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Estimation of cortisol in hospitalized patients with depression

Oszacowanie poziomu kortyzolu w ślinie pacjentek hospitalizowanych z powodu depresji

Cortisol is one of the most important steroid hormones produced by adrenal cortex. The influence of this hormone on body metabolism is significant. Even a small increase of its amount causes a rise of glucoses or amino acids level in blood and rapidness of the gluconeogenesis. Cortisol also has immunosuppressive and antiphlogistic effects [10].

Determination of cortisol can be carried out in different kind of tissues and body fluids. The most popular is blood, where the total amount of hormone can be estimated [1, 3, 5, 12]. For the determination of free and biological active cortisol, different body fluids can be used like urine [15] and saliva [11]. For the estimation of long-term changes of cortisol concentration some biological material can be used, for example hair [16].

Determination of cortisol in saliva is limited to the estimation of free cortisol only, but it is proved that a high correlation between the concentration of cortisol in saliva and its level in blood appeared [12]. The concentration of cortisol is lower in saliva than in serum or urine, but the sampling is quick, non-invasive and moreover stress-free for the volunteers and patients. The subject can collect and store the samples at home and bring for analysis in a greater number.

Cortisol is known as one of the biomarkers of different illnesses. A higher concentration of this hormone was observed during asthma and rhinitis [9]. Measurement of salivary cortisol is popular in diagnosis and screening of the Cushing's syndrome, especially in children [12]. Also, during other illnesses like Addison's [12] and Ménière's [3] the studies were made. Recently lots of studies concerning the determination of cortisol in stress situations like panic attacks [2] and anxiety [14] were conducted. The concentration of salivary cortisol is also estimated in patients with major depression, because a huge influence on the cause and course of the disease of this hormone was observed [1].

For determination of the concentration of cortisol in the saliva, different kinds of methods are used. The most popular are non-separation methods like enzyme immunoassay (EIA) [8] and radioimmunoassay (RIA) [11]. The separation methods are not so often used, but they are more accurate because of smaller possibilities of cross reaction between the different steroids and antibodies. In the group of separation methods the chromatography like liquid chromatography tandem mass spectrometry (LC-MS) [6] or high-performance liquid chromatography with laser-induced fluorimetric detector (HPLC-LIFD) [10] are often used. Also, other separation methods can be used, for example micellar electrokinetic chromatography (MEKC) [5] and microchip capillary electrophoresis (microchip CE) [13].

The aim of this study was to develop a simple, fast and sensitive method for determination of cortisol in human saliva, which would be unstressful especially for patients with major depression.

MATERIAL AND METHODS

Cortisol was purchased from Polpharma (Starogard Gd., Poland) and carbamazepine from Sigma-Aldrich (St. Louis, USA). Both of the standards had the purity accordant with the requirements of FP VI. Dichloromethane and acetonitrile were obtained from Merck (Darmstadt, Germany). For the extraction of cortisol from human saliva, a laboratory shaker, high speed centrifuges and water bath were used. A HPLC system was composed of a pump, an analytical reverse-phase column C-18 (Nucleosil 100-5, 125 x 4 mm), a UV detector and an interface box. All the parts of the HPLC system were produced by Knauer, Germany.

Saliva samples were obtained from 10 healthy volunteers (8 women and 2 men) and collected in the evenings into plastic tubes without any stimulation, stored in the fridge at 4°C until the analysis. The participants refrained from eating and drinking at least 30 min. before collection. The stock solutions of 1 mg mL^{-1} of cortisol and carbamazepine (internal standard) were prepared by dissolving 1 mg of substances in 10 mL of acetonitrile and storing in fridge at 4°C. The stock solutions were further diluted in acetonitrile to desired concentrations before the analysis. 1 mL of saliva was taken, 100 µL of carbamazepine (internal standard, 1 µg mL^{-1}) and an adequate amount of cortisol to appropriate concentration was added. The solution was mixed. For the purpose of extraction, 4 mL of dichloromethane was added and the mixture was shaked for 10 min and centrifuged at 10000 g for 7 min. The dichloromethane layer was carried to a glass tube and evaporated, then the residue was reconstituted in 100 µL of mobile phase, transferred to Eppendorf tube and centrifuged at 4500 g for 4 min. The sample prepared in that way was injected onto the column and analyzed by HPLC system.

