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Column-switching high-performance liquid chromatographic system with a laser-induced fluorimetric detector for direct, automated assay of salivary cortisol

Tatsuya Okumura*, Yuki Nakajima, Tasuku Takamatsu, Masahiro Matsuoka

Analytical Research Group, Safety and Analytical Research Center, R&D Headquarters, Shiseido Co., Ltd., 1050 Nippacho, Kouhoku-ku, Yokohama 223, Japan

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Abstract

In order to measure human stress, an easy and rapid, fully automated method for the determination of cortisol in saliva has been developed, using column-switching high-performance liquid chromatography with laser-induced fluorescence detection, which involves post-column labeling with sulfuric acid. The developed system requires only 0.1 ml of saliva, and a simple pretreatment consisting of dilution and filtration is sufficient. The column-switching system consisted of a Polymer-Coated Mixed-Functional silica (PCMF) column for deproteinization, and a CN column for frontal concentration and separation. An ODS column in place of the CN provided a better separation, but required a post-column make-up of water for safe reaction. Detection limit of cortisol was 8 fmol (signal-to-noise ratio = 3), which is adequate for routine determination of normal levels of cortisol (1–20 pmol/ml). The analysis time was about 40 min and reproducibility was excellent with an R.S.D. of less than 5%.

1. Introduction

Among many endogenous corticosteroids, cortisol has frequently been monitored as a marker of adrenocortical function, as well as adaptation to stress. Assay methods for cortisol have been developed for various biological fluids, such as plasma [1–4], serum [5–10], urine [11–14], amniotic fluid [15] and saliva [16–22]. Saliva is thought to be the most suitable source for monitoring human stress, because saliva sampling is stress-free (in contrast to serum sampling) and saliva reflects biological responses more directly than urine, which affords a kind of cumulative or averaged response. While easy sampling without medical supervision is a major advantage of saliva, the time required for sampling (for example, 30 min for 5 ml of saliva) has been a bottleneck in establishing a convenient assay method. This is partly because the concentration of cortisol is about 10–100-fold lower in saliva than in serum or urine. For this reason, most of the assay methods previously reported for saliva were based upon radioimmunoassay (RIA) [16–19], whereas many different assay methods based upon HPLC have been reported for plasma, serum and urine, including some with a simple and rapid pretreatment, and some that are suitable for automation [12,15,23]. RIA requires only a small amount of saliva, but false-

* Corresponding author.

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positive results due to cross reactions with other steroids have been suspected. HPLC with UV absorbance detection responds not only to cortisol, but also to one of its important metabolites, cortisone [20,21], and its sensitivity for cortisol is insufficient for assay in saliva. In HPLC with fluorescence detection, prelabeling with 9-anthroylnitrile [24] provided 50 pg sensitivity, but the prelabeling procedure is lengthy. Another prelabeling method using sulfuric acid [9,11,12,23] required immediate injection because the reaction is time dependent and the fluorescent derivative is unstable, and is not suitable for routine analysis. The only successful applications of HPLC to saliva are those reported by Sudo [22] and by us [25], both of which employed post-column labeling with sulfuric acid followed by fluorimetric detection. Even in these cases, 2 ml of saliva was required with either dichloromethane extraction for deproteinization and concentration or perchloric acid deproteinization and solid-phase extraction.

Cortisol concentration in saliva shows a circadian rhythm, reaching a peak of around 10–20 pmol/ml in the early morning and decreasing towards midnight to around 1 pmol/ml or less. In order to use cortisol concentration as a parameter of human stress, samplings of saliva should be as frequent as possible to detect clearly the deviation of cortisol concentration from the rhythm, especially if the stress-induced elevation of cortisol is not large in relation to the daily fluctuation. In addition, although corticosterone, another important corticosteroid, has never been found in human saliva, but in rat because of a lack of the specific enzyme in the adrenal gland [26], it is desirable to detect it simultaneously especially when the stress study is expanded to rodents. For this purpose, a fully automated assay system to which the sampled saliva can be applied directly without any complicated or lengthy pretreatment is a prerequisite and the saliva sample size should be less than 1 ml, taking the sampling time into consideration. The method we have developed and automated for routine operation employs a column-switching technique with a PCMF column [27] for deproteinization and a CN column for frontal concentration and separation. Detection is done by post-column labeling with sulfuric acid followed by laser-induced fluorimetry, which is sufficiently sensitive to allow a reduction of saliva sample size to the 0.1 ml level. The saliva sample is injected directly into this system after simple dilution and filtration. Accuracy of the method was fully investigated, including a preliminary experiment on stress detection, with satisfactory results. The system has been in routine operation for more than three months (more than 300 analyses).

