Research Paper

Effects of 11β-hydroxysteroid dehydrogenase 2 activity on the prediction accuracy of plasma unbound cortisol concentration based on salivary cortisol

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Abstract Plasma unbound cortisol concentration is an accurate measure of its physiological activity. As unbound cortisol is excreted from plasma into saliva, it may be possible to non-invasively predict plasma unbound cortisol levels using salivary cortisol levels. However, part of the salivary cortisol is converted to cortisone, which can affect such predictions. Therefore, correlation between salivary cortisol and plasma unbound cortisol was analyzed in healthy subjects (n=9) and the effect of cortisol-to-cortisone conversion on this correlation was investigated. The correlation equation between salivary cortisol concentration (range, 0.26−3.22 ng/mL) and plasma unbound cortisol concentration (range, 0.31−2.74 ng/mL) for all subjects was determined to be y=0.5311x+0.5117 (r=0.51); however, it showed low correlation. Then, as individual differences in the slope of the correlation equation ranged from 0.62 to 2.62, we classified subjects into two groups based on a mean slope value of 1.10 and re-evaluated the correlation. The resulting equations yielded better correlations (y=2.0416x +0.0279, r=0.65, for two subjects and y=0.7417x+0.1195, r=0.77, for seven subjects), and the observed variation in slope was attributed to individual differences in salivary 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) activity. Importantly, mean absolute percentage error in predicting plasma cortisol using salivary cortisol levels using the two correlation equations was 0.6%. Thus, these results suggest that deriving and classifying salivary cortisone/cortisol ratio, along with relevant correlation equations, can be used to non-invasively predict plasma unbound cortisol concentration.

Key words: cortisol, saliva concentration, plasma unbound concentration, GC-MS, 11β-hydroxysteroid dehydrogenase 2

Introduction

Cortisol is the principal corticosteroid in humans and is secreted by the adrenal cortex. Its secretion is controlled by the adrenocorticotropic hormone (ACTH) and is known to exhibit circadian rhythm. As more than 90% of the cortisol is protein-bound in plasma, only less than 10% of the unbound cortisol crosses biological membranes and is bioactive[1,2]. Thus, while plasma unbound concentration of cortisol is an accurate measure of its physiological activity, its measurement is complicated as it involves multiple processes, including equilibrium dialysis, which limit its routine clinical application. Further, although physiological activity can be evaluated by predicting plasma unbound cortisol levels from a simple measurement of total plasma concentration, the relationship between the total and plasma unbound cortisol levels change due to fluctuations in plasma protein concentration and binding affinity. Hence, it has been pointed out that measuring only total concentration when evaluating adrenal function can lead to an errone-

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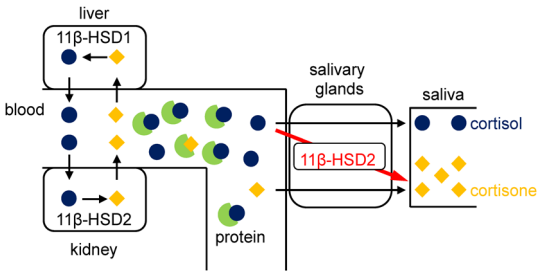

Fig. 1. Schematic representation of transfer of cortisol from blood to saliva. When cortisol is secreted from the plasma into the saliva, a part of it is converted to cortisone by 11*β*-HSD2, which results in a reverse phenomenon in which salivary cortisol is lower than plasma cortisol, but salivary cortisone is higher than plasma cortisone.

ous diagnoses and unnecessary administration of corticosteroids[3,4]. Furthermore, as adrenal function is assessed temporally, i.e., over time during treatment, a simple method for routinely evaluating adrenal function, based on plasma unbound cortisol levels, is useful.

