Study of salivary catecholamines using fully automated column-switching high-performance liquid chromatography

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Abstract

Cortisol and catecholamines are major physiological markers of human stress. In order to establish a fully automated assay system for both cortisol and catecholamines in saliva, which can be sampled without imposing stress, the previously developed system for salivary cortisol [Okumura et al., J. Chromatogr. B, 670 (1995) 11] was modified. The practical sensitivity was around 0.1 pmol ml^{-1} for norepinephrine and epinephrine and 0.5 pmol ml^{-1} for dopamine. The established assay procedure provided R.S.D. values of 2~3% and recoveries of 96~104% at 0.5 pmol ml^{-1}. Measurement of salivary catecholamines in more than 300 samples taken from about 50 healthy volunteers indicated that the normal values of norepinephrine and dopamine were very low, about 0.1 pmol ml^{-1} each. In contrast to cortisol, salivary catecholamine levels did not parallel those in plasma. Nevertheless, since levels of salivary catecholamines may reflect the sympathetic nerve activity in the salivary gland, they were assayed in volunteers making a scientific presentation before a large audience. Four out of eleven volunteers reported strong feelings of fear or anxiety, and their salivary catecholamine levels were about ten times higher than normal. © 1997 Elsevier Science B.V.

Keywords: Catecholamines; Cortisol; Norepinephrine; Epinephrine; Dopamine

1. Introduction

Human stress is generally considered to be reflected by two biochemical (physiological) markers, catecholamines (CA) and coltisol (CS). Canon defined the 'emergency reaction', regarding an accelerated secretion of CA as a marker of an emergency situation [2]. Selye [3] proposed the general adaptation syndrome theory, in which an accelerated secretion of CS is considered as a marker of invasive stimulation. Therefore, in our attempts to measure levels of human stress, CA and CS were selected as targets. Among several biological fluids, we chose to use saliva, because saliva sampling is stress-free, in contrast to blood sampling and saliva should reflect responses to stress more directly and rapidly than urine, which affords a kind of cumulative or averaged response. In our previous paper [1], we described the fully automated assay of salivary CS using a column-switching HPLC system with a polymer coated mixed functional (PCMF) column for deproteinization and a CN or ODS column for frontal concentration followed by gradient separation. The system was equipped with a laser-induced

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fluorimetric detector which provided more than enough sensitivity to determine 0.1–1.0 pmol ml^{-1} of CS in a 400-μl aliquot of 4-fold diluted saliva. Some assay methods for CA in saliva have been reported, but without detailed discussion of the analytical methods [4–6], and with differing results. In order to extend our CS assay system to CA, we therefore started from scratch, although individual technologies necessary for concentration, separation and highly sensitive detection of CA [7–25], especially in blood and urine, are available and are applicable to CA in saliva.

In this report, the results of our extensive examination on each process needed to determine automatically CA in saliva are described. The finally established method was used to investigate the behavior of CA in saliva of normal volunteers and to examine the influence of stress.

2. Experimental

2.1. Chemicals and materials

Norepinephrine (4-(2-amino-1-hydroxyethyl)-1,2-benzenediol, NE), epinephrine (4-[hydroxy-2-(methylamino) ethyl]-1,2-benzenediol, E) and glutathione (N-(N-L-g-glutamyl-L-cysteinyl) glycine (reduced form)) were purchased from Sigma (St. Louis, MO, USA). Dopamine (4-(2-aminoethyl)-1,2-benzenediol, DA) hydrochloride, sodium methylate (28% in methanol) and EGTA (ethyleneglycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid) were from Nacarai Tesque (Kyoto, Japan). 1,2-Diphenylethylenediamine (DPE) was from Tosoh (Tokyo, Japan). Trisodium citrate and potassium ferricyanide were from Wako (Osaka, Japan). Sodium dodecyl sulfate (SDS) of HPLC grade was from Kanto (Tokyo, Japan). Methanol of HPLC grade was from Nacarai Tesque (Kyoto, Japan). Water used in all experiments was filtered through a Milli-Q system (Millipore, Molsheim, France). Salivette and citric acid-impregnated Salivette sampling aids for saliva were obtained from Sarstedt (Rommelsdorf, Nümbreche, Germany).