2. Experimental

2.1. Chemicals and reagents

Cortisol (11,17,21-trihydroxypregn-4-ene-3,20-dione) was obtained from ICN Biomedicals (Cleveland, OH, USA). Corticosterone ((11β)-11,12-dihydroxypregn-4-ene-3,20-dione) was obtained from Fluka (Buchs, Switzerland). Sulfuric acid of analytical-reagent grade and acetonitrile of HPLC grade were from Nacarai Tesque (Kyoto, Japan). Water used in all experiments was filtered through a Milli-Q system (Millipore, Molsheim, France). Salivette as a sampling aid for saliva was obtained from Sarstedt (Rommelsdorf, Nümbrecht, Germany).

2.2. Solutions

Stock solutions of 1 μg/ml of cortisol and corticosterone were prepared in ethanol, and were stored at 4°C. The stock solutions were diluted with the mobile phase A to desired concentrations before use.

2.3. Chromatographic system

A schematic illustration of the column-switching chromatographic system and post-column derivatization system for fluorimetric detection is presented in Fig. 1. It was composed of a pump

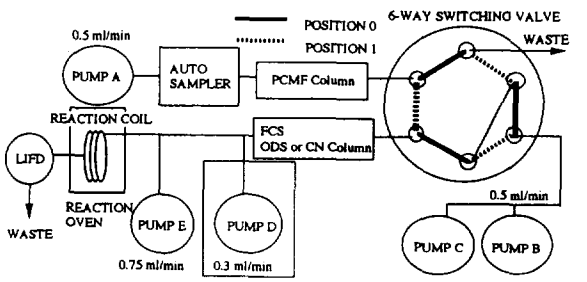

Fig. 1. Schematic diagram of a column-switching HPLC system. Pump A (LC-6A, Shimadzu): 10% acetonitrile, 2 m*M* trisodium citrate, pH 6.5/HCl; pump B (LC-10AD, Shimadzu): 10% acetonitrile; pump C (LC-10AD, Shimadzu): acetonitrile; pump D (L-6300, Hitachi): water (needed when ODS column was used); pump E (L-6010, Hitachi): sulfuric acid; coil: 2.5 m × 0.25 mm I.D. (Dyflon), 105°C; LIFD: laser-induced fluorescence detector (LF-8010, Tosoh).

(Hitachi L-6300, Hitachi, Tokyo, Japan) for the mobile phase of the PCMF column, two pumps (Shimadzu LC-10AD, Shimadzu, Kyoto, Japan) for the gradient mobile phase of the ODS-silica or CN column for frontal concentration and separation, an autosampler (Shimadzu SIL-10A) equipped with a refrigerated sample tray, a PCMF (silicone polymer-coated silica with diol and phenyl groups) column for deproteinization (Capcell pak MF, 100 × 4.6 mm I.D., Shiseido, Tokyo, Japan), a frontal concentration and separation (FCS) column (Capcell pak C_{18} SG, 250 × 4.6 mm I.D. or Capcell pak CN, 250 × 4.6 mm I.D., Shiseido), a column oven (Shimadzu CTO-6A), an acid-resistant pump (Hitachi L-6010) for sulfuric acid as a fluorogenic reagent, a three-way conventional joint of acid-resistant Dyflon, a reaction oven (Shimadzu CRB-6A) with reaction coils of Dyflon (1, 2.5, 5, 10 m × 0.25, 0.5, 0.7 mm I.D.), a fluorescence detector (Hitachi F-1050), and a laser-induced fluorescence detector (10 mW Ar^+ laser: 488 nm, band-pass filter: 537 nm) (Tosoh LF-8010, Tosoh, Tokyo, Japan), both of which were equipped with acid-resistant flow cells, and an integrator (Shimadzu C-R4A).

