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RESEARCH ARTICLE

DEVELOPMENT AND VALIDATION OF LIQUID CHROMATOGRAPHY/STABLE ISOTOPE-DILUTION TANDEM MASS SPECTROMETRY METHOD FOR MEASUREMENT OF DEXAMETHASONE, TOTAL CORTISOL AND FREE CORTISOL IN HUMAN PLASMA

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ABSTRACT

Purpose: Cortisol plays a role in the regulation of metabolic homeostasis. Dexamethasone is a powerful synthetic glucocorticoid that acts as an anti-inflammatory and immunosuppressive agent. Plasma free cortisol, which has a low concentration in the blood, is the cortisol fraction that provides biological activity. It is emphasized that measurement of dexamethasone, total and free cortisol levels can directly affect the results of dexamethasone suppression test, which is routinely used to exclude endogenous hypercortisolemia in patients with adrenal adenoma, and is important in differential diagnosis. In this study, a precise and rapid method was developed for the measurement of dexamethasone, total and free cortisol levels in plasma samples, besides demonstrating the clinical applicability of the method with samples from adrenal adenoma patients.
Material and Methods: Method optimization studies were performed using liquid chromatography-tandem mass spectrometry with stable isotope dilution-multiple reaction monitoring.
Results: The assay demonstrated a good linear dynamic range of 0.5–20 µg/L, 5–200 µg/L and 0.5–100 µg/L for dexamethasone, total and free cortisol, respectively. The values for intra- and inter-day precisions of analytes were ≤6.9% with the accuracies ranging from 91.6% to 113.0%.
Conclusion: Measurement of these parameters can be used reliably to diagnose diseases causing hypercortisolemia.

Keywords: Adrenal adenoma, dexamethasone, free cortisol, LC-MS/MS, method validation, total cortisol

INTRODUCTION

Adrenal lesions are usually detected during imaging of non-adrenal organs in individuals without any adrenal gland-related complaints and signs. Its prevalence is 4-7% in the middle-aged population and 5-10% in individuals aged 70 years or older (1,2). The incidence of adrenal tumors has increased 10-fold over the past two decades, in parallel to the growing number of computed tomography abdominal scans (1). Approximately 90% of adrenal lesions are benign. 75-80% of benign lesions are non-functioning adrenal adenomas and other rare lesions (such as myelolipoma, cyst, etc.), while functional adrenal lesions such as pheochromocytoma, androgen-producing tumor, primary hyperaldosteronism, Cushing's syndrome, or Subclinical Cushing's syndrome are detected in 20-25% of patients (3,4).

Glucocorticoids (GC) are steroid hormones that contribute to the hypothalamic-adrenal-pituitary (HPA) axis. GC exert their effects by binding to glucocorticoid receptors (GR) (5). The most important glucocorticoid secreted in humans is cortisol. It has very important cardiovascular, metabolic, immunological and homeostatic functions (6). Urine and blood cortisol levels are measured to aid in the diagnosis serious adrenal diseases (7). Only 10% of circulating cortisol is free, while the remaining approximately 90% is bound to corticosteroid-binding globulin (CBG), albumin, and erythrocytes in plasma. Free cortisol can pass through cellular membranes and gain access the intracellular GR to generate physiological effects (8).

In most of related studies, free cortisol was measured in urine and saliva in the evaluation of hypercortisolemia and it was found to be significantly higher in Cushing's syndrome patients; therefore has been a part of the diagnostic algorithm (8). Another component of the diagnostic algoritm in hypercortisolemia is the dexamethasone supression test (9). Dexamethasone is a synthetic glucocorticoid, which acts as an anti-inflammatory and immunosuppressant agent and mimics the action of cortisol (10). Simultaneous measurement of dexamethasone and cortisol has been proposed to improve the diagnosis of adrenal diseases.

Glucocorticoids are commonly determined in biological samples by immunoassays due to simplicity of the method and easy automation. Immunoassays are however restricted to single analyte testing, show cross-reactivity with closely related molecules substances and have limited dynamic range. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides a robust platform with sufficient specificity and sensitivity for rapid multiple analyte quantitation (11,12).

In this study, we optimized a stable isotope dilution-multiple reaction monitoring liquid chromatography-tandem mass spectrometry (SID-MRM LC-MS/MS) method with high sensitivity and specificity, for absolute quantification of dexamethasone, total and free cortisol in plasma samples. Also, clinical applicability has been tested of this method with plasma samples from adrenal adenoma patients.

