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Comparison of Electrochemiluminescence and Enzyme Immunoassay Methods for the Measurement of Salivary Cortisol

Tükrük Kortizolü Ölçümünde Elektrokemilüminesan ve Enzimimmünoassay Yöntemlerinin Karşılaştırılması

Gülşah Elbüken, Kader Köse*, Züleyha Karaca, Fatih Tanrıverdi, Kürşad Ünlühizarcı, Gökmen Zararsız**, Fahrettin Keleştimur

Erciyes University Faculty of Medicine, Department of Endocrinology and Metabolism, Kayseri, Turkey
*Erciyes University Faculty of Medicine, Department of Biochemistry, Kayseri, Turkey
**Erciyes University Faculty of Medicine, Department of Biostatistics, Kayseri, Turkey

Abstract

Purpose: The aim of the study was to compare electrochemiluminescence (ECL) and enzyme immunoassay (EI) methods for the measurement of salivary cortisol (SC).
Material and Method: SC levels in 20 healthy subjects were measured by two different methods (EI and ECL).
Results: The results obtained from EI and ECL methods were found to be significantly correlated.
Discussion: Measuring SC by ECL method is faster and easier than EI in routine practice. Therefore, SC measured by ECL rather than EI may be used as a marker of hypothalamic-pituitary-adrenal (HPA) axis function. *Turk Jem 2014; 18: 111-115*
Key words: Salivary cortisol, enzymeimmunoassay, electrochemiluminescence, healthy subjects, HPA axis

Özet

Amaç: Bu çalışmanın amacı tükürük kortizolü (TK) ölçümünde kullanılan elektrokemilüminesan (EKL) ve enzimimmünoassay (Eİ) yöntemlerini birbirleriyle karşılaştırmaktır.
Gereç ve Yöntem: Sağlıklı 20 gönüllüden elde edilen tükürük örneklerinden iki farklı yöntemle (EKL ve Eİ) tükürük kortizolü ölçümü yapıldı.
Bulgular: EKL ve Eİ yöntemleri birbiriyle anlamlı şekilde korele bulundu.
Tartışma: Rutin pratik uygulamada tükürük kortizolünün EKL yöntemiyle ölçülmesi hızlı ve kolay bir yöntemdir. Bu yöntemle ölçülen sonuçlar Eİ yöntemiyle iyi korele bulunmuştur. Bu sebeple EKL yöntemi ile tükürük kortizolü ölçümü, hipotalamus-hipofiz-adrenal (HHA) aks fonksiyonlarının ölçümünde Eİ yerine kullanılabilecek bir yöntemdir. *Turk Jem 2014; 18: 111-115*
Anahtar kelimeler: Tükürük kortizolü, enzim immünoassay, elektrokemilüminesan, sağlıklı kişiler, HHA aks

Introduction

Serum cortisol measured by conventional methods consists of both free and protein-bound forms. Since more than 90 percent of circulating cortisol in serum is protein-bound, any changes in binding proteins can easily alter measured serum total cortisol levels. Salivary cortisol (SC) is also found in free form and this directly diffuses along the capillaries to target tissues. Sampling of saliva is easy and painless. SC, a surrogate biomarker of serum free cortisol (FC), has been increasingly used in scientific studies (1,2,3).

Enzyme-linked immunosorbent assay (ELISA), or enzyme immunoassay (EI), has been frequently used in many studies for the determination of SC levels (3,4,5). There are various other methods for measuring SC, such as assay of Porter-Silber chromogens, competitive protein-binding assay, fluorometric assay, radioreceptor assay, radioimmunoassay (RIA), and other immunoassays (fluorescent, chemiluminescent), and structure-based assays, such as high-performance liquid chromatography (HPLC) and mass spectrometry (5,6,7,8,9,10,11,12,13,14). The disadvantages of EI include its being time-consuming, the cost of the kit, risk of contamination via staff, and need for staff in every step of the method. Therefore, an electrochemiluminescence (ECL) method using a routine automated immunoassay analyser was introduced to measure SC levels. It has been reported that it provides superior analytical performance even in very low concentration of analyte and a shorter turnaround time (15). Although this method seems to be faster and as convenient as EI method, there are not enough studies comparing these two methods.