At valve position 0, samples, injected by an autosampler every 45 min, were introduced into the PCMF column, through which the salivary proteins and cortisol fraction were separated with mobile phase A. Simultaneously, mobile phase B was flowed into the FCS column for initial conditioning. The salivary proteins fraction was discarded, the valve position was switched from 0 to 1, and the cortisol fraction was introduced into the FCS column for frontal concentration. Then, the valve was switched back to position 0 so that cortisol was separated by gradient elution with mobile phases B and C.

2.4. Chromatographic conditions

Mobile phase A was acetonitrile and water (10:90, v/v) with 2 m*M* trisodium citrate, adjusted to pH 6.5 with hydrochloric acid, and used at a flow-rate of 0.5 ml/min. Mobile phase B had the same composition as mobile phase A, but without citrate, and mobile phase C was acetonitrile. For gradient elution using the CN column, the initial ratio of mobile phases B and C was set at 100:0 and the final ratio at 72.3:27.7 (35% acetonitrile). The flow-rate was also 0.5 ml/min. The flow-rate of the fluorogenic reagent solution (concentrated sulfuric acid) was 0.75 ml/min. The time program is shown in Table 1.

2.5. Analytical procedure

Saliva samples were acquired by two simple procedures after mouth rinsing and gargling with plain water followed by a rest period of about 15 min. One was by dribbling freely into a 15 ml polypropylene centrifuge tube without any sampling aid, and the other was by simply holding the cotton swab of a Salivette in the mouth for 2–3 min, putting it back into the tube and centrifuging at 1300 *g* for 30 s. Samples were stocked at −85°C in a freezer until analysis.

The sample solutions were prepared by adding exactly 0.5 ml of water and 1.0 ml of mobile phase A to exactly 0.5 ml of saliva sample thawed at room temperature. The mixture was filtered with a disposable membrane filter, and a 400-μl aliquot of the filtrate was injected into the HPLC system.

Table 1
Time program for the automated system using the CN column for FCS

Time (min)	Condition
0.0	Valve position 0
	Pump C = 0% (10% acetonitrile)
	Total flow of pumps B and C = 0.5 ml/min
7.0	Valve position 1
15.0	Valve position 0
	Pump C = 0% (10% acetonitrile)
20.0	Pump C = 27.7% (35% acetonitrile)
36.0	Pump C = 27.7% (35% acetonitrile)
37.0	Pump C = 0% (10% acetonitrile)
	Stop

Deproteinization column: Capcell pak MF, 100 × 4.6 mm I.D., 40°C; pump A (mobile phase A): 2 m*M* trisodium citrate, 10% acetonitrile (pH 6.5/HCl), 0.5 ml/min; FCS column: Capcell pak CN, 250 × 4.6 mm I.D., 40°C; pump B (mobile phase B): 10% acetonitrile (for gradient); pump C (mobile phase C): acetonitrile (for gradient); LIFD: LF-8010, 10 mW Ar^+ laser, 488 nm, 537-nm band-pass filter; pump E: sulfuric acid, 0.75 ml/min; reaction coil and temperature: 2.5 m × 0.25 mm I.D., 105°C; sample: 4-fold-diluted saliva; injection volume: 400 μl.

3. Results and discussion

3.1. Optimization of post-column reaction and detection

The lowest level of salivary cortisol so far reported was around 1 pmol/ml, and therefore, if the absolute amount of saliva required for analysis is to be limited to 0.1 ml, the required sensitivity is about 100 fmol with a signal-to-noise (S/N) ratio of at least 10. In order to achieve this target sensitivity, the reported sensitivity (300 fmol, S/N = 3) achieved by Sudo [22] with the post-column fluorimetric labeling reaction between 11β-hydroxycorticosteroids and sulfuric acid had to be further increased. We therefore examined optimization of the post-column reaction conditions, and the use of laser-induced fluorimetric detection.

Optimization was carried out with a flow-injection system using the mobile phase of 35% acetonitrile, since higher ratios caused an excessive pressure increase in the column, as described elsewhere in this paper. The flow-rate of the mobile phase was fixed at 0.5 ml/min because it was desired to be as low as possible for routine operation.