MATERIAL AND METHODS

Chemicals and standards

Dexamethasone (D0700000) and cortisol (C106) standards were purchased from Sigma (Sigma-Aldrich Co., USA) and used for validation studies. Dexamethasone-D4 (4,6-ALPHA,21,21-D4, 96%) and cortisol-D4 (9,11,12,12-D4) as internal standards were obtained from Cambridge Isotope Laboratories Inc., USA and MERCK & Co., USA, respectively. Acetonitrile, water, methanol, formic acid, ethyl acetate and 2-propanol were obtained from MERCK & Co., USA. Polymeric Reversed Phase (33 µm-30 mg/1 mL) extraction cartridges from Phenomenex, USA were used for extraction of the samples. Ultrafiltration columns (Cut-off; MA: 30,000 Da) were purchased from Millipore, USA. Albumin from bovine serum (BSA) and estriol were purchased from Sigma, USA. Trifluoroacetic acid (TFA) was purchased from Fisher Scientific, USA.

Plasma Samples

In order to demonstrate the clinical applicability of the developed method, plasma samples from 30 adrenal adenoma patients were included in this study. Ethical approval for this project was obtained from the Dokuz Eylul University Non-invasive Research Ethical Committee (Approval date: 18.06.2015, Decision number: 2015/16-28). All participants were signed the informed consent form. All blood samples were taken between 08:00 and 09:00 after an overnight fast. Four milliliters of venous blood was collected into EDTA tubes. Blood samples obtained for the measurement of dexamethasone, total and free cortisol were centrifuged, divided into aliquots and stored at -80 °C until the analysis.

Sample preparation

1.Pre-extraction procedure of free cortisol

Pre-extraction of free cortisol was performed using an ultrafiltration column. 100 µL D4-cortisol (1000 µg/L) and 600 µL plasma sample or standards were transferred to an ultrafiltration column.

Columns were incubated for 15 min at 37 ˚C and then centrifuged at 37 ˚C for 2 min at 1000xg. The filtrate was discarded, and the column was incubated at 37 ˚C for 15 min. After incubation the column centrifugated for 20 min at 1500xg. The ultrafiltrates were stored at 4 ˚C until used for extraction protocol (13,14).

2.Extraction protocol of total cortisol, free cortisol and dexamethasone

200 µL standard or plasma sample was mixed with 800 µL distilled water and 100 µL labeled standard mix (100 µg/L D4-cortisol and 10 µg/L D4-dexamethasone) for the extraction of total cortisol and dexamethasone. For the free cortisol, 200 µL ultrafiltrate sample was added to 900 µL distilled water. The tube was incubated in room temperature and mixed gently for 15 min. Subsequently, the mixture was pipetted into a polymeric reversed phase (30 mg/1 mL) extraction cartridge which was activated with 500 µL of methanol and 500 µL of distilled water, respectively. The mixture was passed through the cartridge under vacuum. Then washing was then carried out by 1 mL of water and 1 mL of 30% methanol and followed by drying for 15 min under low vacuum. The retained material was eluted by ethyl acetate containing 15% isopropanol (2x500 µL). The eluted analyte was dried in SpeedVacTM (Thermo Scientific, Ohio, USA). Prior to analysis dried analyte was dissolved in 100 µL of water: methanol (3:1) containing 10000 µg/L of estriol and transferred to a liquid chromatography vial (14,15).

Chromatographic and mass spectrometric conditions

Analyses were performed using reversed-phase liquid chromatography (HPLC) (Prominence LC-20A Modular HPLC System, Shimadzu, Japan) triple quadrupole ion-trap mass spectrometer (4000 QTRAP Applied Biosystems, CA, USA) including an atmospheric pressure chemical ionization (APCI) source in positive ionization mode. Samples were separated on a Restek Ultra II® Aromax column (100 x 3.2 mm, 3 µm particle size). Also, C8 guard column (12.5 x 2.1 mm, 5 µm particle size) was used. Mobile phase A was 95% methanol. Mobile phase B was 40% acetonitrile containing 10 mmol/L formic acid and 60% water containing 10 mmol/L formic acid. Mobile phase was delivered at a flow rate of 0.5 ml/min running isocratic (60% aqueous) for the first 4.8 min followed by high organic wash (mobil phase A) for 1 min and re-equilibration to initial conditions. The analysis time was 6.1 min. The injection volume was 30 µL. The system was washed with water: methanol (1:4) containing 0.05% TFA for 2 min, after each measurement. The Applied Biosystems Analyst Software Version 1.5 was used for data analyses(14). A direct infusion method using with standards and internal standards was used for determining mass spectrometric conditions. The final concentration of all standards was 100 µg/L. The flow rate of the infusion pump was 10 µL/min. The flow rate of HPLC was 0.5 mL/min. The compound optimization and manual tuning methods were used for determining of compound and source-dependent parameters of mass spectrometry.