2.2. Solutions

Stock solutions of 1 μmol ml^{-1} of NE, E and DA were prepared in 0.1 M HCl, and were stored at $-85°C$. The stock solutions were diluted to desired concentrations before use with the mobile phase A containing 0.9 mg ml^{-1} glutathione and 0.6 mg ml^{-1} EGTA. An aqueous solution of 90 mg ml^{-1} glutathione and 60 mg ml^{-1} EGTA was used as an inhibitor solution to stabilize CA in saliva.

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 six pumps (Shimadzu LC-10AD, Shimadzu, Kyoto, Japan), A for the
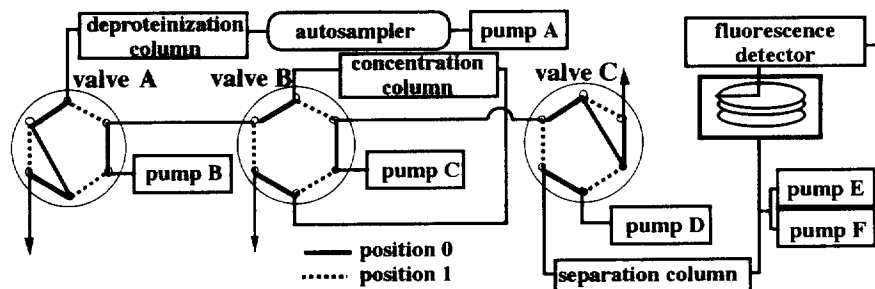

Fig. 1. Schematic diagram of the column switching HPLC system. Pump A (LC-10AD, Shimadzu): 5% methanol, 15 mM trisodium citrate, 2 mM SDS, pH 7.5/HCl; pump B (LC-10AD, Shimadzu): 5% methanol, 40 mM trisodium citrate, pH 7.5/HCl; pump C (LC-10AD, Shimadzu): 5% methanol, 40 mM trisodium citrate, pH 3.5/HCl; pump D (LC-10AD, Shimadzu): 5% methanol, 40 mM trisodium citrate, pH 3.5/HCl; pump E (LC-10AD, Shimadzu): 70% ethanol, 3 mM potassium ferricyanide; pump F (LC-10AD, Shimadzu): 70% ethanol, 5 g l^{-1} DPE, 20 g l^{-1} sodium methylate (28% in methanol); reaction coil: 50 m×0.25 mm I.D. (stainless steel), 95°C; detection: fluorescence detector (F-1080, Hitachi), ex. 350 nm, em. 480 nm.

mobile phase A of the deproteinization (DP) column (TSKprecolumn PW 35×4.6 mm I.D., Tosoh), B and C for the adsorption and desorption mobile phases B and C, respectively, of the clean-up and concentration (C) column (TSKgel Boronate-5PW 75×7.5 mm I.D., Tosoh), D and E for two fluorogenic reagents and F for the separation (S) column (Senshu Pak SCX 1251-N 250×4.6 mm I.D., Senshu Science, Tokyo, Japan), which were connected with three six-way switching valves (LV-306, Lab-Quatec, Tokyo, Japan) and four-way conventional joints. For the other parts of the system, an auto-sampler (Shimadzu SIL-10A) equipped with a refrigerated sample tray, a column oven (Shimadzu CTO-6A), a reaction oven (Shimadzu CRB-6A) with a stainless steel reaction coil (50 m×0.25 mm I.D.), a fluorescence detector (Hitachi F-1080 ex. 350 nm, em. 480 nm, Hitachi, Tokyo, Japan) and an integrator (Shimadzu C-R4A) were used and all of the system parts were controlled by a system controller (Shimadzu SCL-10A).

With the all valve positions at 0, samples, injected by an auto-sampler every 45 min, were introduced into the DP column, through which salivary proteins and CA and CS fractions were separated with mobile phase A. Simultaneously, mobile phase B flowed into column C for initial conditioning. The salivary proteins fraction was discarded and the position of valve A was switched from 0 to 1 to heart-cut the CA fraction and introduce it into column C. Then, valve A was switched back to position 0 to clean column C with mobile phase B. After that, valve B was switched to position 1 to back-flush the concentrated CA with mobile phase C, and the desorbed CA (as a narrow band) was introduced into column S for separation by switching and switching back valve C.