For HPLC analysis, a mixture of acetonitrile and water (35:65; v/v) was used as a mobile phase and the flow-rate was 1.0 mL min^{-1}. The sample in the volume 20 µl was injected onto a reversed – phase column. The separation was made at room temperature and the time of analysis was 10 min. The wavelength of a UV detector was set at 240 nm. In these conditions the retention time of cortisol was 2.9 min and carbamazepine 4.5 min.

RESULTS AND DISCUSSION

OPTIMALIZATION OF THE METHOD

The optimisation of the method consisted of selecting the proper dissolvent of the substances (cortisol and carbamazepine), the way of extraction of the samples, and the adequate composition of mobile phase. In the beginning, the substances under study were dissolved in methanol, but chromatographic preliminary analysis showed that the tops of the peaks were split. That was the cause of dissolving the substances in acetonitrile, which made the satisfactory shape of the peaks.

The second step of optimalization of the procedure was the time of collecting saliva samples. It is known that the lowest concentration of endogenous cortisol is in the evening (about 8 p.m.). For the smallest probability of influence of endogenous cortisol during the preliminary analysis, the saliva samples for the calibration curve were collected in the evenings. For the optimisation of the extraction, two kinds of them were checked – solid phase extraction (SPE) and liquid-liquid extraction (LLE). The SPE as an analytical method revealed that results of cleaning the samples and the limit of the detection for the cortisol were worse than those for the LLE. In addition, preparation of the columns and drying them after loading and washing the samples took more time [4].

For the best results of separation and detection of peaks adifferent composition of the mobile

phase was analyzed. In the beginning, methanol and water was assayed, but shapes and peaks of the peaks were not satisfying enough. We decided to use acetonitrile as a modifier and we started with 45%, but the retention times of the substances were too short. The optimal concentration of the acetonitrile was 35%. Any other substances like phosphoric acid did not influence the shapes and retention time of peaks.

RESULTS

For the described analytical method the detection limit of cortisol was 1 ng ml^{-1} (S/N=3) and the limit of quantification was 3 ng ml^{-1} (S/N=10). The calibration curve of cortisol was made. It was linear in the range 5–200 ng ml^{-1} (r=0.9997). The mean linear regression equations of standard curve was H/H_{IS} = 0.008 (± 0.00005) C – 0.002 (± 0.006), where H/H_{IS} is peak-height of cortisol/peak-height of internal standard and C is the concentration of cortisol. The standard errors of regression coefficients were given in brackets. The calibration curve was constructed from twelve different concentrations of cortisol.

The analytical method was validated. The differences between blank and extracts samples (n=6) were used for determination of specificity of the analytical method. Typical chromatograms of blank saliva extract and saliva extract spiked with cortisol and carbamazepine (internal standard) are shown in Fig. 1. As we can see in Fig. 1A, no interferences were observed in the retention time of the studied substances, which indicates that the reported method is selective. For intra-day and inter-day studies, saliva samples were collected, spiked with cortisol for three different concentrations (15, 75, 125 µg ml^{-1}) and analyzed. During the inter-day study saliva samples between the analyses were kept in fridge at 4°C. The RSD in the intra-day study varied from 1.1 to 9.9% and in inter-day study this parameter did not exceed 6.8% (Table 1).

Table 1. Intra-day and inter-day precision and accuracy assay (n = 6)

Intra-day			Cortisol (ng mL^{-1})	Inter-day		
$\bar{x}$ ±SD	Accuracy (%)	RSD (%)		$\bar{x}$ ±SD	Accuracy (%)	RSD (%)
15.0 ± 1.02	100.0	6.8	15	13.98 ± 0.95	93.2	6.8
76.25 ± 3.55	101.7	4.7	75	70.17 ± 2.28	93.6	4.3
130.0 ± 4.76	104.0	3.0	125	127.90 ± 2.09	102.3	1.6

In addition, for two concentration of cortisol (15, 125 ng ml^{-1}) the extraction efficiency was determined. In this case RSD did not exceed 8.2% for both concentrations. The results of the studies are given in Table 2.