Address for Correspondence: Gülşah Elbüken MD, Erciyes University Faculty of Medicine, Department of Endocrinology and Metabolism, Kayseri, Turkey
Phone: +90 352 437 49 01 E-mail: gelbuken@erciyes.edu.tr **Received:** 14/04/2014 **Accepted:** 26/08/2014

This study was designed to compare ECL and EI methods in the determination of SC levels. And the relationship between these two tests and calculated FC levels was also determined.

Materials and Methods

Method

Twenty healthy volunteers (10 males, 10 females) with a mean age of 37.75±14.23 years (range: 21-61 years) were recruited to the study. The study was approved by the local ethics committee and informed consent was obtained from all volunteers. Exclusion criteria were presence of any disease that can affect hypothalamic-pituitary-adrenal (HPA) axis.

The tests were performed between 08.00-09.00 am, after an overnight fast. Fasting blood glucose levels, serum levels of creatinine and transaminases, and basal hormone levels were measured in all volunteers and were found to be within normal ranges (data not shown).

All participants were evaluated with low (1 µg) and standard (250 µg) dose adrenocorticotropic hormone (ACTH) stimulation tests. The 1 µg ACTH stimulation test was performed on the first day of the study, and the 250 µg ACTH stimulation test was performed on the second day. There was a time interval of 24 hours between the 1 µg and 250 µg ACTH stimulation tests. For a standard dose ACTH stimulation test, 0.25 mg intravenous synthetic ACTH, tetracosactrin, (Synacthène; Defiante Farmacêutica S.A., Funchal, Portugal) was used. The 0.25 mg tetracosactrin was injected into a 250 ml 0.9% NaCl solution, and preserved at +4 °C for not more than 4 months. For the 1 µg ACTH stimulation test, 1 ml of this mixed solution was used. On the first day, blood and saliva samples were obtained before and 30 and 60 minutes after 1 µg ACTH injection with the patient in a supine position. On the second day of the study, blood and saliva samples were obtained before and 30, 60, and 90 minutes after 250 µg ACTH injection. Both stimulation tests were completed succesfully in all participants. No adverse events were reported during both doses of the ACTH stimulation tests. The samples were centrifuged, aliquoted and stored at -80 °C until analysis. A basal and stimulated concentration of TC, cortisol binding globulin (CBG), and SC were measured; then free cortisol index (FCI), and calculated free cortisol (cFC) were calculated, by using TC and CBG, respectively.

Salivary Samples

Within sixty minutes before the test, individuals were not allowed to smoke, eat, drink liquids or brush their teeth. Saliva samples were collected using oral swabs (Salimetrics oral swab; Salimetrics Europe Ltd., Suffolk, United Kingdom) made of a non-toxic, inert polymer shaped into a 30x10 mm cylinder leading to a passive droll of saliva. It also helps to filter mucus and other matter from the sample. The participants placed the oral swabs under the front of the tongue and waited for 1 or 2 minutes until they are saturated completely. After removal of the moisturised swab, it was kept in a swab storage tube measuring 17x100 mm (Salimetrics saliva stogare tube; Salimetrics Europe Ltd., Suffolk, United Kingdom), which consists of a capped, conical polypropylene centrifuge tube with a separate insert that allows saliva to be centrifuged into the bottom of the conical tube. When they were still at room temperature, the tubes were vortexed, and then were immediately centrifuged for 15 minutes at approximately 3.000 RPM. After centrifugation, the swab and small insert were thrown away, and the large outer tube was stored at -80 °C until analysis.

