7.7	-9.0
360	321.2 (10.7)	3.3	-10.8	320.2 (10.7)	3.3	-11.1
Urine sample						
1.0	1.0 (0.1)	9.7	-2.5	1.1 (0.1)	10.2	5.2
3.0	2.7 (0.1)	3.5	-11.5	2.9 (0.3)	10.4	-2.6
100	103.5 (10.3)	10.0	3.5	106.5 (8.9)	8.4	6.5
180	181.4 (15.8)	8.7	0.8	186.5 (17.3)	9.3	3.6

SD: Standard deviation. CV: Coefficient of variation=standard deviation divided by mean measured concentration×100. Bias=measured level - nominal level divided by nominal level×100

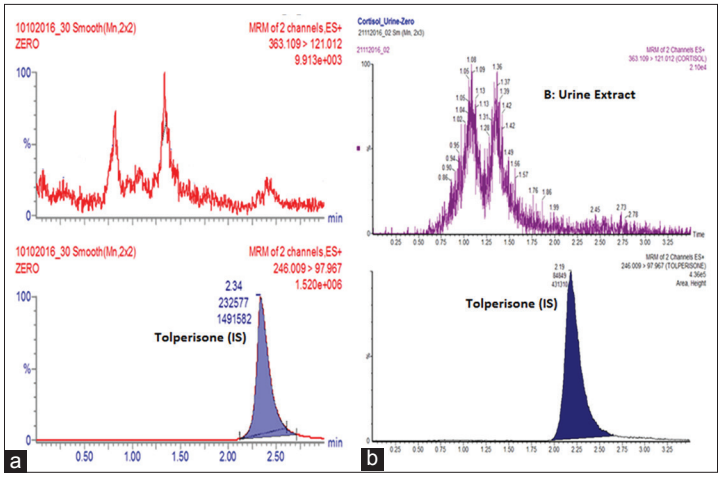

Fig. 2: MRM chromatogram of blank human plasma (a) and urine (b) (spiked with 100 µl internal standard) that were used in preparing calibrating standard and quality control samples

