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Determination of cortisol in human saliva by automated in-tube solid-phase microextraction coupled with liquid chromatography–mass spectrometry

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Abstract

We developed a simple, rapid, and sensitive method for determination of cortisol levels in human saliva. Cortisol was analyzed by on-line in-tube solid-phase microextraction (SPME) coupled with liquid chromatography–mass spectrometry (LC/MS). Cortisol was separated within 5 min by HPLC using an Eclipse ZDB-C8 column and 1% acetic acid/methanol (50/50, v/v) at a flow rate of 0.2 mL/min. Electrospray ionization conditions in the positive ion mode were optimized for MS detection of cortisol. The optimum in-tube SPME conditions were 20 draw/eject cycles with a sample size of 40 μL using a Supel Q PLOT capillary column as the extraction device. The extracted compounds could be desorbed easily from the capillary by passage of the mobile phase, and no carryover was observed. Using the in-tube SPME LC/MS method, good linearity of the calibration curve ($r = 0.9977$) was obtained in the concentration range 50–2000 pg/mL of cortisol in saliva, and the limit of detection (S/N = 3) was 5 pg/mL. The method described here showed 48-fold higher sensitivity than the direct injection method (5 μL injection). The within-run and between-day precisions (relative standard deviations) were below 4.6% and 8.9% ($n = 5$), respectively. This method was applied successfully to the analysis of saliva samples without interference peaks. The recoveries of cortisol spiked into saliva samples were above 95%, and the relative standard deviations were below 6.0%. This method was used to analyze the changes in salivary cortisol level according to stress load.

Keywords: In-tube solid-phase microextraction; Cortisol; Saliva; Liquid chromatography–mass spectrometry; Stress load

1. Introduction

The stress system coordinates the adaptive responses of the organism to various types of stressors, and stress can trigger off various illnesses. The main hormones of the stress system are the corticotrophin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and norepinephrine. The hormonal neurons in these systems innervate each other and show reciprocal activation. The hypothalamic/pituitary/adrenal axis is controlled by several feedback loops that tend to normalize the time-integrated secretion of cortisol (hydrocortisone) [1]. Thus the cortisol concentrations in biological samples, such as serum, urine, and saliva, have been used as biomarkers for endocrinological stress [2–6]. However, the sampling of blood may itself induce stress, and the collection of urine represents a cumulative or averaged response. On the other hand, cortisol in saliva has been suggested to be a good biomarker for evaluation of stress objectively because it can be collected easily in a stress-free manner without medical supervision, it allows direct measurements, and it is strongly correlated with plasma cortisol levels [6–8]. However, the cortisol level in saliva is lower than those in serum and urine. The reference concentrations of salivary cortisol are 1–8 ng/mL in the morning and 0.1–1 ng/mL in the evening, which are about two orders of magnitude lower than those in serum [9]. Therefore, it is necessary to develop a simple, sensitive, and accurate method for determination of salivary cortisol level.

Determination of cortisol in saliva has been carried out by radioimmunoassay [10,11], enzyme immunoassay [12,13], high performance liquid chromatography (HPLC) [14], and LC with tandem mass spectrometry (MS/MS) [6]. The immunological methods are sensitive, but have cross-reactivity with other steroids and give relatively high concentrations [6]. The HPLC method using column switching shows interference by coexisting material [14]. Although the LC/MS/MS method is highly selective and sensitive, it requires evaporation to dryness of saliva sample prior to analysis.