Method Validation Studies

1.Calibration procedures, linearity, limit of detection (LOD) and limit of quantification (LOQ)

Method optimization studies were performed in accordance with the C62A and C50A protocols offered by the Clinical & Laboratory Standards Institute (16,17). The concentration of each analyte was calculated using calibration curve graphs. Since free and total cortisol concentrations are different in serum samples, we prepared separate calibration curves for free and total cortisol. Also, we prepared the calibration curve for dexamethasone. The standards were prepared in 0.1% BSA dissolved in phosphate buffer saline (1X PBS). Spiked internal standard concentrations were 10 µg/L for D4-dexamethasone ve 100 µg/L for D4-cortisol. Calibration standards were prepared in triplicate with the standard concentrations of 0.5, 1, 2.5, 5, 10 and 20 µg/L for dexamethasone; 5, 10, 25, 50, 100 and 200 µg/L for total cortisol, and 0.5, 1, 5, 10, 50, 100 µg/L for free cortisol. All measurements were performed on three independent days as six replicates. Different standard concentrations (1, 10, 50, 100, 200, 400 µg/L for dexamethasone, 10, 50, 100 200, 400, 800, 1000 µg/L for total and free cortisol) were prepared in order to determine linearity and run in the same way with calibration standards. The linearity was assessed by calculating the correlation coefficient (R square) and the regression

Table 1. Optimal mass spectrometric conditions of the method for LC-MS/MS

		Cortisol m/z 363.1 → m/z 121.1	d4-Cortisol m/z 367.4→ m/z121.1	Dexamethasone m/z 393.5 → m/z 373.4	d4-Dexamethasone m/z 397.3→ m/z 377.3
Compound-dependent parameters	DP	55	67	55	44
	EP	13	11	8	10
	CE	31	30	15	16
	CXP	2	6	16	10
Source-dependent parameters	Source temp.	300 °C	300 °C	300 °C	300 °C
	Nebulizer current	3	3	3	3
	Curtain gas	10	10	10	10
	Ion source gas	35	35	35	45
Dwell time		100 ms /transition			

m/z: mass/charge, DP: Declustering potential; EP: entrance potential; CE: collision energy; CXP: cell exit potential.

Table 2. Dexamethasone, total cortisol and free cortisol calibration curve and linearity results for plasma

	Dexamethasone			Total Cortisol			Free Cortisol		
	Slope	Intercept	R^2	Slope	Intercept	R^2	Slope	Intercept	R^2
Calibration curve results	0.0149	-0.0080	0,9989	0.0357	0.0155	0,9997	0.0069	0.0029	0,9995
Linearity results	0.0486	0.1054	1.000	0.0575	-1.1056	0,9919	0.0199	0.3531	0,9982

equation. The LOD was calculated as 3 x (standard deviation of the intercept (SD)/slope). The LOQ was calculated as 10 x (SD/slope).

2. Precision and Accuracy

Two different levels of standards (1 and 20 µg/L for dexamethasone, 5 and 200 µg/L for total cortisol, 5 and 100 µg/L for free cortisol) were prepared. Precision was calculated as between run (one measurement in three different days) and within run (three measurements in a single day). The [(standard deviation/mean) x 100] formula used for calculation of precision. The results were stated as coefficient of variation% (CV%) values. Accuracy was expressed as the deviation percentage of measured concentration and nominal using the two different concentrations of the standards via the formula [(measured concentration/nominal concentration) x 100].

3. Recovery

Five different plasma samples were mixed and used for the recovery study. The plasma matrix was spiked with standards for both cortisol and dexamethasone at 50 µg/L and 5 µg/L, respectively. Recovery was evaluated by comparing peak areas received before and after spike protocol and it is expressed as percentages.

4. Carry-over effect

The carry-over effect was evaluated with the blank sample (0.1% BSA) that was measured following the highest standard.

5. Verification of retention times

100 µg/L standard for free cortisol or 10 µg/L dexamethasone and 100 µg/L cortisol standard mix was added to extracted plasma samples separately to verify the retention times.

