Simultaneous quantitative analysis of salivary cortisol and cortisone in Korean adults using LC-MS/MS

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The levels of salivary cortisol and cortisone in Korean adults were measured for the first time using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The salivary cortisol and cortisone were separated within 10 min. The regression coefficients (*r*) of the calibration curves were greater than 0.999 for the two steroids. The limits of quantitation (LOQ) were 0.2 ng/ml for cortisol and 1 ng/ml for cortisone. The intra-day precisions of the assay were <3.9% and 8.6% for cortisol and cortisone respectively, and the inter-day precisions were <1.9% and 4.3% for cortisol and cortisone, respectively. The salivary cortisone concentrations were approximately 4-9 times higher than those of salivary cortisol during the daytime. Diurnal rhythms, during which the cortisol and cortisone concentrations were higher in the morning than in the afternoon, were also observed. The present assay may be useful for the diagnosis of several adrenal dysfunctions in clinical biochemistry. [BMB reports 2010; 43(7): 506-511]

INTRODUCTION

The assay of saliva specimens is an increasing area of research with implications for basic and clinical purposes and saliva analysis provides important information about the functioning of various organs within the body (1). Saliva can also be collected through a simple and noninvasive method without medical supervision. These features of saliva make it possible to monitor several biomarkers in humans.

Recently, salivary cortisol has received much attention as a biomarker of psychological stress and levels may be related to mental or physical diseases (2). Salivary cortisol and cortisone are steroid hormones synthesized from cholesterol by a multi-

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enzyme cascade in the adrenal glands. It is well known that 11β-hydroxysteroid dehydrogenase (11β-HSD) isoenzymes reversibly catalyze the interconversion between cortisol and cortisone. Endogenous cortisol is reversibly converted to its inactive metabolite, cortisone by 11β-HSD type 1 (3). On the contrary, 11β-HSD type 2 predominantly catalyzes the conversion of cortisol to cortisone mostly in mineralocorticoid target tissues (4-7).

The 11β-HSD type 2 and the cortisol-cortisone shuttle are of importance for the corticosteroid regulation of sodium homeostasis, the pathophysiology of hypertension and an inherited genetic disease caused by an enzyme defect (8).

Salivary cortisol and cortisone have been considered as useful targets in evaluation of mineralcorticoid or glucocorticoid excess, congenital adrenal hyperplasia (CAH) and adrenal insufficiency. Cortisol is the main glucocorticoid in humans and is increased in Cushing's syndrome or decreased in Addison's disease (9-11). Also, cortisol can be associated with cardiovascular disease (12) and anti-inflammatory and immunosuppressive effects (13, 14).

Evaluation of salivary free cortisol has been found to be a good indicator of unbound concentrations of cortisol in serum or plasma (15-20).

Thus, a highly sensitive and specific analytical tool is needed to determine the very low concentrations of cortisol and cortisone existing in saliva. Actually, the concentration of cortisol in saliva is about 10 times lower than that of cortisol in plasma or serum. Several quantitative methods have been developed for the measurement of salivary cortisol (17, 21-23). Among them, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been considered to provide a fast, highly sensitive and specific method for steroid quantification in clinical samples and can measure several steroids simultaneously. LC-MS/MS also provides the specificity needed to overcome interference by similar steroids. The salivary levels of cortisol and cortisone in an English population were recently reported using LC-MS/MS (19).

Although salivary cortisol and dehydroepiandrosterone (DHEA) levels in a Korean population were investigated using radioimmunoassay (RIA) (24), no study has been reported on the simultaneous determination of salivary cortisol and cortisone in

Korean adults by LC-MS/MS assay.

The purpose of the present study was to validate an LC-MS/MS assay method for measuring cortisol and cortisone in human saliva, measure salivary cortisol and cortisone levels simultaneously in Korean healthy adults using LC-MS/MS, and to confirm the diurnal rhythms of the two steroids in basal condition.

RESULTS AND DISCUSSION

LC-MS/MS characteristics
Salivary cortisol and cortisone were eluted within 10 min under an efficient gradient method used in this study. Cortisol, cortisone and cortisol-d_4 were fully separated in the chromatogram of an extracted saliva sample (Fig. 1A). The retention times were 8.42 min for cortisol, 8.25 min for cortisone and 8.39 min for cortisol-d_4, respectively. No ion suppression was observed.