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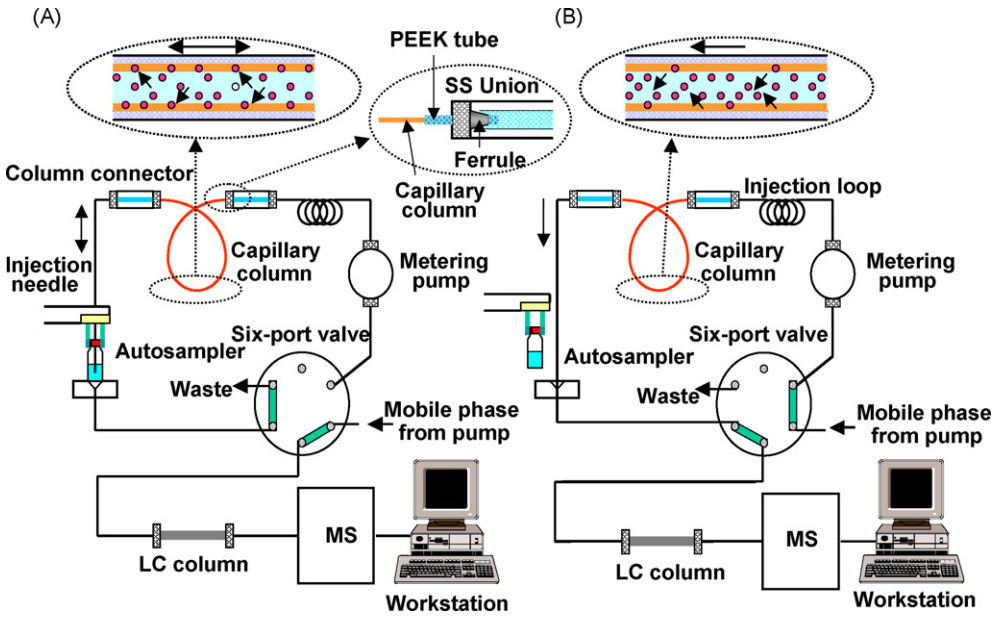

Fig. 1. Schematic diagram of the on-line in-tube SPME LC/MS system: (A) extraction step; (B) desorption step.

In-tube solid-phase microextraction (SPME) [16], using an open tubular fused-silica capillary with an inner surface coating as the SPME device, is simple and can be coupled easily on-line with HPLC and LC/MS. In-tube SPME allows convenient automation of the extraction process, which not only reduces the analysis time, but also provides better accuracy, precision, and sensitivity than manual off-line techniques. We have developed an in-tube SPME method for determination of various compounds, such as drugs and environmental contaminants, by coupling with HPLC [17,18], LC/MS [19,20], and LC/MS/MS [21,22]. The details of the in-tube SPME technique and its applications have also been summarized in a number of reviews [23–25]. In this paper, we report an automated on-line in-tube SPME LC/MS method for determination of cortisol in saliva samples. Using this method, we also analyzed the changes in salivary cortisol level with experimental stress load.

2. Experimental

2.1. Materials

Cortisol was purchased from Sigma–Aldrich Japan (Tokyo, Japan) and was dissolved in methanol to make a stock solution at a concentration of 1 mg/mL. The solution was stored at 4 °C and diluted to the required concentrations with pure water prior to use. LC/MS grade methanol and distilled water used as mobile phases were purchased from Kanto Chemical (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Instrument and analytical conditions

The LC/MS system was a Model 1100 series LC coupled with an atmospheric pressure electrospray ionization (ESI) MS (Agilent Technologies, Boeblingen, Germany). An Eclipse ZDB-C8 column (50 mm × 2.1 mm i.d., particle size of 3.5 μm) from Agilent Technology was used for LC separation. LC conditions were as follows: column temperature, 30 °C; mobile phase, 1% acetic acid/methanol (50/50, v/v); flow rate, 0.2 mL/min. ESI-MS conditions were as follows: nebulizer gas, N_2 (55 psi); drying gas, N_2 (12 L/min, 350 °C); fragmentor voltage, 110 V; capillary voltage, 4500 V; ionization mode, positive mode; mass scan range, 100–400 amu; selected ion monitoring (SIM), *m/z* 363 ($M+H$); dwell times for the ions in SIM, 580 ms. LC/MS data were processed using an HP ChemStation.