RESULTS

Determination of the method characteristics for mass spectrometry

The mass spectrometric conditions were summarized in Table 1. The acquisition was performed in MRM acquisition mode. The transitions m/z 363.1 → m/z 121.1 and m/z 367.4 → m/z 121.1 were used for both cortisol and D4-cortisol, respectively (Figure 1). The transitions for dexamethasone and D4-dexamethasone were m/z 393.5 → m/z 373.4 and m/z 397.3 → m/z 377.3, respectively (Figure 2).

Method Validation

1. Calibration curve, linearity, LOD and LOQ

Calibration curves and linearity graphics for dexamethasone, total and free cortisol were built separately for plasma. R square, slope and intercept values of the calibration and linearity graphics for all matrices were given in Table 2. LOD and LOQ values calculated using calibration curve graphics were given in Table 3.

2. Precision and accuracy

Precision and accuracy were determined using two different concentrations of the standards (1 and 20 µg/L for dexamethasone, 5 and 200 µg/L for total cortisol, 5 and 100 µg/L for free cortisol). According to the results obtained, accuracy was between 91.6%-113.0% for all parameters at different levels of concentration. The CV% of the intra- and inter-day precision were calculated to range from 1.5% to 6.9% for dexamethasone and range from 2.0% to 3.7% for total and free cortisol, which all fell within the acceptable limit (15%) (18).

3. Recovery

Levels of recovery were evaluated for cortisol and dexamethasone independently, and determined as 114% and 92%, respectively in the plasma matrix.

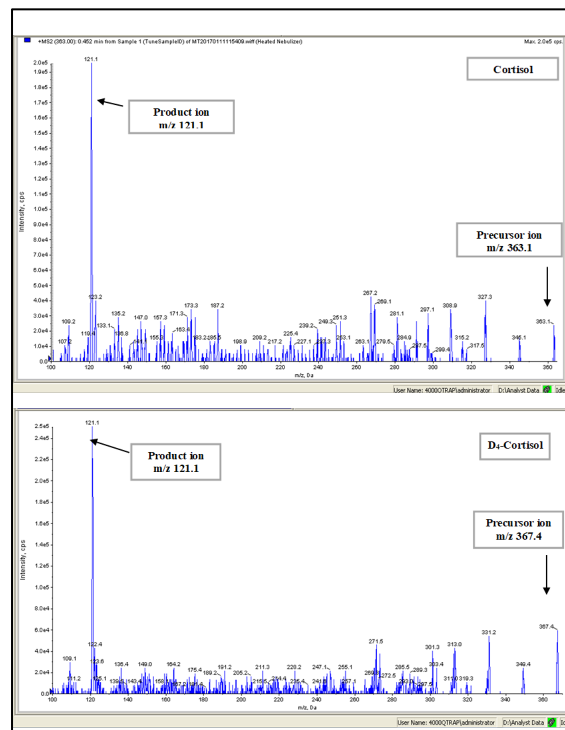

Figure 1. Fragmentation spectrums of cortisol and D4-cortisol

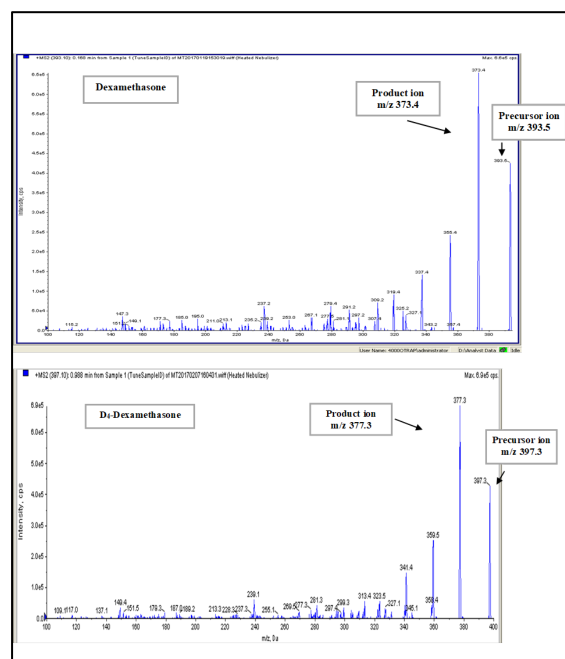

Figure 2. Fragmentation spectrums of dexamethasone and D4-dexamethasone

4. Carry-over effect

There was no detectable peak on the blank sample (0.1% BSA) chromatogram.