Method performance
A simultaneous quantitative assay method for cortisol and cortisone in human saliva using LC-MS/MS was validated. The calibration curves were linear in the range of 0.2-25 ng/ml for cortisol (y = 0.13271x + 0.00643) (Fig. 1B) and 1.0-50 ng/ml for cortisone (y = 0.09931x − 0.01150) (Fig. 1C). The weighed (1/X) least-squares regression lines were greater than 0.999 for the two steroids, indicating excellent linearity. The limit of quantitation (LOQ) was 0.2 ng/ml (0.55 nmol/L) for cortisol and 1 ng/ml (2.77 nmol/L) for cortisone. Precision and accuracy of the present method were determined by analyzing three QC samples at three different concentrations for cortisol

and cortisone (Table 1). Intra-assay (n = 5) precisions (% CV) ranged from 0.9 to 3.9 for cortisol and 6.4 to 8.6 for cortisone, while accuracies (% bias) ranged from 98.2 to 100.4 for cortisol and 98.1 to 102.9 for cortisone. Inter-assay (n = 5) precisions ranged from 1.5 to 1.9 for cortisol and 3.8 to 4.3 for cortisone, while accuracies ranged from 96.2 to 104.0 for cortisol and 101.5 to 105.2 for cortisone. These results indicate that the precisions and the accuracies were within internationally accepted limits. The average recoveries of cortisol in triplicate were 81.1 ± 2.7% for 1.0 ng/ml spike, 102.5 ± 0.4% for 10.0 ng/ml spike and 99.9 ± 1.5% for 25.0 ng/ml spike. The average recoveries of cortisone, in triplicate, were 66.5 ± 5.4% for 5.0 ng/ml spike, 80.8 ± 1.3% for 20.0 ng/ml spike and 83.0 ± 0.4% for 50.0 ng/ml spike (Table 1).

Saliva sample measurements in healthy volunteers
The levels of salivary cortisol and cortisone were higher in the morning than in the afternoon (6:00-7:00, a.m. vs. 4:30-5:30, p.m., P < 0.0001 for cortisol and P < 0.0001 for cortisone). The average concentrations of cortisol and cortisone were 1.91 ± 0.72 ng/ml and 7.57 ± 1.84 ng/ml at 6:00-7:00, a.m., 0.67 ± 0.41 ng/ml and 4.68 ± 1.83 ng/ml at 1:00-2:00, p.m., and 0.30 ± 0.24 ng/ml and 2.80 ± 1.32 ng/ml at 4:30- 5:30, p.m., respectively (Table 2 and Fig. 2A). The concentrations of salivary cortisone were about 4 times higher than cortisol at 6:00-7:00, a.m., 7 times higher at 1:00-2:00, p.m. and 9 times higher at 4:30-5:30, p.m. During the daytime (6:00, a.m.-5:30, p.m.), typical diurnal rhythms of salivary cortisol and cortisone were observed, in agreement with previously reported results in healthy adults (19, 25, 26). The cortisol/cortisone ratio was also highest at 6:00-7:00, a.m. and then the ratio gradually de-
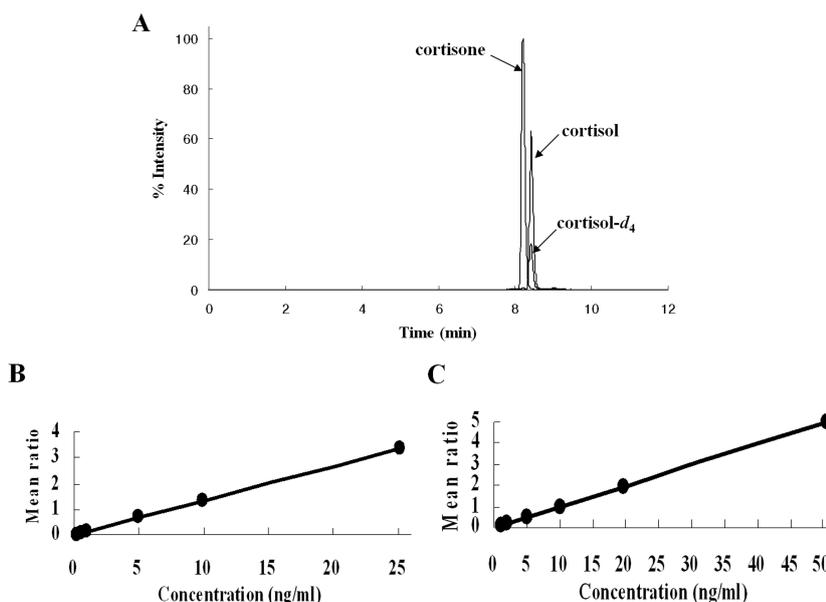