2.3. In-tube solid-phase microextraction

As shown in Fig. 1, a Supel-Q PLOT capillary column (60 cm × 0.32 mm i.d., 12 μm film thickness; Supelco, Bellefonte, PA, USA) was used as the in-tube SPME device. The column was placed between the injection loop and injection needle of the autosampler, and the injection loop was retained in the system to avoid fouling of the metering pump. Capillary connections were facilitated by use of a 2.5-cm sleeve of 1/16 in. polyetheretherketone (PEEK) tubing at each end of the capillary (1 in. = 2.54 cm). A PEEK tubing (330 m i.d.) was found to be suitable to accommodate the capillary used. Normal 1/16 in. stainless steel nuts, ferrules, and connectors were then used to complete the connections. The autosampler software was programmed to control the in-tube SPME extraction, desorption and injection. Vials (2 mL) were filled with 0.5 mL of sample for extraction, and set into the autosampler programmed to control the SPME extraction and desorption technique. In addition, 1.5-mL aliquots of methanol and water in 2-mL autosampler vials with a septum were set on the autosampler. The capillary column was washed and conditioned by two repeated draw/eject cycles (40 μL each) of these solvents, and then a 50-μL air plug was drawn prior to the extraction step. The extraction of cortisol onto the capillary coating was performed by 20 repeated draw/eject cycles of 40 μL of sample at a flow rate of 150 μL/min with the

six-port valve in the LOAD position (Fig. 1(A)). After washing the tip of the injection needle by one draw/eject cycle of 2 μL of methanol, the extracted compounds were desorbed from the capillary coating with mobile phase flow. Then, the compounds were transported to the LC column by switching the six-port valve to the INJECT position (Fig. 1(B)), and detected by the MS system with SIM mode. During the analysis, the SPME capillary was washed and conditioned with mobile phase for the next extraction.

2.4. Sample preparation

Saliva samples were collected in Salivette tubes containing a polyester wool swab (Sarstedt, Nümbrecht, Germany), and the tubes were centrifuged at 2500 × *g* for 5 min to elute the saliva. Saliva solutions (0.1–0.2 mL) were added to 50 μL of 0.2 M acetate buffer (pH 4) and the total volume was made up to 0.5 mL with distilled water. The mixtures were heated at 80 °C for 5 min to precipitate proteins, and, after centrifugation, the liquid samples were used for the following in-tube SPME LC/MS analysis. Standard cortisol was added to pooled saliva sample at the concentration of 0, 50, 100, 200, 500, 1000 and 2000 pg/mL, and a calibration curve was constructed by subtraction of endogenous cortisol concentration in non-additioned saliva from cortisol concentrations in added-saliva samples.

2.5. Experimental stress load test and saliva collection

Brachialis muscle ankylosis (persistent flexing of the arm causing the muscle to stand out), skin clipping (persistently picking up the skin of the arm with a clip), and running were tested as stressors. The aim of the experiment was explained to the subjects and consent was obtained after confirmation that they fully understood the experiment. The subjects for brachialis muscle ankylosis and skin clipping tests were 22 female volunteers and the test was conducted between 14:00 and 16:00, 1 h or more after lunch. Saliva was sampled just before, immediately after, and 10 min after the start of brachialis muscle ankylosis. In the skin clipping test, saliva was sampled 10 min before, just before, immediately after, and 10 min after the start of skin clipping. The subjects in the running test were 51 male volunteers, and saliva was collected before, during, and after 30 min of running between 20:30 and 21:00, 1 h or more after supper. The collected samples were stored at −30 °C until assayed.

3. Results and discussion

3.1. LC/MS analysis of cortisol

For MS operation, ESI positive ion mode was evaluated for determination of cortisol. To select the monitoring ion for cortisol, the ESI mass spectrum was initially analyzed by LC/MS with direct liquid injection into the column. Cortisol gave *m/z* 363.2 $[M+H]^+$ as a base ion and *m/z* 385.2 $[M+Na]^+$ in the mass scan range of 100–400 amu. Parameters, including nebulizer gas pressure, drying gas flow rate, fragmentor voltage, and capillary voltage, were optimized by flow injection analysis.