Table 3. Calculated LOD and LOQ values for dexamethasone, total cortisol and free cortisol

	LOD (µg/L)	*LOQ (µg/L)*
Total cortisol	0.30	0.70
Dexamethasone	0.50	1.00
Free cortisol	0.30	0.70

LOD: limit of detection, LOQ: limit of quantification

Table 4. Clinical applicability study: The median total and free cortisol, dexamethasone concentrations of the patients in plasma samples

	Total cortisol concentration median (min-max)	Free cortisol concentration median (min-max)	Dexamethasone concentration median (min-max)
Plasma (µg/dL)	3.3 (1.3-15.2)	1.6 (0.09-4.8)	2.4 (0.4-3.8)

5. Verification of retention times

Previously extracted plasma samples were spiked with 100 µg/L cortisol standard for free cortisol, or 10 µg/L dexamethasone and 100 µg/L cortisol standards mix and exact retention times of total cortisol, free cortisol, dexamethasone were verified as 2.42, 2.45 and 3.35 minutes, respectively (Figure 3).

Clinical Applicability

Thirty adrenal adenoma patients were included in the study. Simultaneous measurement of dexamethasone and total cortisol, and selective measurement of free cortisol from plasma samples of these patients was performed using the optimized method. The concentrations were given in Table 4. These findings showed that the validated method we developed was substantially applicable to plasma samples in clinical.

DISCUSSION

It is highly important to develop high-sensitivity methods for the measurement of plasma dexamethasone, total and free cortisol levels, which may have a direct impact on the results of the dexamethasone suppression test routinely used to exclude endogenous hypercortisolemia in patients with adrenal adenoma. In this study, we optimized and developed a method for selective and specific measurement of dexamethasone, total and free cortisol in human plasma.

Thus far, comprehensive validation data using mass spectrometry for measurement of total and free cortisol in plasma have been presented in a few studies (8,19). Huang et al. (8), developed a protocol to separate free cortisol using ultrafiltration with human plasma samples. Indeed, we used this method in our study to obtain the total and free cortisol fractions separately in plasma samples. In our study, method optimization studies were carried out for total and free cortisol measurement independently. Huang et al. showed intra- and inter-assay precision as between 4.8%-13.1% and the recovery was >80% for both total and free cortisol. The LOD of cortisol was 0.25 ng/mL and the LOQ was 0.50 ng/mL. The intra- and inter-assay accuracy for the low and high quality controls varied from 97.6% to 113% and 98.1% to 103% of nominal concentration, respectively. Total analytical run time of their method was 8 min. McWhinney et al. (19) presented a methodology for both measurement of cortisol and dexamethasone with mass spectrometry in plasma and plasma ultrafiltrate. Total analytical run time of their method was 3 min. In this study intra- and inter-day precision values were detected as <10% for all analytes in all matrices. LOQ values of total cortisol, dexamethasone and free cortisol were found as 3.75 nmol/L, 1 nmol/L and 0.6 nmol/L, respectively. Overall, our accuracy, intra- and inter-day precision, recovery results are close to those reported previously. Accuracy was between 91.6%-113.0% while intra- and inter-run precisions were determined as 1.5%-6.9% for two different concentrations of cortisol and dexamethasone. Furthermore, the calculated LOD and LOQ values, for total cortisol, free cortisol and dexamethasone demonstrated that our method indicated a higher sensitivity. According to the linearity results, we showed that our method was linear up to 1000 µg/L for total and free cortisol, and 400 µg/L for dexamethasone. In our study, calibration curves and linearity graphics for total and free cortisol were built separately unlike other studies. In this way, the sensitivity of plasma free cortisol measurement, which is found in lower concentration than total cortisol, was increased. We demonstrated the clinical applicability of our method for plasma samples obtained from adrenal adenoma patients. Moreover, in our previous study, we also conducted a larger sample-size (n=110) clinical trial with this validated method (14). Therefore, we have developed