Cortisol, like the unbound forms of many drugs and endogenous substances, is secreted into the saliva, and thus, it might be possible to predict plasma unbound cortisol concentration based on salivary levels when evaluating adrenal function[5,6]. Notably, when cortisol is secreted from plasma into saliva, part of it is converted to cortisone by 11*β*-hydroxysteroid dehydrogenase type 2 (11*β*-HSD2)[7,8], which leads to a reverse phenomenon wherein salivary cortisol is lower than plasma cortisol but salivary cortisone is higher than plasma cortisone (Fig. 1)[5]. Consequently, when predicting the plasma unbound cortisol concentration based on salivary levels, it is necessary to consider the concentration of both cortisol and cortisone in saliva. However, as no reports to date have investigated this, we investigated if plasma unbound cortisol levels can be predicted based on salivary cortisol concentration by developing a correlation equation between these two variables using data from healthy subjects and by exploring the effect of cortisol-to-cortisone conversion on such predictions.

Experimental

Chemicals and reagents

[1,2,4,19-$^{13}C_4$]cortisol (cortisol-$^{13}C_4$) and [1,2,4,19-$^{13}C_4$] cortisone (cortisone-$^{13}C_4$) were synthesized in our laboratory for use as internal standards[9] and the isotopic composition of the labeled compounds were determined to be greater than >98%. Cortisol, cortisone, and heptafluoro-*n*-butyric anhydride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical-reagent grade purchased from Kanto Chemicals (Tokyo, Japan).

Sample collection

Plasma and saliva were collected from nine healthy volunteer subjects (Subjects A–I, eight male and one female, age range 22–42 years). Plasma and saliva were collected for 3 days between 10:00 and 18:00 h randomly (6–12 times for each subject, total 81 times) according to the subject's schedule. The subjects did not smoke or take medication during the study period. Additionally, the subjects did not have oral diseases, such as periodontal disease. The study was approved by the Human Subjects Review Board of the Tokyo University of Pharmacy and Life Sciences, and written informed consent was obtained from all participants. Plasma and saliva samples were stored frozen at −20°C until analysis.

Measurement of plasma unbound cortisol concentration

A Nanosep 10K Omega Membrane (Nihon pall, Tokyo, Japan) was used for ultrafiltration. After adding 0.5 mL of distilled water to the device, centrifugation was performed at 2500 g for 10 minutes and this process of distilled water removal was repeated twice. Plasma samples were thawed and centrifuged at 800 g for 10 minutes, and 500 μL of plasma sample was added to the device and incubated at 37°C for 30 minutes. Unbound cortisol was filtered by centrifugation at 2500 g for 60 minutes in a centrifuge heated to 37°C.

Measurement of saliva cortisol and cortisone

Saliva samples were collected using Salimetrics oral swabs (SOS) and swab storage tubes (SST) purchased from Salimetrics (Carlsbad, CA, USA). The Salimetrics indicated that salivary cortisol concentrations measured with the collection devices are almost identical to passive drool concentrations (https://salimetrics.com/rigor-and-reproducibility-for-collecting-saliva-samples/). Saliva was collected 30 minutes after eating and 10 minutes after rinsing their mouth. Tooth brushing was prohibited for at least 2 hours before saliva collection. Saliva was collected by placing SOS under the tongue for 1–2 minutes (maximum 5 minutes) and asking the participants to suck in saliva. The swab was kept immobile to prevent blood from mixing with the

saliva. The SOS was removed from the mouth, inserted into the SST, and centrifuged at 800 g for 15 minutes. After centrifugation, both SOS and SST were discarded, and the collected saliva was immediately frozen and stored at −20°C until analysis. For the analysis of the stored saliva sample, the collected saliva sample was thawed at room temperature and centrifuged at 800 g for 15 minutes.

Extraction

We have previously described a method for measuring plasma and urinary cortisol and cortisone concentrations using GC−MS[10−12] and this method was used in this study for measuring saliva and plasma unbound cortisol. Cortisol-$^{13}C_4$ (13.60 ng) and cortisone-$^{13}C_4$ (31.45 ng) used for internal standards were added to the saliva sample (0.75 mL) or the plasma unbound ultrafiltration sample from the Nanosep 10K Omega Membrane. A Sep-Pak C18 Plus column (360 mg/0.7 mL, Waters, Milford, MA, USA) was first washed with 6 mL of ethyl acetate and 5 mL of distilled water. Next, the sample was injected, washed with 8 mL of distilled water, and eluted with 4 mL of ethyl acetate. The solvent was evaporated to dryness under a stream of nitrogen and the residue was derivatized.