LC separation of cortisol was performed using an Eclipse ZDB-C8 column. Cortisol was eluted within 5 min using 1% acetic acid/methanol (50/50, v/v) as the mobile phase, at a flow rate of 0.2 mL/min. Cortisol could be selectively detected by SIM mode selected at *m/z* 363 $[M+H]^+$.

3.2. Optimization of in-tube solid-phase microextraction and desorption

To optimize the extraction of cortisol by in-tube SPME, several parameters, such as the stationary phase of the in-tube SPME capillary column and number and volume of draw/eject cycles, were investigated. Four different capillary columns, CP-Sil 5CB, CP-Sil 19CB, and CP-Wax 52CB (Varian Inc., Lake Forest, CA, USA) and Supel Q PLOT were evaluated for extraction efficiency. As shown in Fig. 2(A), the extrac-

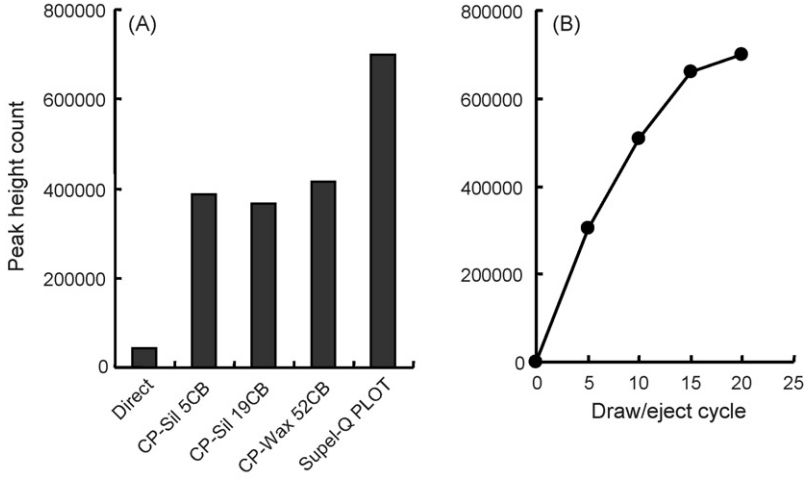

Fig. 2. Effects of (A) capillary coatings and (B) draw/eject cycle on the in-tube SPME of cortisol. Cortisol was extracted by draw/eject of 40 μL of standard solution (10 ng/mL) at a flow rate of 150 μL/min.

tion efficiency of the porous polymer-type capillary column (Supel Q PLOT) was better than those of the other columns. As the PLOT column has a large adsorption surface area, the amount extracted was greater than that with liquid-phase type columns.

With in-tube SPME, the extraction time, flow rate, and sample pH are related to the amounts of compounds extracted. To monitor the extraction time profile of cortisol by in-tube SPME, the number of draw/eject cycles was varied from 0 to 20 using a Supel Q PLOT capillary column. As shown in Fig. 2(B), the extraction reached almost equilibrium after 20 draw/eject cycles of 40 μL of sample at a draw/eject rate of 150 μL/min. Although extraction equilibrium is incomplete, it is possible to cease extraction before equilibrium to reduce the analysis time, because quantitative reproducibility is obtained by fixing SPME conditions using an autosampler. The effect of the pH of the sample matrix on extraction of cortisol was examined using several buffer solutions. Acetate buffer (pH 4) was most effective, and the optimal concentration of this buffer was 20 mM. The absolute amounts of cortisol extracted by the SPME capillary column were calculated by comparing peak area counts with the corresponding direct injection of the sample solution onto the LC column. At a sample concentration of 0.1 ng/mL (sample volume, 0.5 mL), 21.5 pg (43%) of cortisol was extracted onto the Supel Q PLOT column by in-tube SPME. Although the extraction yield of cortisol was relatively low, its reproducibility was good (R.S.D. < 3%) thanks to the autosampler.