The effect of the flow-rate of sulfuric acid was examined for a temperature range of 60–120°C using 1, 2.5 and 5.0 m reaction coils with the same 0.25 mm I.D. Maximum peak height was obtained with 0.75 ml/min, while flow-rates of 0.5 and 1.0 ml/min did not show either a maximum or plateau under temperatures over 100°C, and this was fixed for the following experiments. A 10-m coil with 0.25 mm I.D. provided a 5 times higher response than a 2.5 m × 0.5 mm I.D. coil with the same volume. The effect of a 0.25 mm I.D. coil length was also examined for the same temperature range. The peak height obtained with the 1-m coil was the highest, but a plateau was not reached. The 2.5- and 5-m coils showed a plateau over 100°C. Hence a 2.5 m × 0.25 mm I.D. coil was chosen, and the reaction temperature was set at 105°C, which was in the middle of the plateau phase.

These optimized post-column labeling conditions were applied in isocratic HPLC with a CN column as employed by Sudo [22] for comparison. The volume of the flow cell was also increased from 12 μl to 40 μl (this was confirmed to have little effect on the separation). The detection limit achieved was 30 fmol with a S/N of 3. Although this was 10 times higher than that of Sudo, it was still not high enough for our purpose. Application of the laser-induced fluorimetric detector provided a 10-fmol detection limit, which was adequate. With the final

column-switching system, the detection limit was about 8 fmol with a S/N of 3. Calibration curves of cortisol and corticosterone obtained by the final system showed excellent linearity from 0.5 pmol/ml to 20 pmol/ml ($y = 2.647 \cdot 10^4 + 1.1309 \cdot 10^6 x$, $R^2 = 1.000$ and $y = 3.2012 \cdot 10^4 + 8.5025 \cdot 10^5 x$, $R^2 = 1.000$, respectively).

When an ODS column was used instead of the CN column, the final acetonitrile concentration in the gradient elution was approximately 80% and this caused a marked pressure elevation, leading to bursting of the reaction coil. In this case, a make-up with water at 0.3 ml/min flow-rate before addition of sulfuric acid was necessary, and the sensitivity declined to about one-third due to dilution and decrease in reaction efficiency.

3.2. Sampling of saliva and pretreatment

In the beginning of this study, whole saliva instead of partial saliva taken from a specific salivary gland was acquired without any sampling aid. However, several volunteers took 20–30 min even to generate a very small amount of saliva. Later in this study, Salivette, which consists of a cotton swab and a centrifuge tube with a removable insert, into which the swab is placed after use, was employed as a sampling aid. When the cotton swab was held in the mouth for 2 min, at least 1 ml of saliva was acquired consistently. Another advantage of this sampling aid was that the acquired saliva was clear and had lower viscosity. Results for pooled saliva obtained with and without the Salivette are shown in Fig. 2. Although the nature of the acquired saliva was quite different, the only changes with the Salivette sample were a few percent loss of cortisol, some minor interfering peaks around cortisol and a slight base line elevation, as indicated by arrows. As mentioned above, saliva has a high viscosity and some floating insoluble substances such as food residues and dead cells, so at least 2-fold dilution and filtration were needed before injection into HPLC. The influence of the dilution ratio (2-, 4- and 8-fold) of saliva acquired from 5 volunteers at 10 to 11 AM is shown in Table 2. In addition, the influence of injection