On the day of measurement, the samples were brought to room temperature. Assays were performed using only clear saliva,

Table 1. The median hormone levels (min and max), obtained in the test minutes of 1 µg adrenocorticotropic hormone (ACTH) stimulation test

1 µg ACTH stimulation test					
Minutes	**0**	**30**	**60**	**Peak**	**p1 value**
SC -EI (µg/dL)	0.40 (0.15-1.43)	1.43 (0.65-2.51)	0.89 (0.46-2.06)	1.43 (0.65-2.51)	<0.001
SC-ECL (µg/dL)	0.24 (0.08-1.51)	1.13 (0.54-3.26)	0.77 (0.31-3.05)	1.13 (0.54-3.26)	<0.001
cFC (µg/dL)	0.50 (0.14-3.11)	1.11 (0.35-3.27)	0.62 (0.16-7.26)	1.32 (0.43-7.26)	<0.001
p2 value	0.086	0.350	0.086	0.705	

SC-EI: Salivary cortisol measured by enzyme immunoassay, SC-ECL: Salivary cortisol measured by electrochemiluminescence, cFC: Calculated free cortisol, p1: p value between basal (min 0) and peak hormone levels, p2: p value between 3 different methods.

Table 2. The median (min and max) hormone levels, obtained in the test minutes of 250 µg adrenocorticotropic hormone (ACTH) stimulation test

250 µg ACTH stimulation test						
Minutes	**0**	**30**	**60**	**90**	**Peak**	**p1 value**
SC -EI (µg/dL)	0.46 (0.13-2.75)	1.44 (0.87-2.29)	2.00 (1.43-3.58)	2.35 (1.33-5.20)	2.35(1.43-5.20)	<0.001
SC-ECL (µg/dL)	0.23 (0.08- 1.02)	1.19 (0.75-2.82)	2.00 (1.11-5.10)	2.38 (1.29-6.36)	2.38 (1.29-6.36)	<0.001
cFC (µg/dL)	0.28 (0.13-3.1)	1.03 (0.50-2.41)	1.35 (0.39-7.63)	2.10 (0.50-7.90)	2.93 (0.92-7.90)	<0.001
p2 value	0.096	0.819	0.165	0.486	0.350	

SC-EI: Salivary cortisol measured by EI, SC-ECL: Salivary cortisol measured by ECL, cFC: Calculated free cortisol, p1: p value between basal (min0) and peak hormone levels, p2: p value between 3 different methods.

avoiding any sediment present in the bottom of the tube. We divided saliva samples into 2 sets: the first set was for EI and the other set was for ECL. Salivary cortisol was measured by using a high-sensitivity EI kit (Salimetrics; Inc, State College, PA, USA), according to the manufacturer's instructions. The lower limit of detection for the assay was 0.06 µg/dL. Samples exceeding 3 µg/dL, the upper limit of the standard curve, were re-analysed after dilution. The intra and interassay coefficients (CVs) were both <6%. The second part of salivary samples was used to measure SC levels by using an Elecsys Cortisol assay in the same way as for serum or plasma specimens. Two hundred millilitres of salivary samples were transferred to an Elecsys sample cup and measured by a "cobas e 601" an analyser (Roche Diagnostics GmbH; Mannheim, Germany). The reference values for salivary cortisol were as follows: morning hours 8-10 am: <19.1 nmol/L (<0.69 µg/dL), afternoon hours 2:30-3:30 pm: <11.9 nmol/L (<0.43 µg/dL).

Table 3. Median basal and peak hormone levels of 1 and 250 µg adrenocorticotropic hormone (ACTH) stimulation tests

		Basal	Peak
SC -EI (µg/dL)	1 µg ACTH stimulation test	0.40 (0.15-1.43)	1.43 (0.65-2.51)
	250 µg ACTH stimulation test	0.46 (0.13-2.75)	2.35 (1.43-5.20)
	p value	0.765	< 0.001
SC-ECL (µg/dL)	1 µg ACTH stimulation test	0.24 (0.08-1.51)	1.13 (0.54-3.26)
	250 µg ACTH stimulation test	0.23 (0.08- 1.02)	2.38 (1.29-6.36)
	p value	0.455	< 0.001
cFC (µg/dL)	1 µg ACTH stimulation test	0.50 (0.14-3.11)	1.32 (0.43-7.26)
	250 µg ACTH stimulation test	0.28 (0.13-3.1)	2.93 (0.92-7.90)
	p value	0.313	< 0.001

SC-EI: Salivary cortisol measured by EI, SC-ECL: Salivary cortisol measured by ECL, cFC: Calculated free cortisol.