The mobile phase was found to be suitable for desorption of cortisol extracted into the stationary phase of the capillary column. Dynamic desorption of cortisol from the capillary could be readily achieved by switching the six-port valve (Fig. 1(B)). The desorbed cortisol was transported to the LC column by mobile phase flow.

Air plugging before the extraction step was carried out to prevent not only sample mixing but also desorption of analyte from the capillary coating by the mobile phase during the ejection step. No carryover was observed because the capillary column was washed and conditioned by draw/eject cycles of methanol and mobile-phase prior to extraction. The extraction and desorption of cortisol by the in-tube SPME method were accomplished automatically within 25 min, and automated analysis of about 56 samples per day was possible by overnight operation.

3.3. Sensitivity, linearity, and precision

Cortisol provided an excellent response in ESI-MS. Under our LC/MS conditions, and the detection limit (S/N = 3) was 5 pg/mL. The in-tube SPME method was 48-fold more sensitive than the direct injection method (5 μL injection), because cortisol was concentrated in the capillary column during draw/eject cycles. The calibration curve for cortisol was linear in the range 50–2000 pg/mL saliva (six-point). The calibration line in human saliva was $y = 6.80x + 201$ with $r = 0.9977$ ($n = 18$) (y, peak height count; x, concentration (pg/mL) of cortisol; r, correlation coefficient). The within-run and between-day precisions (relative standard deviations, R.S.D.) were below 4.6% and 8.9% ($n = 5$), respectively (Table 1).

Table 1
Within-run and between-day precisions for the analysis of cortisol

Spiked cortisol (pg/mL)	Within-run R.S.D. (%)[a]	Between-day R.S.D. (%)[a]
50	4.6	8.9
500	2.0	2.8
2000	1.5	3.1

[a] $n = 5$.

3.4. Analysis of saliva samples and changes of salivary cortisol level by stress load

Saliva samples were collected easily using Salivette tubes containing a polyester wool swab, and the samples were heated at 80 °C to remove the proteins. The recovery rate of cortisol from the Salivette device was 79.0 ± 3.5% ($n = 3$) by comparison spiked saliva sample with pure standard sample. The lower recovery was corrected by using a calibration curve of cortisol spiked into the pooled saliva as described above. The saliva samples were analyzed successfully without interference peaks using the established in-tube SPME LC/MS method with SIM mode detection. The quantification limit of cortisol in saliva samples was 17 pg/mL (S/N = 10). To confirm the validity of this method, known amounts of cortisol were spiked into pooled saliva samples, and their recoveries were calculated. The recoveries of cortisol were 97.2 ± 5.8% ($n = 3$) for 0.2 ng/mL spike and 95.5 ± 4.9% ($n = 3$) for 2 ng/mL spike. Typical chromatograms obtained from saliva samples are shown in Fig. 3.

Salivary cortisol content is known as a useful biomarker to evaluate stress [6,7,10–14]. In order to evaluate utility of the developed method, we analyzed the changes in salivary cortisol levels with several stress load tests. These tests were performed in the afternoon, because salivary cortisol content is relatively high until 1 h after awakening [6]. As shown in Fig. 4(A), the cortisol content doubled after 10 min of brachialis muscle ankylosis. This was considered to depend on the results of patience for 10 min. In the skin clipping test, the cortisol