2.4. Chromatographic conditions

The same composition of methanol and water (5:95, v/v) was used for all mobile phases, A, B, C and D. Mobile phase A contained 15 mM trisodium citrate and 2 mM SDS adjusted to pH 7.5 with HCl. The mobile phases B, C and D all contained 40 mM trisodium citrate of pH 7.5, 3.5 and 3.5 respectively. The flow-rates of the mobile phases A, B, C and D were 1.0, 1.5, 0.8 and 0.8 ml min^{-1}, respectively. The flow-rates of the two fluorogenic reagent solu-

Table 1

Time program of switching valves and conditions for the automated system

Time (min)	Condition
0.0	All valve positions 0
1.4	Valve A position 1
5.2	Valve A position 0
9.2	Valve B position 1
13.2	Valve C position 1
15.2	Valve C position 0
20.7	Valve B position 0
	Stop

Deproteinization column: TSK precolum PW, 35 mm×4.6 mm I.D., 40°C; pump A (mobile phase A): 5% methanol, 15 mM trisodium citrate, pH 7.5/HCl, 1.0 ml min^{-1}; concentration column: TSKgel Bronate-5PW, 75 mm×7.5 mm I.D., 40°C; pump B (mobile phase B): 5% methanol, 40 mM trisodium citrate, pH 7.5/HCl, 1.5 ml min^{-1}; pump C (mobile phase C): 5% methanol, 40 mM trisodium citrate, pH 3.5/HCl, 0.8 ml min^{-1}; separation column: Senshu Pak SCX, 250 mm×4.6 mm I.D., 40°C; pump D (mobile phase D): 5% methanol, 40 mM trisodium citrate, pH 3.5/HCl, 0.8 ml min^{-1}; pump E (oxidizing reagent): 70% ethanol, 3 mM potassium ferricyanide, 0.3 ml min^{-1}; pump F (fluorigenic reagent): 70% ethanol, 5 g l^{-1} DPE, 20 g l^{-1} sodium methylate (28% in methanol), 0.3 ml min^{-1}; reaction coil and temperature: 50 m×0.25 mm I.D., 95°C; fluorescence detector: Hitachi F-1080, ex. 350 nm, em. 480 nm; sample: 2-fold-diluted saliva; injection volume: 800 μl.

tions were 0.3 ml min^{-1}. The time program is shown in Table 1.

2.5. Analytical procedure

Saliva samples were acquired by simply holding the cotton swab of a Salivette in the mouth for 2–3 min, putting it back in the tube and centrifuging at 1300 g for 30 s, after mouth rinsing and gargling with plain water followed by a rest period of about 15 min. After immediate addition of a 10-μl aliquot of the inhibitor solution, 90 mg ml^{-1} glutathione and 60 mg ml^{-1} EGTA, per 1 ml of saliva, acquired saliva samples were stocked at −85°C in a freezer until analysis.

The sample solutions for free CA measurement were prepared by adding exactly 1.0 ml of mobile phase A with inhibitor to exactly 1.0 ml of saliva sample thawed at room temperature. For total CA (free CA+conjugated CA) measurement, the thawed saliva was hydrolyzed with hydrochloric acid accord-

ing to the method reported by Demasieux et al. [26] and then diluted in the same manner as above. The mixture was filtered with a disposable membrane filter, and an 800-μl aliquot of the filtrate was injected into the HPLC system.