Serum TC

For each sample, 2 ml of blood was collected via venous catheter. Sera were stored at -80 °C until analysed. Serum TC levels were measured by RIA method (Immunotech; Prag, Czech Republic) with an intraassay coefficient of variation: 5.1%, interassay coefficient of variation: 9.2% and sensitivity of 10 nmol/L. To convert nmol/L to ng/mL and µg/dL, the result was multiplied by 0.362 and 0.1, respectively. Serum CBG concentrations were analysed with radioimmunoassay (BioSource Europe S.A.; Nivelles, Belgium). Intraassay and interassay coefficients of variation were 3.9% and 5.5%, respectively. To convert to µg/dl from µg/mL, all results were divided by 100.

The FCI was calculated by dividing serum TC by the CBG.

The Coolens method was used to calculate cFC concentration: U2K (1+N)+U [1+N+K (G-T)] -T=0, where K= 3×10-7 M-1 (affinity of CBG) to cortisol at 37 °C, G=CBG, U=unbound cortisol, T=cortisol, and N=ratio of albumin bound to free cortisol (1.74). T levels were divided to 52 for converting to µM from µg/mL. And also C levels were divided to 36.2 converting to µg/dL from µM. U was calculated as follows:

$$U=\sqrt{Z^2+\frac{T}{(1+N)\,K}}-Z$$

where Z=0.0167+ 0.182 (CBG-TC) (16). The unbound (cFC) was obtained as µg/dL by multiplying with 36.2.

Peak TC, FCI and cFC were determined as the maximum achieved during the testing.

Table 4. Summary of data regarding salivary cortisol levels in healthy subjects

Reference number	Number of subjects	Method of measurement	Time of measurement		Salivary cortisol levels	
					nmol/L	µg/dL
20	83	ECL	Morning 10.48±0.05		9.38±0.05	0.34±0.002
		ELISA			0.38±0.002	
5	197	RIA	08.00 am		3.5-27.0	0.13-0.98
			10.00 pm		<6.0	<0.22
22	58	ECL	Morning		13.4±3.2	0.49±0.12
			Late night		3.55±0.94	0.13±0.03
23	20 male, 20 female	RIA	08.00 am	Male	8.3±5.0	0.30±0.18
				Female	9.5±4	0.34±0.15
				All	8.7±4.8	0.32±0.17
3	31	EI (Salimetrics®)	Morning		5.24±3.0	0.19±0.11
			After 250 µg ACTH		41.12±20.42	1.49±0.74

ECL: Electrochemiluminescence, ELISA: Enzyme-linked immunosorbent assay, RIA: Radioimmunoassay, EI: Enzyme immunoassay, ACTH: Adrenocorticotropic hormone.

Statistical Analysis

All statistical analyses were done by the Statistical Package for Social Sciences (SPSS, version 15 for Windows; Chicago, IL, USA).