Gas chromatography-mass spectrometry/selective ion monitoring (GC-MS/SIM)

Capillary GC-MS/SIM analysis was done on a Shimadzu QP2010 GC-MS. GC-MS used a SPB-1 fused-silica capillary column (15 m×0.25 mm i.d.) with the stationary phase coated to a film thickness of 0.25 μm (Supelco, Bellefonte, PA, USA). As cortisol and cortisol-$^{13}C_4$ were used as bis-methylenedioxy-heptafluoro-*n*-butyryl (BMD-HFB) derivatives, *m/z* 582 and 586 peaks (cortisol) were monitored and their peak area ratios were determined. Similarly, cortisone and cortisone-$^{13}C_4$ were used as BMD-HFB derivatives, *m/z* 598 and 602 peaks were monitored, and their peak area ratios were determined.

Data analysis

Correlation equation for predicting plasma unbound cortisol concentration from salivary cortisol levels was derived as a linear regression model with plasma unbound cortisol concentration on the *x*-axis and salivary cortisol on the *y*-axis. The correlation coefficient (*r*) was used as the index for evaluating the strength of the correlation. A linear regression model passing through the origin was derived for each subject, and the equations were used to compare individual slope values. Correlations were divided into two groups (high and low) based on the mean value of the slope (1.10). Mean absolute percentage error (MAPE) was used for calculating the prediction accuracy and it was estimated using an internal verification method that reapplies the data used for model creation.

Results

Measurement accuracy for salivary cortisol and cortisone, and plasma unbound cortisol, using GC-MS/SIM

The lower limit of detection (LOD) for cortisol and cortisone was 10.2 pg and 5.45 pg, respectively, when the *s/n* ratio was greater than 3. The limit of quantification (LOQ) was set at 0.41 ng for cortisol and 0.44 ng for cortisone, and the relative error (RE) and relative standard deviation (RSD) were calculated −1.41% and 0.32%, respectively, indicating that samples near the quantification limit could be measured with good accuracy. Calibration curves were constructed for cortisol from 0.406 to 10.16 ng/mL and for cortisone from 4.360 to 21.80 ng/mL, and linearity was obtained with a correlation coefficient of 0.999 or higher. For determining the accuracy of salivary cortisol and cortisone measurements, 12 pooled salivary samples (750 μL each) were prepared, and 6 of them were spiked with known concentrations of cortisol (0.81 ng) or cortisone (6.54 ng). The cortisol concentration in 750 μL of saliva sample was determined to be 1.95±0.10 ng while the cortisone concentration was 8.26±0.14 ng. Next, as the concentration of cortisol and cortisone in the six spiked samples was determined to be 2.62±0.06 ng/750 μL and 14.41±0.31 ng/750 μL, respectively, RE was calculated to be −4.91% and −2.67% for cortisol and cortisone, respectively, whereas the RSD was 1.7−4.9%.

For determining the accuracy of plasma unbound cortisol levels, plasma was filtered with an ultrafiltration device (Nanosep 10K Omega Membrane), and a known amount of cortisol was added to the filtrate. RE was calculated to be 1.58%, whereas RSD ranged from 2.8 to 3.0%.

Correlation between salivary cortisol and plasma unbound cortisol in nine healthy subjects

In the nine healthy subjects (Subjects A−I), salivary cortisol concentration ranged from 0.257 to 3.216 ng/mL, salivary cortisone ranged between 3.624 and 16.95 ng/mL, whereas

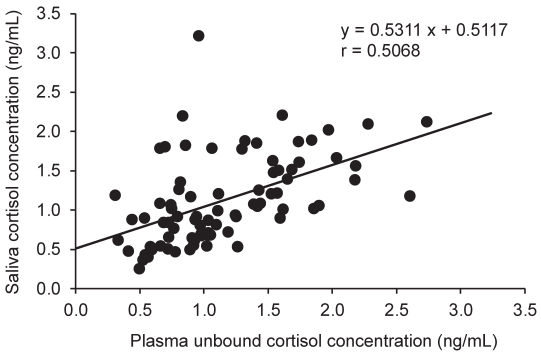

Fig. 2. Correlation between salivary and plasma unbound cortisol concentration in nine healthy subjects (Subjects A–I). The correlation equation between saliva concentration and plasma unbound concentration was determined y=0.5311x+0.5117, and the correlation coefficient was low at 0.5068.