Table 2. Estimation of precision and accuracy assay for determination of cortisol in saliva spiked with standards before and after extraction

Saliva spiked with standards						
Before extraction			Cortisol (ng mL^{-1})	After extraction		
$\bar{x}$ ±SD	Accuracy (%)	RSD (%)		$\bar{x}$ ±SD	Accuracy (%)	RSD (%)
18.33±1.51	100.0	8.2	15	17.29±1.23	122.2	7.1
129.79±4.36	104.0	3.4	125	131,67±3.42	103.8	2.6

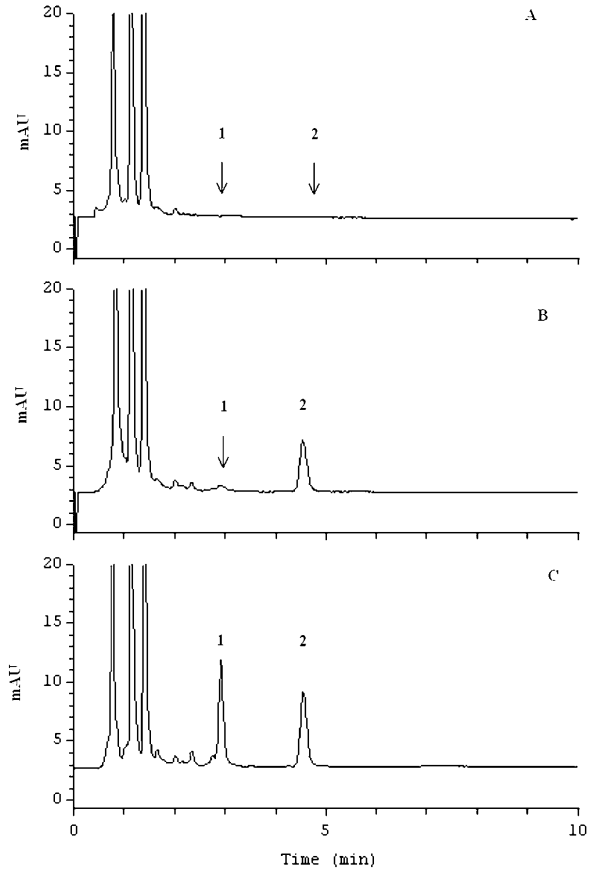

Fig. 1. Chromatograms (A) Blank human saliva extract (B) Human saliva extract spiked with cortisol concentration 10 ng mL^{-1} (1) and 100 ng mL^{-1} of carbamazepine (internal standard) (2) (C) Human saliva extract spiked with cortisol concentration 150 ng mL^{-1} (1) and 100 ng mL^{-1} of carbamazepine (internal standard) (2)

For three concentrations (15, 75,125 ng ml^{-1}) of cortiosl the stability of the substance was checked. Saliva spiked with an appropriate amount of cortisol was exposed to three freezing and thawing cycles each for one week. The results of freeze-thaw stability indicated that the substance is stable in saliva during the freezing process to -20°C and thawing to room temperature.

ANALYSIS OF THE SAMPLES

For the utility of the developed method the level of the hormone in depressed persons was analyzed. The saliva samples were collected into plastic tubes about 10 a.m. when the concentration of the endogenous hormone is the highest. As is shown in Fig. 2, cortisol is detected in a saliva sample from a person with hypercorticism and a person without this illness.

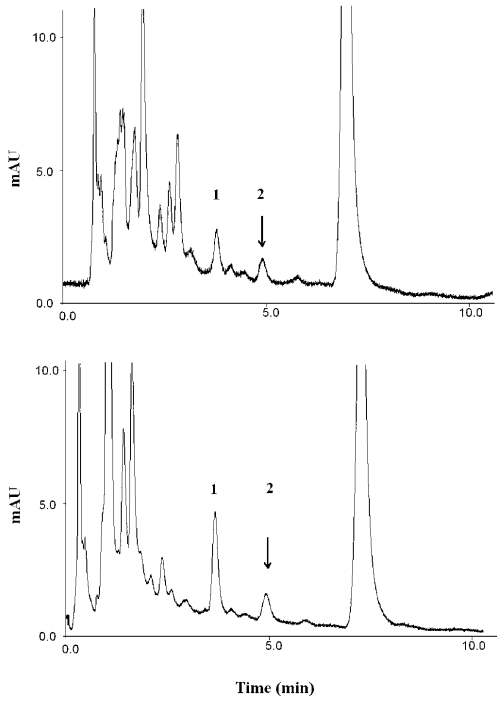

Fig. 2. Chromatograms of depressed persons' saliva extracts; 1 – cortisol, 2 – carbamazepine (100 ng mL^{-1})