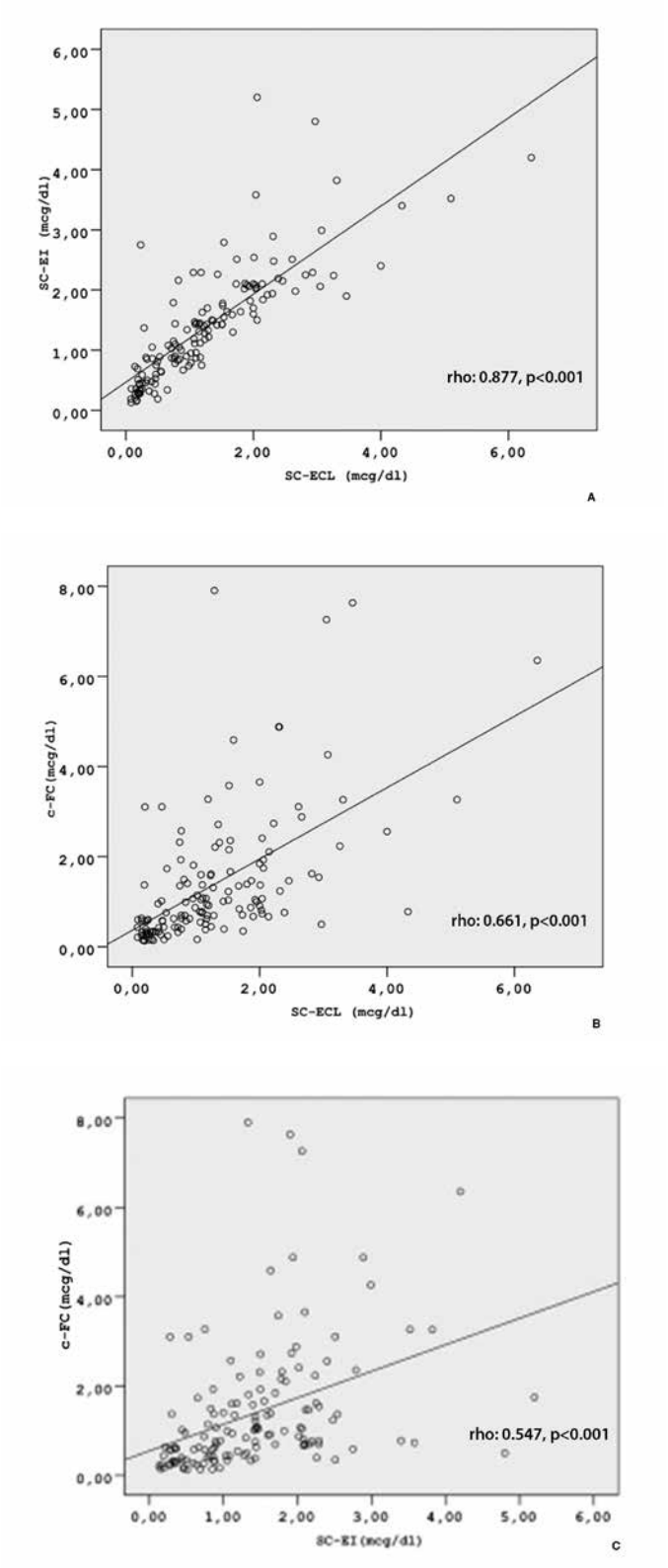

Figure 1. Correlations of SC-EI and SC-ECL (1a), c-FC and SC-ECL (1b), c-FC and SC-EI (1c)

To determine whether the distribution of data was normal, the Shapiro-Wilk test was used. Since the data were not distributed homogenously, statistical analysis was done by nonparametric tests. The results are presented as median, minimum and maximum levels. Comparisons between the groups of data were evaluated by use of Friedman's nonparametric ANOVA and the Wilcoxon signed-ranks tests. The relationship between continuous variables was tested by Spearman's rho correlation analysis. Statistical significance was set at a p-value of less than 0.05.

Results

In 20 healthy volunteers (10 men, and 10 women), the mean age was 37.75±14.23 years (range: 21-61 years). The mean age in the male and female groups were 43.80±14.77 (range: min:26, max:61) and 31.70±11.31 (range: min:21, max:59) (p=0.055), respectively.

Peak hormone responses obtained during the 1 and 250 µg ACTH stimulation tests were found to be higher than basal levels by SC-EI, SC-ECL and cFC methods (Tables 1, 2). Median basal and peak levels of SC-EI, SC-ECL and cFC during 1 µg and 250 µg ACTH stimulation tests are presented in Tables 1 and 2. Each method revealed similar simultaneous hormone levels in the test minutes of both doses of ACTH stimulation tests (Tables 1, 2). In both doses of ACTH stimulation tests, all peak hormone levels were found to be higher than their basal levels (p1<0.001, Tables 1, 2). Neither SC-EI and SC-ECL levels which were obtained by the same salivary samples, nor cFC levels which were calculated by Coolens' equation were found to be different during both doses of ACTH stimulation tests (p2>0.05, Tables 1, 2). Although there was no difference between the basal hormone levels of 1 and 250 µg ACTH stimulation tests, peak hormone levels during 250 µg ACTH stimulation test were found to be significantly higher than in 1 µg ACTH stimulation test (p<0.001, Table 3).