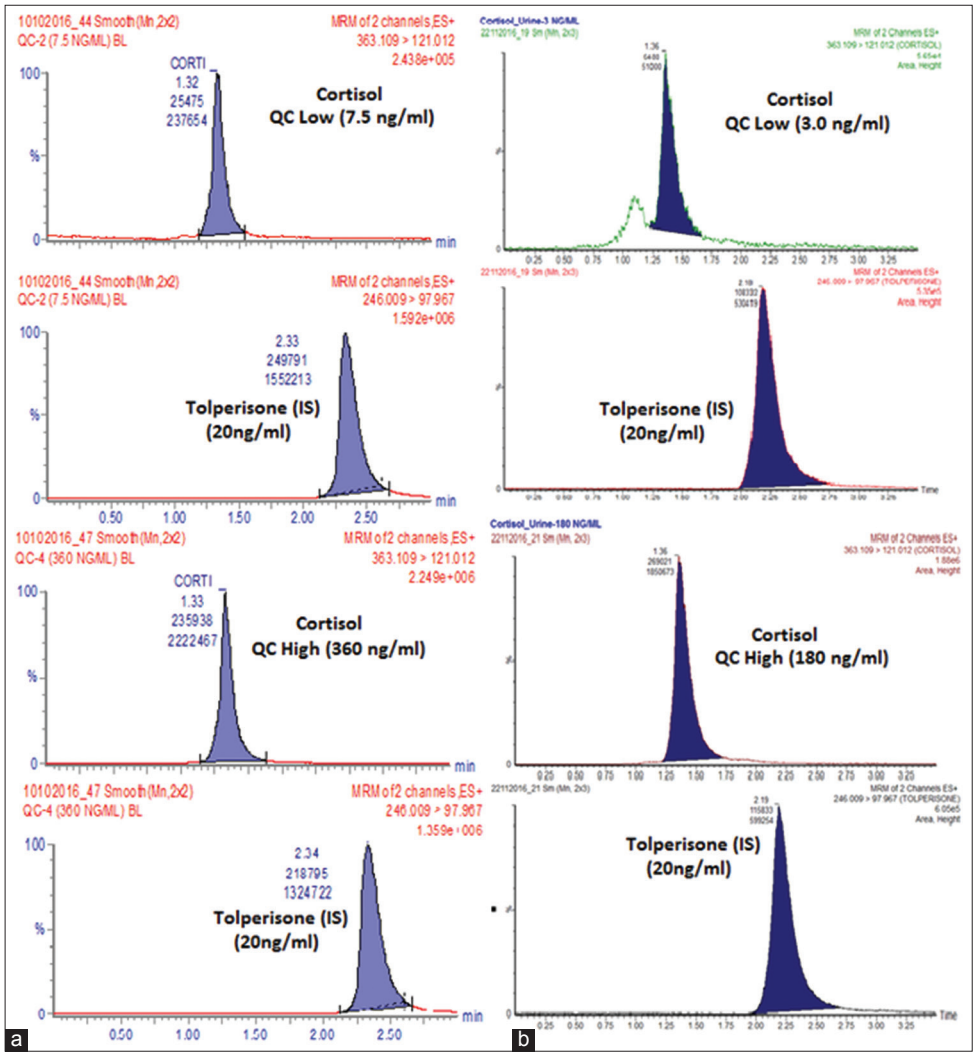

Fig. 3: MRM chromatograms of two quality control concentrations 7.5 and 360 ng/ml in plasma (a) and 3.0 and 180 ng/ml in urine (b) samples, all spiked with tolperisone (internal standard, 20 ng/ml)

samples. Cortisol was ≥86% stable when stored at room temperature for 24 h or at −20°C for 24 weeks in plasma samples and ≥93% stable when stored at room temperature for 24 h or −20°C for 16 weeks in urine samples.

CONCLUSION

We described a validated, clinically relevant, simple, and rapid UPLC-MS/MS assay of cortisol level that utilizes 0.5 ml human plasma

Table 3: Stability of cortisol in different matrix and conditions

Storage condition	Matrix: Plasma			Matrix: Urine		
	Nominal level (ng/ml)	Measured level mean (SD) (ng/ml)	Stability (%)	Nominal level (ng/ml)	Measured level mean (SD) (ng/ml)	Stability (%)
Baseline						
	7.5	7.9 (0.6)		3.0	2.7 (0.1)	
	360	329 (38)		180	165 (9.4)	
Processed samples 24 hr (RT)						
	7.5	7.6 (0.8)	96	3.0	2.6 (0.1)	96
	360	296 (10.7)	90	180	154 (8.2)	93
48 h (−20°C)	7.5	7.4 (0.7)	94	3.0	2.6 (0.1)	96
	360	295 (7.5)	90	180	167 (6.9)	101
Unprocessed samples 24 h (RT)						
	7.5	6.8 (0.3)	86	3.0	2.7 (0.1)	100
	360	311 (2.2)	95	180	154 (3.3)	93
24 weeks (plasma) (−20°C)	7.5	7.1 (0.4)	90			
	360	312 (5.7)	95			
16 weeks (Urine) (−20°C)						
				3.0	2.8 (0.1)	104
				180	166 (4.0)	101
FT Cycle-1	7.5	7.6 (0.8)	96	3.0	2.8 (0.2)	104
(−20°C)	360	274 (10)	83	180	165 (5.3)	100
FT Cycle-2	7.5	7.1 (0.5)	90	3.0	2.5 (0.1)	93
(−20°C)	360	318 (5.8)	97	180	181 (9.1)	110
FT Cycle-3	7.5	7.1 (0.5)	90	3.0	2.6 (0.5)	96
(−20°C)	360	283 (5.6)	86	180	176 (8.9)	107

Stability (%) = mean measured level at the indicated time divided by mean measured level at base line×100 (n=5). RT: Room temperature (20±2°C). FT: Freeze-thaw cycle; samples were frozen at−20°C and thaw at RT

or 1.0 ml urine. The assay involves the use of a readily available IS (tolperisone), and it takes 30 min to complete the analysis. It was used to study cortisol stability under various clinical laboratory conditions.

AUTHOR'S CONTRIBUTIONS

LCMS/MS method development, validation, data analysis and manuscript preparation done by Dr. Syed N Alvi (Scientist).

The manuscript reviewed and revised by Dr. Muhammad M Hammami (Co-Author), Chairman, Department.

CONFLICTS OF INTEREST

The authors declared that they had no conflicts of interests.

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