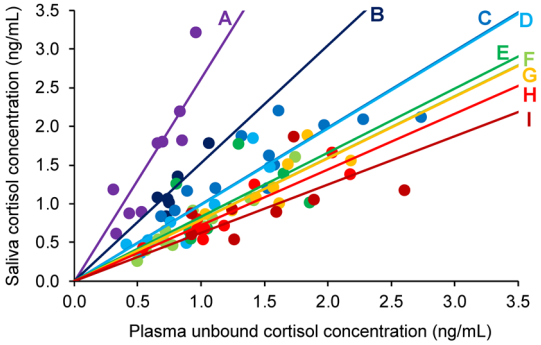

Fig. 3. Slope values for individual subjects for the correlation between salivary and plasma unbound cortisol levels in nine healthy subjects (Subjects A–I). Prediction equations were designed for each subject with the intercept set as 0, and the slope values ranged from 0.62 to 2.62.

plasma unbound cortisol concentrations were between 0.307 ng/mL and 2.737 ng/mL. The correlation equation between saliva concentration and plasma unbound concentration was determined to be y=0.5311x+0.5117; however, the correlation coefficient was low at 0.51 (Fig. 2).

Classification of correlation based on slope of the equation

Prediction equations were created for each subject with the intercept set as 0, and the slope values ranged from 0.62 to 2.62 (Fig. 3). The average slope value was 1.10, and subjects were divided into groups based on this value. The average of salivary cortisone/cortisol ratios of two subjects (Subjects A and B; greater slope value than the average) and of the other seven subjects (Subjects C–I; lesser slope value than the average) was 5.17 and 8.60, respectively (Table 1). Further, due to this difference in slope values, correlation equations for the two groups were derived again, and while that for the group including Subjects A and B was y=2.0416x+0.0279 (r=0.65), the same for the other group with Subjects C–I was y=0.7417x+0.1195 (r=0.77) (Fig. 4).

Table 1. Slope values for the correlation equation and salivary cortisone/cortisol ratio in nine healthy subjects (Subjects A–I). The average of salivary cortisone/cortisol ratios was 5.17 (Subjects A and B) and 8.60 (Subjects C–I), respectively.

Subject	Slope value	Salivary cortisone/cortisol ratio
A	2.622	5.203±1.479
B	1.524	5.132±0.961
C	0.994	5.688±0.847
D	0.987	10.325±2.855
E	0.828	8.490±1.509
F	0.798	9.473±2.028
G	0.795	7.669±1.848
H	0.720	7.996±1.897
I	0.624	10.546±2.236

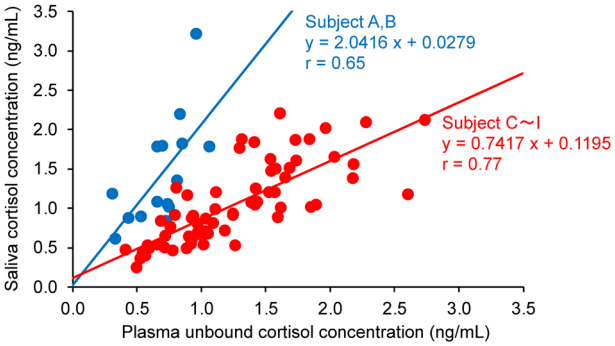

Fig. 4. Correlation lines based on which the study subjects were classified into two groups for predicting plasma unbound cortisol concentration from salivary cortisol concentration. The correlation equations for the two groups were derived, and while this equation for the group, including Subjects A and B was y=2.0416x+0.0279 (r=0.65), the same for the other group with Subjects C–I was y=0.7417x+0.1195 (r=0.77).

Improvement in prediction accuracy of the correlation after classification based on slope values

When plasma unbound concentration was predicted using the correlation equation before classification based on slope (y=0.5311x+0.5117), the MAPE value was −20.7%.