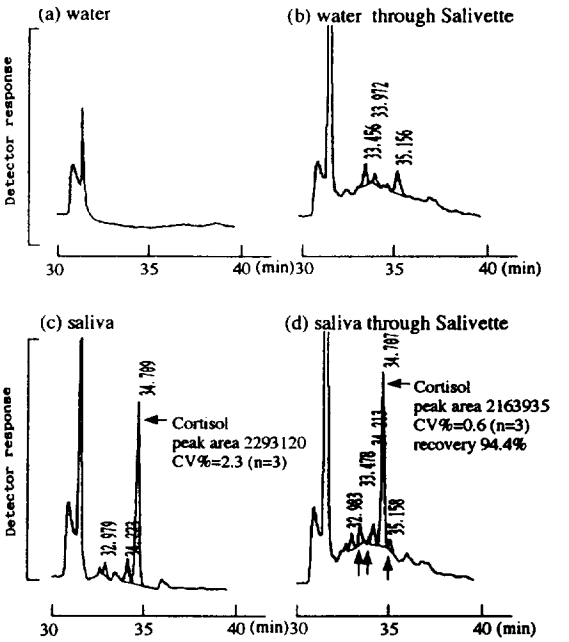

Fig. 2. Chromatograms of saliva and water taken with and without use of the Salivette by the developed automated system. (a) Water: 400 μl injected; (b) saliva: 400 μl injected after 4-fold dilution by the mobile phase; (c) an amount of 2 ml of water was applied to the Salivette, the centrifuged supernatant was diluted 4-fold with the mobile phase; (d) an amount of 2 ml of saliva was applied to the Salivette, the centrifuged supernatant was diluted 4-fold with the mobile phase. Gradient condition: aqueous acetonitrile 10–35% (gradient ratio 10%/min), other analytical conditions were the same as in Table 1.

volume (200, 400 and 800 μl) of filtrate from 2-, 4- and 8-fold diluted saliva, respectively (initial volume of saliva, 100 μl), is shown in Table 3. The results and coefficient of variation were not significantly influenced by either of these factors. Therefore, from the viewpoints of ease of operation, sensitivity and precision, a 400-μl injection after 4-fold dilution was selected as standard procedure.

3.3. Deproteinization on the PCMF column

Proteinaceous substances are present in saliva at a fairly low level, 0.5–1.0% [28], but nevertheless should be removed to ensure stability of the system, because of their hydrophobic adsorption on the reversed-phase column. Per-

Table 2
Influence of dilution ratio of saliva on determination results

Dilution ratio[a]		Cortisol found ($n = 5$) (pmol/ml saliva)				
		Subject A	Subject B	Subject C	Subject D	Subject E
Two-fold	Average	2.832	1.622	3.822	3.829	8.469
	R.S.D. (%)	4.69	9.53	5.29	3.39	2.22
Four-fold	Average	2.886	1.602	3.777	3.325	7.960
	R.S.D. (%)	4.82	7.17	7.75	4.85	3.23
Eight-fold	Average	3.365	1.572	4.056	3.471	7.961
	R.S.D. (%)	3.99	7.79	4.17	9.33	5.74

[a] Injection volume was fixed at 400 μl.

chloric acid deproteinization in combination with ODS bonded silica solid-phase extraction provided an effective clean-up of substances eluted around cortisol as reported in our previous work [25]. For the automated system, a PCMF column, having diol and phenyl groups bonded to silica, was chosen; it was shown to be compatible with repeated (500 times) injections of about 10 ml of serum [27].

The dependence of the retention times of cortisol and corticosterone on the concentrations of acetonitrile and citrate was investigated. Retention times of cortisol and corticosterone decreased from about 22 and 38 min to about 11 and 17 min, respectively, with an increase of the acetonitrile ratio from 0 to 10%, but were not influenced by citrate concentration in the range of 0–40 m*M* (data not shown). Since the cortisol and corticosterone fraction eluted from the PCMF column was to be concentrated at the front of the reversed-phase column, we wished to keep the acetonitrile ratio as low as possible, consistent with a short retention time. Therefore, the acetonitrile concentration was set at 10% and the amount of citrate was set at 2 m*M*. The salivary proteinaceous substances detected by UV were eluted almost at the dead volume as shown in Fig. 3, and the spiked cortisol and corticosterone were well separated from them, with retention times of 11.3 and 16.5 min, respectively. Thus, the fraction from 7 to 15 min for cortisol or from 7 to 22 min for both cortisol and corticosterone was passed to the reversed-phase column for FCS.