Fig. 1. (A) Multiple reaction monitoring (MRM) chromatogram of extracted saliva sample containing 25 ng/ml of cortisol and 50 ng/ml of cortisone. Calibration curves of (B) cortisol and (C) cortisone, constructed from steroid-free saliva. The ranges of the calibration curves were 0.2-25 ng/ml for cortisol (y = 0.13271x + 0.00643; r^2 = 0.99997) and 1.0-50 ng/ml for cortisone (y = 0.09931χ − 0.01150; r^2 = 0.99986), respectively.

creased (Table 2). In basal condition, the averaged ratios from different collection times showed a similar pattern to the ratios measured by LC-MS/MS in English adults (19). The concentrations of cortisol in saliva were also compared with those of cortisone (Fig. 2B). The curve showed a tendency towards a plateau at higher concentration levels, probably implicating the saturation of the 11β-HSD type 2 at high concetrations of the substrate (19). These results show the status of cortisol-cortisone shuttle in saliva during the daytime and also give evidence for the activity of 11β-HSD type 2, that catalyzes the irreversible conversion of hormonally active cortisol to inactive cortisone (19, 25).

In this study, we validated a fast, sensitive and robust LC-MS/MS assay for simultaneous measurement of cortisol and cortisone in saliva and evaluated the concentration levels of salivary cortisol and cortisone in Korean adults. Using the validated assay method, we also confirmed the concentration levels of cortisol and cortisone in saliva and the diurnal rhythms of the two steroids during the daytime. From all the data, it is suggested that the present assay method has sufficient sensitivity and reliability for the simultaneous quantitation of salivary cortisol and cortisone in clinical samples. Because accurate measurement of salivary cortisol and cortisone levels can give valuable information about 11β-HSD activity, the present analytical method has potential as a noninvasive tool in the study of the cortisol-cortisone shuttle. The present LC-MS/MS assay could also be an alternative method for the diagnosis of several glucocorticoid related disorders.

Table 2. Concentrations (ng/ml) of salivary cortisol and cortisone at different collection times

Collection time	Cortisol	Cortisone	Cortisol/ cortisone ratio
6:00-7:00, a.m.	1.91 ± 0.72	7.57 ± 1.84	0.25 ± 0.39
1:00-2:00, p.m.	0.67 ± 0.41	4.68 ± 1.83	0.14 ± 0.22
4:30-5:30, p.m.	0.30 ± 0.24	2.80 ± 1.32	0.10 ± 0.18

Table 1. Intra and inter-run accuracy, precision, limits of quantitation (LOQ) and recovery of the LC-MS/MS method for the simultaneous analysis of saliva cortisol and cortisone