However, when the plasma unbound concentrations were predicted using correlation equations derived after classification (i.e., groups with two subjects and seven subjects, respectively) the MAPE value was found to be 0.6%.

Discussion

Salivary and plasma unbound concentrations of cortisol have been previously quantified by LC-MS[13,14] or immunoassays[3,15]. However, even though the GC-MS method has excellent sensitivity, specificity, and robustness, no reports have used this method to measure salivary or plasma unbound cortisol levels. We have previously established a method for measuring total plasma and urinary cortisol and cortisone concentrations by GC-MS[10–12], and we used this method to measure saliva and plasma unbound concentrations. As a result of validation, the sensitivity of LOD and LOQ was sufficient to measure saliva and plasma unbound concentrations. The calibration curve had a correlation coefficient of more than 0.999, indicating sufficient linearity for quantification. Importantly, the low RE and RSD values (RE −4.91% and −2.67%; RSD 1.7–4.9%) demonstrate that cortisol and cortisone in saliva can be quantified with high accuracy using the GC-MS method. Plasma samples were ultrafiltered using the Nanosep 10K Omega Membrane because this device can filter substances with a molecular weight (MW) of 10,000 or less; thus, cortisol bound to the plasma albumin (MW 66,000) and CBG (MW 52,000) is retained in the sample receiver and only unbound cortisol is filtered. Similar to saliva samples, accuracy of cortisol estimation in plasma after ultrafiltration was deemed as high as both RE (+1.58%) and RSD (2.8–3.0%) were low.

Values for salivary cortisol concentration (0.257–3.216 ng/mL), cortisone concentration (3.624–16.95 ng/mL), and plasma unbound cortisol (0.307–2.737 ng/mL) among the nine healthy subjects of this study are comparable to values reported for healthy subjects[13,14]. The correlation equation between salivary cortisol and plasma unbound cortisol concentration among nine healthy subjects (Subjects A–I; y=0.5311x+0.5117), derived using all measurement points, yielded a low correlation coefficient. Additionally, data from a few subjects did not fit the correlation equation, suggesting that predictions based on this equation would lead to overestimation in some subjects. Perogamvros et al. and Arafah et al. have evaluated the correlation between salivary and plasma unbound cortisol in 537 samples and 31 subjects, respectively, and report high correlation coefficients of 0.96 and 0.91, respectively[5,6]. The data in these studies were obtained from the samples of the ACTH supplementation test, and plasma levels of cortisol were higher after the ACTH supplementation test. Therefore, the correlation coefficient in these studies with a wide range of concentrations is considered good. However, Meulenberg et al. studied the correlation in pregnant women[16]. In these reports without ACTH supplementation test, the correlation between plasma unbound cortisol and salivary cortisol was y=0.432x+1.00 (r=0.5949) and y=0.324x+0.55 (r=0.6969) in the morning and evening, respectively, which is close to our results. In the present study, the concentration range was narrow only in healthy subjects without ACTH loading test, which might have reduced the correlation coefficient. Therefore, to investigate the cause of apparent non-conformity of data to the correlation equation, we created individual correlation equations for each subject using multiple sampling points. To identify subjects whose data did not fit the correlation line, we first evaluated the slope of the correlation equation for each subject by setting the intercept to 0 (as the concentration range was narrow) and found that slope values showed individual differences (Table 1). We interpreted the large slope values to indicate lower conversion of cortisol to cortisone when unbound cortisol was secreted from plasma to saliva, i.e., salivary cortisol tended to be higher. Notably, this difference in slope values also implies that the conversion of cortisol to cortisone affects the correlation between salivary and plasma unbound cortisol in individual subjects. Further, as the conversion of cortisol to cortisone is carried out by 11β-HSD2 present in the salivary glands, we hypothesized that the individual difference in 11β-HSD2 activity caused the observed non-conformity to the correlation equation.