When we pooled all the salivary and blood samples of the subjects into 3 methods, 140 samples were obtained for each method. The relationship between the methods was tested by Spearman's rho correlation analysis. There were significant positive correlations between EI, ECL and cFC levels. Rho and p values between SC-EI and SC-ECL; cFC and SC-ECL; and cFC and SC-EI were determined as follows: rho: 0.877, p<0.001; rho: 0.661, p<0.001; and rho: 0.547, p<0.001, respectively (Figure 1a, 1b, 1c).

Discussion

Serum cortisol free fraction is responsible for its physiological function. Serum FC diffuses easily into saliva. Therefore, measurement of SC may reflect serum FC more accurately than serum TC (1,2,3). Most previous clinical studies have used EI and RIA for the measurement of SC levels (3,4,5,6). Recently ECL has been proposed as a new method for measuring SC (15). In several clinical studies, SC levels obtained by ECL were found to be highly correlated with ELISA (17,18,19,20). However, there are not enough data to compare these two methods. Therefore, we aimed to compare ECL and EI methods in the same salivary samples. We also aimed to compare these two methods by their cFC levels determined simultaneously during the test minutes of both doses of ACTH stimulation tests.

In the present study, after both doses of ACTH stimulation tests, all median peak hormone levels were found to be higher than their basal levels. In addition, their peak hormone responses after 250 µg ACTH stimulation tests were significantly higher than 1 µg ACTH stimulation. It shows that HPA axis was stimulated enough after both doses of ACTH and all these hormone results were compatible with the current data (21).

Some studies including SC levels, which were collected from healthy subjects by different methods, are summarized in Table 4. Both basal and stimulated SC levels found in the present study are similar to the current data.

When we analysed SC and cFC levels in 20 healthy volunteers, SC-EI and SC-ECL levels were found to be positively correlated. Previously, it was found that SC measured by ELISA did not differ from that measured by ECL, and there was a positive correlation between SC values measured by both assays (20).

In another study by Aardal et al., SC levels (the central 95%), which were collected from 197 healthy volunteers, were estimated as 0.13-0.98 µg/dL at 8.00 am and <0.22 µg/dL at 10.00 pm (5). Although they have measured SC by RIA method (different method from ours), two SC measurements were very similar. Thus, it strengthens the hypothesis that choosing one of these methods, which gives faster result, does not affect the test results.

In a study by Arafah et al., SC levels were measured by Salimetrics® EI kit which was also used in the present study. FC was measured directly and basal and stimulated levels were found to be 0.76±0.40 and 3.29±1.51 µg/dL, respectively (3). In the present study, we carried out 1 µg ACTH stimulation test in addition to 250 µg ACTH stimulation test. Although we have obtained cFC results by an indirect method, the results were also compatible with Arafah's measured FC results. Therefore, it also shows that cFC method is being a surrogate marker for the determination of FC levels. Furthermore, cFC and SC levels were found to be well-correlated supporting the idea that SC levels indirectly reflect FC by cFC.

The ECL technique has several advantages over EI: it is widely available, cheaper, and easy to use. In addition, it has a rapid turnaround time in a large number of samples, requires small volumes of saliva, and has high analytical accuracy (15). Because of its practical usage and providing faster results, we could obtain SC levels easily. Therefore, due to the lack of staff-originated errors, the ECL method seems to be better for biological or clinical studies.

In conclusion, in the present study, SC was found to reflect cFC. Additionally, ECL method was highly correlated with EI method in the measurement of SC. Therefore, ECL may be considered as an alternative and practical method to determine SC levels in routine clinical practice.

Conflicts of Interest

There are no conflicts of interest.