3.4. Chromatographic separation

For efficient concentration and separation, a polymer-coated ODS-silica reversed-phase column was expected to be suitable, and a Capcell

Table 3
Influence of dilution ratio of saliva and injection volume on determination results

Injection volume[a] (μl)	Dilution ratio		Cortisol found ($n = 5$) (pmol/ml saliva)				
			Subject F	Subject G	Subject H	Subject I	Subject J
200	Two-fold	Average	3.280	0.953	7.370	4.674	2.793
		R.S.D. (%)	4.98	6.51	4.99	7.63	6.45
400	Four-fold	Average	3.597	0.867	7.626	5.082	3.085
		R.S.D. (%)	4.24	8.66	1.48	2.35	7.45
800	Eight-fold	Average	3.838	0.899	7.578	4.866	3.152
		R.S.D. (%)	3.11	12.90	1.40	3.88	5.45

[a] The volume of saliva was fixed at 100 μl.

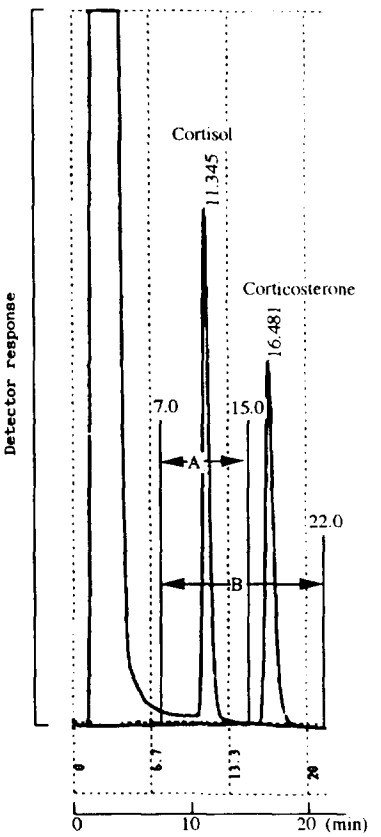

Fig. 3. PCMF chromatogram of saliva spiked with cortisol and corticosterone: (a) introduced into FCS column for analysis of only cortisol; (b) introduced into FCS column for cortisol and corticosterone analysis. Column: Capcell pak MF, 100 × 4.6 mm I.D.; mobile phase: 2 m*M* trisodium citrate, 10% acetonitrile (pH 6.5/HCl), 0.5 ml/min; UV detector: LC-10AD (Shimadzu), 270 nm; sample: 4-fold-diluted saliva spiked with cortisol and corticosterone 100 nmol each/ml; injection volume: 400 μl.

pak C_{18} SG [29] was initially studied. A preliminary study showed that a stepwise gradient from 10 to 80% acetonitrile concentration was unsuitable, but a linear gradient provided well-shaped peaks of cortisol and corticosterone. Selection of the final gradient conditions was done by injecting the saliva sample, based on the need to separate an unknown peak that eluted before cortisol (the structure of this compound is under study). As described earlier, the system using an ODS column required make-up with water for safe post-column reaction. Therefore, in order to simplify the system, we examined the use of a CN column, as employed in our previous study [25] for isocratic separation. The initial and final acetonitrile ratios were set at 10% and 35%, respectively, and it was subjected to the same procedure for selecting the final conditions. In Fig. 4, chromatograms obtained by both systems under their optimal separating conditions are shown for comparison. Obviously, the ODS system provided a better result for the target separation, although the CN system also provided satisfactory results even with several interfering components from the Salivette. The purity of the cortisol peak under the final separating conditions could be ensured with the far inferior sensitivities of sulfuric acid labeling and fluorimetric detection to related compounds such as cortisone and prednisolone, which were found to elute very close to cortisol. The molar sensitivities of cortisone and predonisolone were found to be about 1/27 000 and 1/130 of cortisol, respectively. The level of cortisone in human saliva has been reported to be 2–5 times higher than cortisol [30]. In addition, SIM at *m/z* 363 for cortisol and 361 for cortisone by HPLC–atmospheric pressure chemical ionization (APCI)-MS using 10 ml of saliva with dichlorome-