To verify our hypothesis, we evaluated individual differences in 11β-HSD2 activity in the salivary glands by using the salivary cortisone/cortisol ratio as the readout for 11β-HSD2 activity[17,18]. In support of our hypothesis, the salivary cortisone/cortisol ratio was lower in the group with the larger slope values (5.17, Subjects A and B) than that for the other seven subjects (8.60, Subjects C–I, small slope values). The results of the t-test showed a significant difference between the two groups (t=−2.726, df=7, p=0.0295). These two groups can be classified as the low 11β-HSD2 and normal 11β-HSD2 activity groups. The group with low 11β-HSD2 activity may have decreased enzyme activity

due to genetic mutations or some subject-related background factors, such as disease, diet, etc. Moreover, it has been reported that some genetic mutations in 11β-HSD2 activity cause essential hypertension and that the intake of glycyrrhetinic acid causes pseudohyperaldosteronism[19]. We believe that investigating the factors related to decreased 11β-HSD2 activity in these subjects will be a challenge in the future. Based on these results, we hypothesized that the prediction accuracy could be improved by accounting for the difference in 11β-HSD2 activity, i.e., the salivary cortisone/cortisol ratio (around 5.5), and creating separate correlation equations for the two groups for predicting the plasma unbound cortisol concentration. Therefore, prediction correlation equations were re-created for two groups, i.e., for Subjects A and B with slope values greater than the mean value of 1.10, and for Subjects C−I, whose slope values were smaller. The resulting correlation equations had an intercept close to the origin and yielded higher "r" values of 0.65 and 0.77, respectively, implying a better correlation between the two variables. However, subject C had a salivary cortisone/cortisol ratio of 5.688, which was similar to that of Subjects A and B. Therefore, we calculated the correlation between Subjects A−C. The correlation line was y=0.5217x+0.9468 (r=0.52). The slope and intercept were close to the correlation line from Subjects A−I, and the correlation did not improve. The fact that some subjects, such as subject C, had a large slope and a small salivary cortisone/cortisol ratio suggests that further studies with more subjects are needed for grouping using the salivary cortisone/cortisol ratio.

The relationship between salivary cortisone and plasma unbound cortisone was also investigated. The correlation line of all subjects was y=2.9167x+3.661 (r=0.55), showing the same degree of linearity as that of cortisol in all subjects. Since a part of salivary cortisone is produced from cortisol by 11β-HSD2, the relationship between the correlation slope and salivary cortisone/cortisol ratio was also investigated, but no relationship was found. Some reports have suggested that salivary cortisone is a better biomarker of the adrenal cortex than salivary cortisol[5,7]. Possibly, the salivary concentration of cortisone (3.624−16.95 ng/mL) is more than that of cortisol (0.257−3.216 ng/mL) and may be less susceptible to the conversion of cortisol-to-cortisone by 11β-HSD2.

Finally, we used an internal verification method to examine the accuracy of the correlation equation that predicted plasma concentration based on salivary concentration of cortisol and found that predictability had improved when two correlation equations, corresponding to two sets of subjects, were used because the MAPE value recovered from −20.7 to 0.6%.

In conclusion, the results of this study show that it is necessary to consider individual differences in 11β-HSD2 activity present in the salivary glands while predicting plasma unbound cortisol levels using salivary cortisol. Further, classifying subjects based on the salivary cortisone/cortisol ratio and using a correlation equation can result in more accurate predictions. However, while it is conceivable to divide the correlation line when the salivary cortisone/cortisol ratio is approximately 5.5, further studies are needed to classify 11β-HSD2 activity based on the salivary cortisone/cortisol ratio. Undoubtedly, studies with larger sample size that use external evaluation methods are needed to improve prediction accuracy based on correlation equations.

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Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1) Ionita IA, Akhlaghi F: Quantification of unbound prednisolone, prednisone, cortisol and cortisone in human plasma by ultrafiltration and direct injection into liquid chromatrography tandem mass spectrometry. *Ann Clin Biochem* 47: 350−357, 2010.
2) Vogeser M, Möhnle P, Briegel J: Free serum cortisol: quantification applying equilibrium dialysis or ultrafiltration and an automated immunoassay system. *Clin Chem Lab* 45: 521−525, 2007.
3) Duplessis C, Rascona D, Cullum M, Yeung E: Salivary and free serum cortisol evaluation. *Mil Med* 175: 340−346, 2010.
4) Hamrahian AH, Oseni TS, Arafah BM: Measurements of serum free cortisol in critically ill patients. *N Engl J Med* 350: 1629−1638, 2004.