References

1. Dunn JF, Nisula BC, Rodbard D. Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid binding globulin in human plasma. J Clin Endocrinol Metab 1981;53:58-68.
2. Brien TG. Human corticosteroid binding globulin. Clin Endocrinol (Oxf) 1981;14: 193-212.
3. 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 Endocr Metab 2007;92:2965-2971.
4. Kirschbaum C, Hellhammer DH. Salivary cortisol in psychoneuroendocrine research: recent developments and applications. Psychoneuroendocrinology 1994;19:313-33.
5. Aardal E, Holm AC. Cortisol in saliva: reference ranges and relation to cortisol in serum. Eur J Clin Chem Clin Biochem 1995;33:927-932.
6. Walker RF, Riad-Fahmy D, Read GF. Adrenal status assessed by direct radioimmunoassay of cortisol in whole saliva or parotid saliva Clin Chem 1978;24: 1460-1463.
7. Laudat MH, Cerdas S, Fournier C, Guiban D, Guilhaume B, Luton JP. Salivary cortisol measurement: A practical approach to assesss pituitary-adrenal function, J Clin Endocrinol Metab 1988;66:343.
8. Nelson DH, Samuels LT. A method for the determination of 17-hydroxycorticosteroids in blood; 17- hydroxycorticosterone in the peripheral circulation, J Clin Endocrinol Metab 1952;12:519.
9. Murphy BE. Some studies of the protein-binding of steroids and their application to the routine micro and ultramicro measurement of various steroids in body fluids by competitive protein binding radioassay. J Clin Endocrinol Metab 1967;27:973.
10. Murphy BE. Non-chromatographic radiotransinassay for cortisol: Application to human adult serum, umbilical cord serum, and amniotic fluid. J Clin Endocrinol Metab 1975;41:1050.
11. Mattingly D. A simple fluorimetric method for the estimation of free 11-hydroxycorticoids in human plasma. J Clin Pathol 1962;15:374-379.
12. Ballard PL, Carter JP, Graham BS, Baxter JD. A radioreceptor assay for evaluation of the plasma glucocorticoid activity of natural and synthetic steroids in man. J Clin Endocrinol Metab 1975;41:290.
13. Guo T, Taylor RL, Singh RJ, Soldin SJ. Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry. Clin Chim Acta 2006;372:76-82.
14. Taylor RL, Grebe SK, Singh RJ. Quantitative, highly sensitive liquid chromatography-tandem mass spectrometry method for detection of synthetic corticosteroids. Clin Chem 2004;50:2345-2352.
15. Chiu SK, Collier CP, Clark AF, Wynn-Edwards KE. Salivary cortisol on ROCHE Elecsys immunoassay system: pilot biological variation studies. Clin Biochem 2003;36:211-214.
16. Coolens JL, van Baelen H, Heyns W. Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroid-binding globulin. J Steroid Biochem 1987;26:197-202.
17. Carrozza C, Corsello SM, Paragliola RM, Ingraudo F, Palumbo S, Locantore P, Sferrazza A, Pontecorvi A, Zuppi C. Clinical accuracy of midnight salivary cortisol measured by automated electrochemiluminescence immunoassay method in Cushing's syndrome. Ann Clin Biochem 2010;47:228-232.
18. Carrasco CA, García M, Goycoolea M, Cerda J, Bertherat J, Padilla O, Meza D, Wohllk N, Quiroga T. Reproducibility and performance of one or two samples of salivary cortisol in the diagnosis of Cushing's syndrome using an automated immunoassay system. Endocrine 2012;41:487-493.
19. Deutschbein T, Broecker-Preuss M, Flitsch J, Jaeger A, Althoff R, Walz MK, Mann K, Petersenn S. Salivary cortisol as a diagnostic tool for Cushing's syndrome and adrenal insufficiency: improved screening by an automatic immunoassay. Eur J Endocrinol 2012;166:613-618.
20. Saiyudthong S, Suwannarat P, Trongwongsa T, Srisurapanon S. Comparison between ECL and ELISA for the detection of salivary cortisol and determination of the relationship between cortisol in saliva and serum measured by ECL. Science Asia 2010;36:169-171.
21. Karaca Z, Lale A, Tanriverdi F, Kula M, Unluhizarci K, Kelestimur F. The comparison of low and standard dose ACTH and glucagon stimulation tests in the evaluation of hypothalamo-pituitary-adrenal axis in healthy adults. Pituitary 2011;14:134-140.
22. Lo MS, Ng ML, Azmy BS, Khalid BA. Clinical applications of salivary cortisol measurements. Singapore Med J 1992;33:170-173.
23. van Aken MO, Romijn JA, Miltenburg JA, Lentjes EG. Automated measurement of salivary cortisol Clin Chem 2003;49:1408-1409.