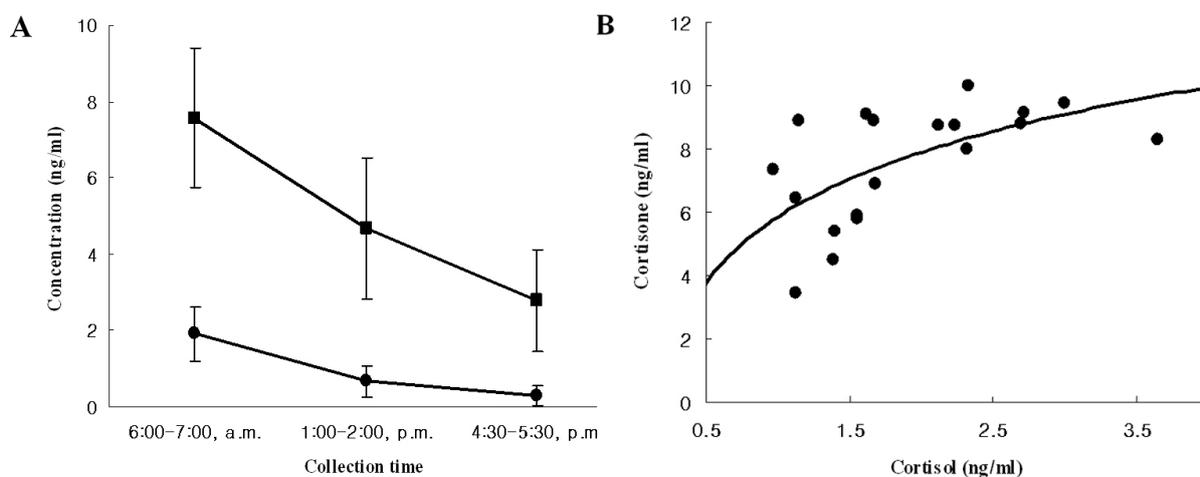|	Concentration (ng/ml)	Intra-assay (n = 5)		Inter-assay (n = 5)		Recovery ± SEM[a] (%)	LOQ (ng/ml)
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)		
Cortisol	1.0	98.2	3.9	104.0	1.9	81.1 ± 2.7	
	10.0	97.8	2.0	96.2	1.5	102.5 ± 0.4	0.2
	25.0	100.4	0.9	100.3	1.6	99.9 ± 1.5	
Cortisone	5.0	102.9	6.4	105.2	3.7	66.5 ± 5.4	
	20.0	101.6	8.6	101.5	3.8	80.8 ± 1.3	1.0
	50.0	98.1	7.7	104.1	4.3	83.0 ± 0.4	

[a]SEM: Standard error of mean

Fig. 2. (A) Diurnal patterns of salivary cortisol (●) and cortisone (■) in healthy subjects (n=22) at different collection times. (B) Comparison of the concentration levels between morning cortisol and cortisone in saliva.

MATERIALS AND METHODS

Chemicals

Cortisol and cortisone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated cortisol (cortisol-9,11,12,12-d_4, 98%) as an internal standard, was from Cambrideisotope Laboratories, Inc (MA, USA). All chemicals were of analytical-grade and all solvents, including methanol, acetonitrile and water were of HPLC-grade. All solvents were filtered through Advantec$^®$ membranes (pore size 0.45 μm, Toyo Roshi Kaisha, Ltd., Japan) before use.

LC-ESI-MS/MS

HPLC was performed using an Agilent 1200 series (Palo Alto, CA, USA) equipped with an auto-injector. All separations were achieved on a Shiseido MGII C18 (2.0 × 50 mm) with a 3 μm particle size from Shiseido, Tokyo, Japan. The initial mobile phase was composed of water and methanol (80 : 20, v/v). Cortisol and cortisone were separated by gradient elution at a flow rate of 0.2 ml/min. The initial mobile phase at time 0 min was gradually changed to 100% methanol for 3 min and the elution with 100% methanol was maintained at time 1 min. The gradient was then returned to the initial condition (water-methanol (80 : 20, v/v)) for 1 min and held for 7 min before injecting the next sample. Sample injection volume was 5 μl. Column temperature was maintained at 20°C. Mass spectra were obtained on a triple-quadruple mass spectrometer, API-4000^{TM} equipped with a TURBO V^{TM} source and a TurboIonSpray$^®$ probe (ABI/MDS Sciex, CA, USA) operated in positive ion mode. The following m/z multiple reaction monitoring (MRM) transitions were selected: $363.2 \rightarrow 121.2$ for cortisol, $361.2 \rightarrow 163.2$ for cortisone, and $367.1 \rightarrow 121.1$ for cortisol-d_4. For cortisol, the declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (EXP) were optimized to achieve the highest response for the respective signals as followings: 71 V, 10 V, 30 eV and 10 V. For cortisone, the parameters were optimized as followings: 111 V, 10 V, 33 eV and 30 eV, and for cortisol-d_4, the parameters were as followings: 79 V, 10 V, 33 eV and 6 V. Ionspray voltage (IS = 5500 V), temperature (TEM = 450°C), gas 1 (GS1 = 60), gas 2 (GS2 = 45), curtain gas (CUR = 20) and collision gas (CAD = 4) were optimized to maximize the sensitivity for the steroids. Peak areas of each steroid and the internal standard were obtained using Sciex Analyst 1.5 data processing software. The calibration curves were constructed using peak area ratios of each analyte to the internal standard of the calibration standards and applying a weighted (1/X) linear regression.