CONCLUSIONS

The developed method can be used for detection, identification and quantification of the endogenous cortisol in human saliva. The applied procedure and chromatographic parameters allowed for separation of cortisol and carbamazepine from pollutions descendent from biological matrix. The device method is selective, sensitive and fast for routine analysis of endogenous concentration of cortisol, for example in monitoring the changes of this hormone in depressed persons during hospitalization, or for diagnosis of other illnesses like Cushing's syndrome.

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SUMMARY

The aim of this study was to develop the analytical method of the isolation and determination of cortisol from the saliva of depressed women. The material for introduct study was obtained from 10 healthy subjects and was collected in the evening hours, about 8 p.m. when the concentration of cortisol is the lowest. The extraction of the cortisol from saliva was made by liquid-liquid extraction (LLE) by dichloromethane. The level of the hormone was estimated by HPLC with UV detection at 240 nm. The mobile phase contained acetonitrile and water (35:65; v/v) at a flow of 1 mL min^{-1}. The time of retention for cortisol was 2.9 min and for carbamazepine (internal standard) was 4.9 min. The estimated analytical method was validated. Linearity of the calibration curve (r = 0.9997) was obtained in the concentration range of 5–200 ng mL^{-1} of cortisol in saliva and the limit of detection (S/N = 3) was 1 ng mL^{-1}. The RSD for the intra-assay study varied between 1.1 and 9.9% and in the inter-assay study did not exceed 6.8%. In the intra-assay and inter-assay of the study the accuracy was about 100. The efficiency of the method was made for two different concentrations of cortisol 15 and 125 ng ml^{-1}. The precision for the lower concentration was 7.1% and 8.2% adequate for cortisol

spiked after and before the extraction. For the concentration 125 ng ml^{-1} the efficiency was 3.4% and 2.6%. The estimated method of extraction was applied to analysis of saliva samples without interference peaks. It gave satisfying results and may be a useful toole for monitoring the changes in salivary cortisol.

STRESZCZENIE

Opracowano metodę analityczną pozwalającą na wyizolowanie i oznaczenie poziomu kortyzolu w ślinie kobiet chorych na depresję. Materiał, który posłużył do prób wstępnych, pochodził od 10 ochotniczek i gromadzony był w godzinach wieczornych, około godziny 20.00, kiedy poziom kortyzolu jest najniższy. Ekstrakcja śliny została przeprowadzona przy pomocy dichlorometanu. Poziom kortyzolu został oznaczony przy pomocy HPLC z detekcją UV przy długości fali 240 nm. Fazę ruchomą stanowił układ binarny acetonitryl-woda (35:65; v/v), przy przepływie fazy ruchomej 1 ml min^{-1}, czas retencji kortyzolu wyniósł 2,9 min, natomiast karbamazepiny (wzorzec wewnętrzny) 4,9 min. Opracowana metoda analityczna została poddana walidacji. Liniowość wyznaczono w zakresie stężeń 5-200 ng ml^{-1} (r=0.9997). Granica wykrywalności dla kortyzolu wyniosła 1 ng ml^{-1} (S/N=3). Następnie wyznaczono powtarzalność oraz odtwarzalność metody. W obu przypadkach precyzja nie przekroczyła 10%. Sprawdzono również wydajność ekstrakcji, określając precyzję oraz dokładność dla dwóch wybranych stężeń 15 i 125 ng ml^{-1}. Precyzja dla stężenia 15 ng ml^{-1} wyniosła 7,1% oraz 8,2% odpowiednio dla wzorca dodanego po i przed ekstrakcją. Natomiast dla stężenia 125 ng ml^{-1} 3,4% oraz 2,6%. Stwierdzono, iż opracowana metoda jest prosta w wykonaniu, specyficzna i pozwala na oznaczanie poziomu kortyzol w ślinie zdrowych ochotników oraz osób z podwyższonym poziomem tego hormonu, np. chorych na depresję.