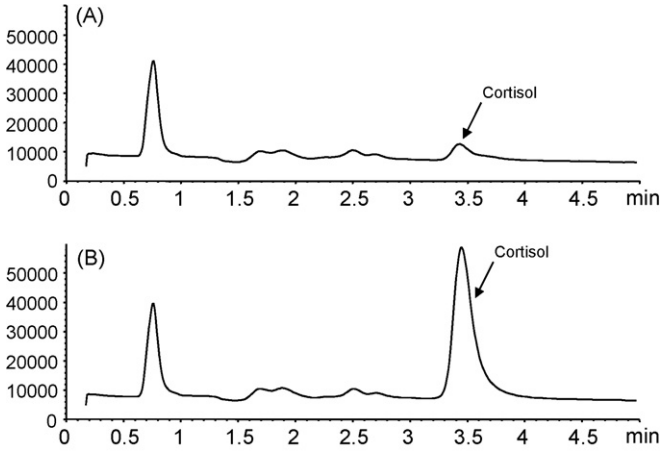

Fig. 3. Chromatograms obtained from (A) saliva and (B) saliva spiked 1.0 ng/mL of cortisol. The *m/z* 363 ion was selected. LC/MS conditions: see Section 2.

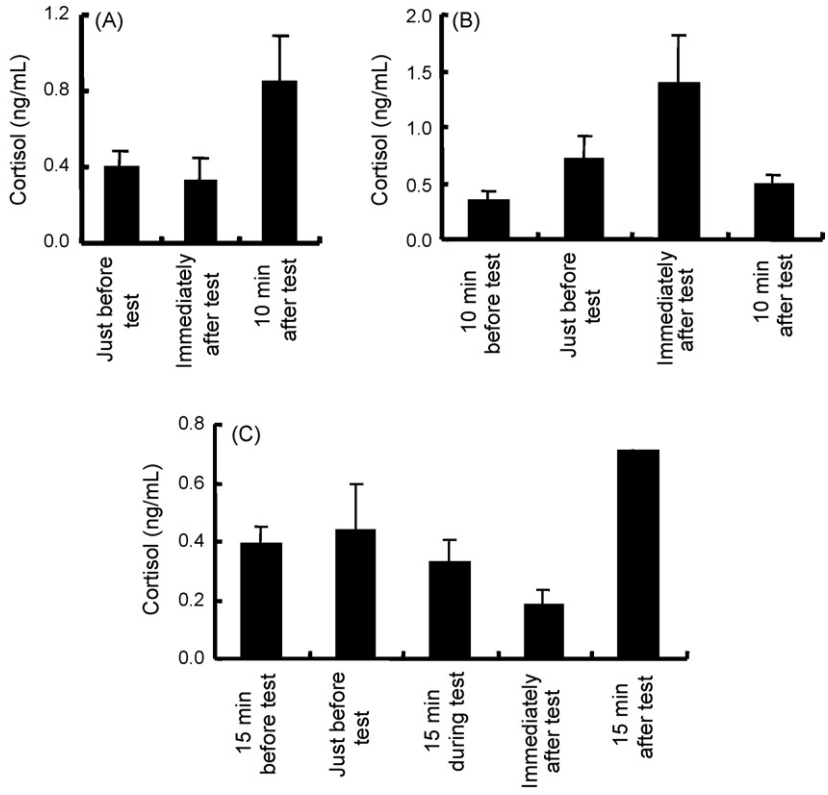

Fig. 4. Changes in salivary cortisol contents by several stress load tests: (A) brachialis muscle ankylosis, (B) skin clipping, and (C) running. The data shown are the means of three independent measurements. Details of each test are given in Section 2.

content increased three-fold as compared to the normal level immediately after beginning the test (Fig. 4(B)). This was considered to depend on the results of mental stress by association with pain. On the other hand, the cortisol content decreased during running, but increased 15 min after the running test (Fig. 4(C)). This was considered to be due to physical fatigue. These results suggest that the secretion time of cortisol is different by source and strength of stress. Stronger stress such as skin clipping may raise a level of salivary cortisol by earlier response.

4. Conclusions

The on-line in-tube SPME LC/MS method developed in the present study can continuously perform extraction and concentration of cortisol from saliva samples and then allow analysis by LC/MS. This method is automated, simple, rapid, selective, and sensitive, and can be applied easily to the analysis of saliva samples. We believe that this method is a very useful tool for determination of cortisol in saliva samples and for the evaluation of stress.

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