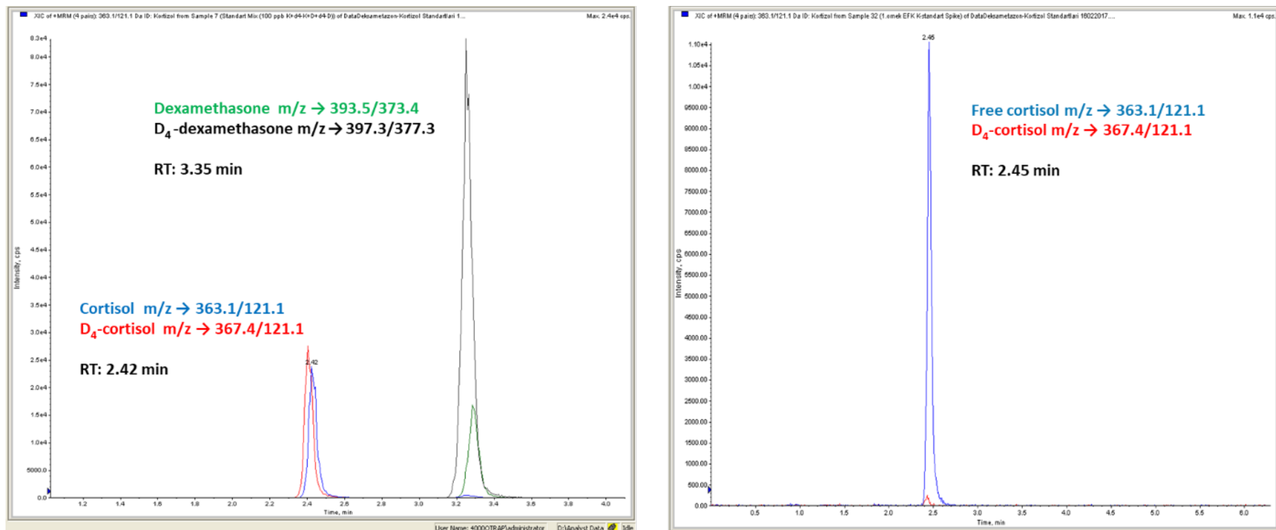

Figure 3. Ion chromatograms and retention times (RT) of dexamethasone, total and free cortisol

a selective and specific method for quantification of dexamethasone, total and free cortisol in plasma samples. The LC-MS/MS analysis time was 6.1 min. The relatively long analysis time compared to few studies performed is the limitation of this study (19).
Liquid chromatography-tandem mass spectrometry methods are now commonly used in most clinical laboratories for the simultaneous analysis of various compounds, including steroids, requiring very high sensitivity (12). Mass spectrometry can overcome some common diagnostic challenges, such as identification of exogenous corticosteroids or simultaneous assessment of appropriate dexamethasone levels in suppression tests (20). Also, LC-MS/MS has sufficient sensitivity in combination with ultrafiltration to measure plasma free cortisol accurately. The indirect method to estimate free cortisol concentration based on total cortisol and CBG in plasma is consider problematic when CBG binding reaches saturation or when there are changes in protein binding affinity (8). It is more accurate to use ultrafiltration coupled with LC-MS/MS to measure the concentration of unbound cortisol in plasma. Also, compared with equilibrium dialysis, ultrafiltration is simpler and faster to perform pre-extraction of free cortisol. Stable isotope labelled LC-MS/MS with MRM acquisition mode provides greater analytical specificity than immunoassays (IA). The Endocrine Society Clinical Practice Guidelines recommend LC-MS/MS as a reference method for the analysis of steroid hormones at particularly low concentrations (21). Cross-reactivity of IA methods with prednisolone, cortisone, and 11-deoxycortisol has been described and this non-selectivity of the IA may partly explain the overestimation of plasma cortisol at high levels (19). The majority of recent studies demonstrate major non-linearity between cortisol measurements by IA and MS and a lack of accuracy of different IA (22–24). But, the mass spectrometry technology is more complex than IA and required specialist skill. Therefore, if possible, research and clinical diagnostics of cortisol secretion should move toward the MS-based methodology.

CONCLUSION

In conclusion, we presented a SID-MRM LC-MS/MS based method with highly sensitive, high throughput, short run time and wide dynamic range for absolute quantification of dexamethasone, total and free cortisol. We believe that this method will be notably beneficial for diagnostic and prognostic purposes as well as follow-up of the treatment regimens.

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Author contribution: G.T., S.Y. and G.H.İ designed the study; S.Y. performed diagnostic interviews, clinical assessments; G.T., M.K. and M.A. performed sample storage and laboratory analyses. G.T., G.H.İ., M.K. and M.A. carried out statistical analysis. G.T. wrote the first draft. All authors contributed to the final manuscript.
Conflict of interests: The authors declare that they have no conflicts of interest.
Ethical approval: Ethical approval for this project was obtained from the Dokuz Eylul University Non-invasive Research Ethical Committee (Approval date: 18.06.2015, Decision number: 2015/16-28). All participants were signed the informed consent form.
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