Sample collection and sample preparation

Saliva samples were obtained from twenty-two healthy volunteers, with informed consent (male = 6, female = 16, average age = 42.6 ± 8.1), who had not received any hormone supplementation. Samples were collected in commercial Salivette tubes containing a cotton swab with citric acid preparation (Sarstedt, Nümbrecht, Germany) and stored at −70°C until assay. The volunteers did not consume food or beverage within 1 h before the sample collection and did not brush their teeth within 1 h before the collection. To measure diurnal variation in salivary cortisol and cortisone excretion, all samples used in this study were collected at 6:30-7:30, 1:00-2:00 pm, and 4:30-5:30 pm, respectively. The saliva tubes were centrifuged at 4,000 rpm for 5 min. The supernatants were stored at −70°C until analysis. Stock solutions of cortisol and cortisone were prepared at concentrations of 100 μg/ml in 100% methanol and stored at −20°C. The stock solutions were used to spike calibration standards and quality control samples. Saliva pooled from healthy volunteers was stirred with 1 g of activated charcoal (Sigma-Aldrich, St. Louis, MO, USA) overnight and then centrifuged at 4,000 rpm for 15 min. The supernatant was used as the steroid-free saliva, in which cortisol and cortisone were not detected. Working calibrators were prepared by dilution of the stock solutions in the steroid-free saliva. Calibration standards and quality control samples were stored at −20°C until analysis.

Sample extraction

The saliva specimens were thawed at room temperature. A 100 μl aliquot of the internal standard solution, with a concentration of 100 ng/ml, was added to the saliva specimens (1,000 μl). The extraction was carried out using Oasis$^®$ HLB 1 cc solid phase extraction (SPE) columns (Waters, MA, USA). Each saliva sample (1,000 μl) spiked with 100 μl of the internal standard was applied to the SPE column, which was pre-equilibrated with 1 ml methanol followed by 1 ml water. After washing with 50% methanol, cortisol and cortisone were eluted with 100% methanol. The eluates were evaporated to dryness under a gentle nitrogen stream and then reconstituted with 100% acetonitrile. The analytes were injected into LC-MS/MS. The calibration standards and the quality control samples, spiked with 100 μl aliquot of the internal standard, were also extracted using the same method as above. The analytes were injected into LC-MS/MS.

Method validation

All validation steps were performed following the international guidelines (27). The linearities were assessed by the correlation coefficients (r^2) on the calibrators with six different concentrations ranging 0.2-25.0 ng/ml for cortisol and 1.0-50.0 ng/ml for cortisone. The limit of quantification (LOQ), with a signal-to-noise (S/N) ratio of 10, was defined as the lowest concentration on the calibration curve, with acceptable precision, in which relative standard deviation (R.S.D.) was <20%. Method precision (expressed as % coefficient of variation (CV)) and accuracy (expressed as % bias) were determined from QC samples at three different concentrations of the two steroids (1, 10 and 25 ng/ml for cortisol, and 5, 20 and 50 ng/ml for cortisone). The intra-assay accuracy and precision were evaluated

by analyzing the three QC samples 5 times on 1 day. The inter-assay accuracy and precision were evaluated by analyzing the samples over five different days. Recoveries (%) were calculated by dividing the measured concentrations by the spiked concentrations with three different concentrations for cortisol (1, 10 and 25 ng/ml) and cortisone (5, 20 and 50 ng/ml). Interference studies were performed by analyzing pooled saliva spiked with 10 ng/ml of corticosterone, 11-deoxycortisol, progesterone, testosterone, 17-hydroxyprogesterone, dihydrotestosterone, aldosterone and dehydroepiandrosterone.

Statistical analysis

Data processing and graphic presentation were performed with a Microsoft Office Excel 2002 (Microsoft Inc.). Diurnal variations in salivary cortisol and cortisone levels were evaluated statistically using the Student t-test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad software (QuickCalcs, La Jolla, CA).

Acknowledgements

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