5) Perogamvros I, Keevil BG, Ray DW, Trainer PJ: Salivary cortisone is a potential biomarker for serum free cortisol. *J Clin Endocrinol Metab* 95: 4951−4958, 2010.
6) Arafah BM, Nishiyama FJ, Tlaygeh H, Hejal R: Measurement of salivary cortisol concentration in the assessment of adrenal function in critically ill subjects: A surrogate marker of the circulating free cortisol. *J Clin Endocrinol Metab* 92: 2965−2971, 2007.
7) Raff H, Findling JW: Biomarkers: Salivary cortisol or cortisone? *Nat Rev Endocrinol* 6: 658−660, 2010.
8) Antonelli G, Ceccato F, Artusi C, Marinova M, Plebani M: Salivary cortisol and cortisone by LC-MS/MS: Validation, reference intervals and diagnostic accuracy in Cushing's syndrome *Clin Chim Acta* 451: 247−251, 2015.
9) Furuta T, Eguchi N, Yokokawa A, Shibasaki H, Kasuya Y: Synthesis of multi-labeled cortisols and cortisones with ^{2}H and ^{13}C for study of cortisol metabolism in humans. *Steroids* 65: 180−189, 2000.
10) Yokokawa A, Takasaka T, Shibasaki H, Kasuya Y: Kawashima S, et al: The effect of water loading on the urinary ratio of cortisone to cortisol in healthy subjects and a new approach to the evaluation of the ratio as an index for in vivo human 11β-hydroxysteroid dehydrogenase 2 activity. *Steroids* 77: 1291−1297, 2012.
11) Furuta T, Eguchi N, Shibasaki H, Kasuya Y: Simultaneous determination of endogenous and ^{13}C-labelled cortisols and cortisones in human plasma by stable isotope dilution mass spectrometry. *J Chromatogr B Biomed Sci Appl* 738: 119−127, 2000.
12) Shibasaki H, Furuta T, Kasuya Y: Stable isotope dilution mass spectrometry for the simultaneous determination of cortisol, cortisone, prednisolone and prednisone in plasma. *J Chromatogr* 579: 193−202, 1992.
13) Perogamvros I, Owen LJ, Newell-Price J, Ray DW, Trainer PJ, et al: Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography-tandem mass spectrometry: application in basal and stimulated conditions. *J Chromatogr B Analyt Technol Biomed Life Sci* 877: 3771−3775, 2009.
14) Pretorius CJ, Galligan JP, McWhinney BC, Briscoe SE, Ungerer JP: Free cortisol method comparison: Ultrafiltration, equilibrium dialysis, tracer dilution, tandem mass spectrometry and calculated free cortisol. *Clin Chim Acta* 412: 1043−1047, 2011.
15) MacMahon W, Thompson J, Bowers W, Sgoutas, D: A simplified ultrafiltration method for determination of serum free cortisol. *Clin Chim Acta* 131: 171−184, 1983.
16) Meulenberg PM, Hofman JA: Differences between concentrations of salivary cortisol and cortisone and of free cortisol and cortisone in plasma during pregnancy and postpartum. *Clin Chem* 36: 70−75, 1990.
17) Kargl C, Arshad M, Salman F, Schurman RC, Del Corral P: 11β-hydroxysteroid dehydrogenase type-II activity is affected by grapefruit juice and intense muscular work, *Arch Endocrinol Metab* 61: 556−561, 2017.
18) Wilson M, Thayer Z: Maternal salivary cortisone to cortisol ratio in late pregnancy: An improved method for predicting offspring birth weight. *Psychoneuroendocrinology* 78: 10−13, 2017.
19) Mariniello B, Ronconi V, Sardu C, Pagliericcio A, Galletti F, et al: Analysis of the 11beta-hydroxysteroid dehydrogenase type 2 gene (HSD11B2) in human essential hypertension. *Am J Hypertens* 18: 1091−1098, 2005.