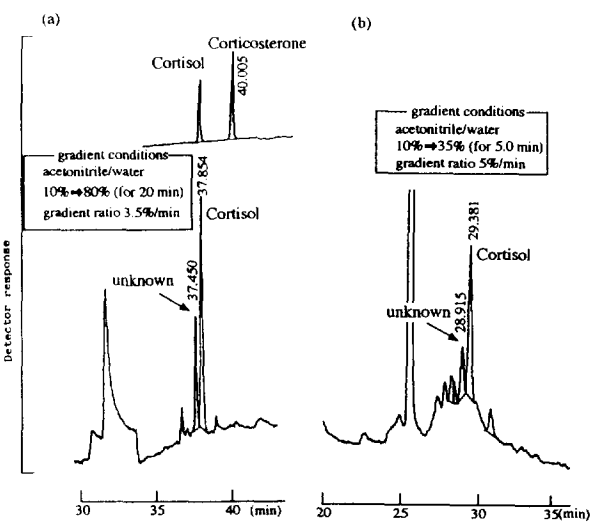

Fig. 4. Separation of cortisol and unknown peaks by the developed system using (a) an ODS column and (b) a CN column. Analytical conditions were the same as shown in Table 1, except for the FCS column and gradient condition.

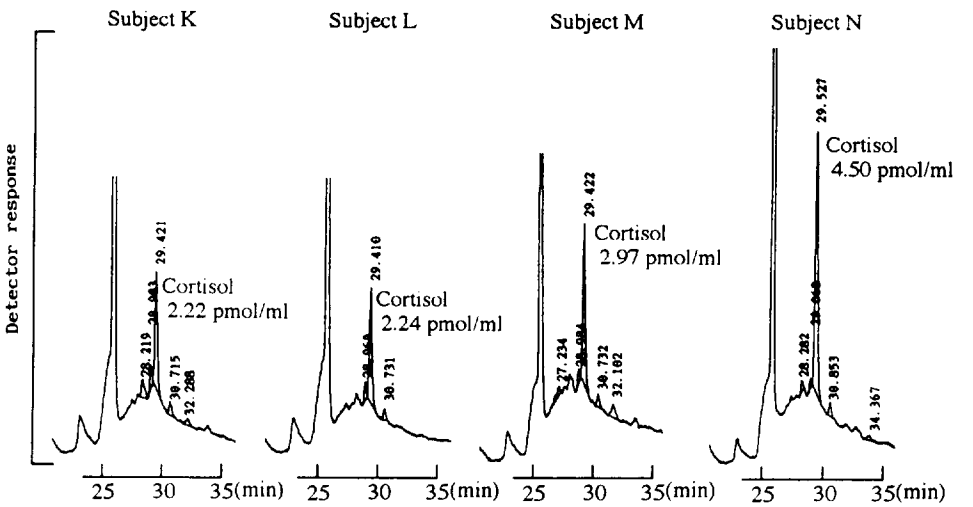

Fig. 5. Several chromatograms of saliva taken by the developed system using a CN column under the final conditions.

thane extraction, confirmed that the cortisol peak and the cortisone peak eluted just after cortisol. The CN system was finally chosen for routine analyses. Since the difference between initial and final acetonitrile concentration was smaller than that in the case of the ODS column, the time required for reconditioning at the initial conditions became 20 min shorter. During this study, involving a total of 17 subjects (Fig. 5, Tables 2–4), there was no indication of the presence of corticosterone at more than the detection limit (80 fmol/ml). The introduction time of cortisol from the PCMF to the CN column could also be shortened by 7 min. With all the above improvements, the analysis time was about 40 min. The results for saliva samples obtained with the final CN system are illustrated in Fig. 5.

Chromatograms of plasma and urine obtained with the ODS system are shown in Fig. 6 with a 100- and 40-fold dilution, respectively. The levels of cortisol were determined as 143.8 and 78.3 pmol/ml, respectively, which are consistent with reported levels. In the case of plasma, there were a few interfering peaks, while in urine, a group of unknown peaks eluted around the

Table 4
Results of recovery studies of salivary cortisol

		Cortisol found ($n = 5$) (pmol/ml saliva)		
		Subject O	Subject P	Subject Q
(1) Saliva	Average	2.481	3.254	4.363
	R.S.D. (%)	2.37	2.38	4.97
(2) Saliva spiked[a]	Average	5.342	6.333	7.299
	R.S.D. (%)	3.84	2.46	1.90
(2) − (1)		2.861	3.079	2.936
Recovery (%)		95.4	102.6	97.9

[a] 5 ml of saliva was spiked with 15 pmol of cortisol (3 pmol/ml saliva).

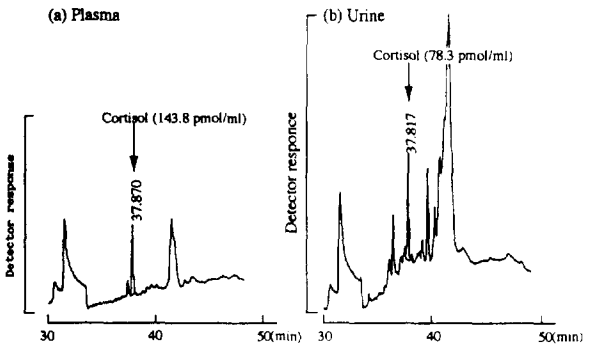

Fig. 6. Chromatograms of plasma and urine samples taken by the developed system. (a) Plasma was diluted 100-fold with mobile phase A and water; (b) urine was diluted 40-fold with mobile phase A and water. Analytical conditions were the same as shown in Table 1, except for the FCS column and gradient condition. FCS column: Capcell pak C_{18}, 250 × 4.6 mm I.D., 40°C; gradient condition: see Fig. 4a. Analytical conditions were the same as shown in Table 1.

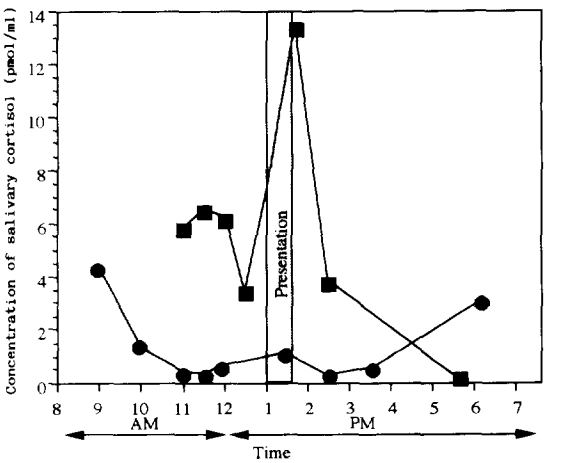

Fig. 7. Result of a preliminary experiment on stress detection. Analytical conditions were the same as shown in Table 1. Subject: male (age 31); ■ = Day of presentation; ● = day after presentation.

retention time of cortisol and corticosterone, which may indicate an advantage of the ODS system.

3.5. Reproducibility and recovery

Cortisol in saliva samples acquired with the Salivette at 10 AM from three healthy volunteers was determined by the automated system. The analytical results are summarized in Table 4. The levels of cortisol at 10 AM were found to be 2.5 to 4.4 pmol/ml, in good agreement with the values by Sudo who has used the same detection system and reported 4.35 pmol/ml as an average of nine healthy volunteers [22]. The recoveries of 3 pmol/ml of cortisol added to the above saliva samples were excellent, as shown in Table 4. The relative standard deviation was less than 5% at the above cortisol level.

3.6. Stability of cortisol in saliva

Saliva samples spiked with 9 pmol/ml cortisol were stored at −85°C and 4°C, and the stability of cortisol was examined. No loss of cortisol was detected at −85°C for at least 19 days, and at 4°C in a refrigerator for at least 18 days. No change in the chromatographic pattern was observed.

3.7. Preliminary experiment on stress detection

In order to confirm further the accuracy of this system and the ability of the system to detect stress, cortisol levels in saliva of a person who was presenting a research report to an audience of over 150 were examined every 30–60 min. A comparison was made between the day of presentation and the day after, taken as a control. As shown in Fig. 7, on the day of presentation, the level was considerably increased even before the start of the presentation, and the maximum (14 pmol/ml) was obtained at the end of the presentation, while the control data followed a circadian rhythm except for a slight increase in the evening. This indicated that the developed system is sensitive and accurate enough for stress detection.

4. Conclusions

The described automated method is suitable for routine analyses of cortisol in saliva. It

requires only a simple and brief sampling of saliva, followed by dilution and filtration, and the analysis time is about 40 min. It is accurate and sensitive enough to allow investigation of the relationship between cortisol level and stress level with only a sample size of 0.1 ml of saliva.

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