3. Results and discussion

3.1. Deproteinization on the gel permeation column

For direct assay of components in biological fluids by HPLC, deproteinization is indispensable to ensure stability of the system, although proteinaceous substances are present in saliva at the fairly low level of 0.5–1.0% [27]. In our automated assay of salivary CS [1], a PCMF column based on silica [28] was successfully employed without deterioration during more than 1000 analyses. Therefore, the composition of the mobile phase was modified to allow retention of CA by simply adding an ion pairing agent; this provided a very good separation of proteinaceous substances, CA and CS, as shown in Fig. 2. However, when it was incorporated into the system, the recovery of 1 pmol of CA was very low, around 10%. This phenomenon was thought to be due to oxidation of CA caused by a trace amounts of metals contained in the packing material. This was confirmed by injecting E at the nmol level and observing its UV spectrum with a photodiode array detector; E was converted to its oxidized form, adrenochrome. In order to prevent such oxidation during deproteinization, the use of a gel permeation column (PW column) was studied under a similar mobile phase condition. A chromatogram obtained under optimized conditions is shown in Fig. 3, where CA was well separated from both proteinaceous substances and CS with retention times of 2.4, 2.4 and 3.3 min for NE, E and DA, respectively. So the CA fraction from 1.4 to 5.2 min was heart-cut and passed to the concentration column discussed in Section 3.2. The CS fraction was found as a broad peak from 5.2–11.7 min, though it should be concentrated at the front of either an ODS or a CN column. Namely, the PW column can also be used in our previously developed CS system [1].
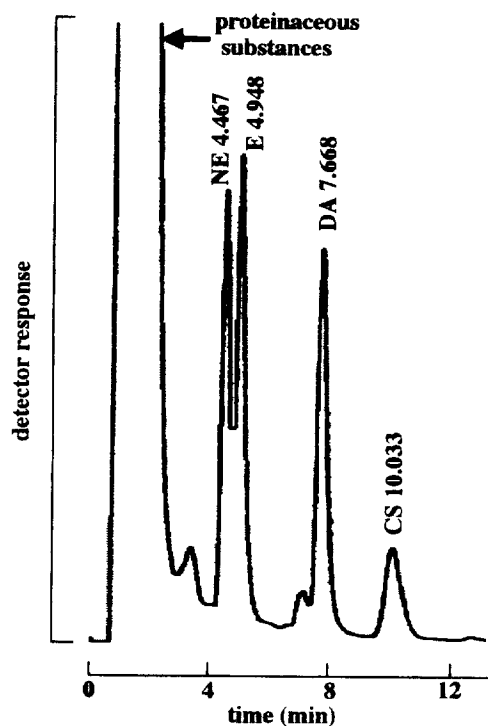

Fig. 2. PCMF column chromatogram of saliva spiked with catecholamines and cortisol. Column: Capcell pak MF, 100×4.6 mm I.D.; mobile phase: 5% methanol, 2 mM SDS, 30 mM trisodium citrate, pH 7.5/HCl; 1.0 ml min⁻¹; UV detector: SPD-10AV (Shimadzu), 270 nm; sample: 4-fold-diluted saliva spiked with catecholamines and cortisol 10 nmol each ml⁻¹; injection volume: 400 μl.

3.2. Concentration on the phenylboronate column

Manual concentration of CA in biological fluids has frequently been achieved using alumina [8,20,21], but always with a low recovery of around 60%. Other methods reported include the use of a cation-exchange resin [13,17], boric acid [22], boronic acid gel [23,24], etc. As described elsewhere, we selected for CA separation a cation-exchange column under the mobile phase condition of citrate buffer (pH 3.5), so a different mode of concentration was thought to be better for clean-up purposes. One alumina column (LiChrosorb Alox T (Merck, Darmstadt, Germany)), and two phenylboronate (PB) columns, silica gel-based (PB-s column: SelectiSpher-10 (Hyclone, Lund, Sweden) packed by ourselves) and polymer gel-based (PB-p column:
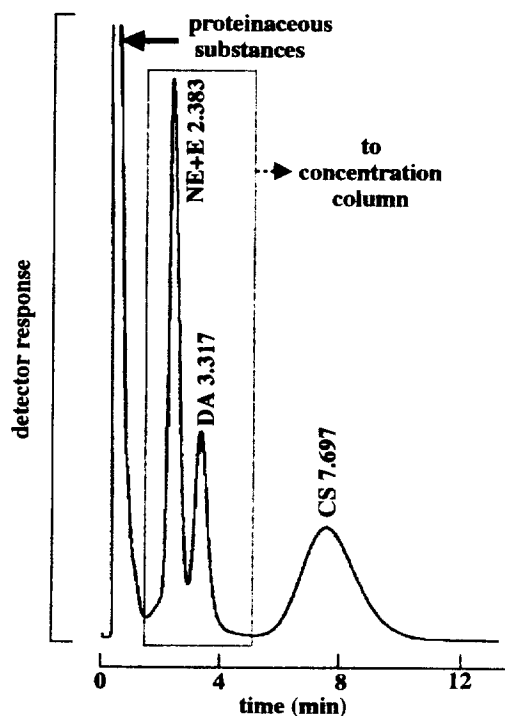
Fig. 3. PW column chromatogram of saliva spiked with catechol-amines and cortisol. Column: TSKprecolumn PW, 35×4.6 mm I.D.; mobile phase: 5% methanol, 2 mM SDS, 15 mM trisodium citrate, pH 7.5/HCl; 1.0 ml min^{-1}; UV detector: SPD-10AV (Shimadzu), 270 nm; sample: 4-fold-diluted saliva spiked with catecholamines and cortisol 10 nmol each ml^{-1}; injection volume: 400 μl.
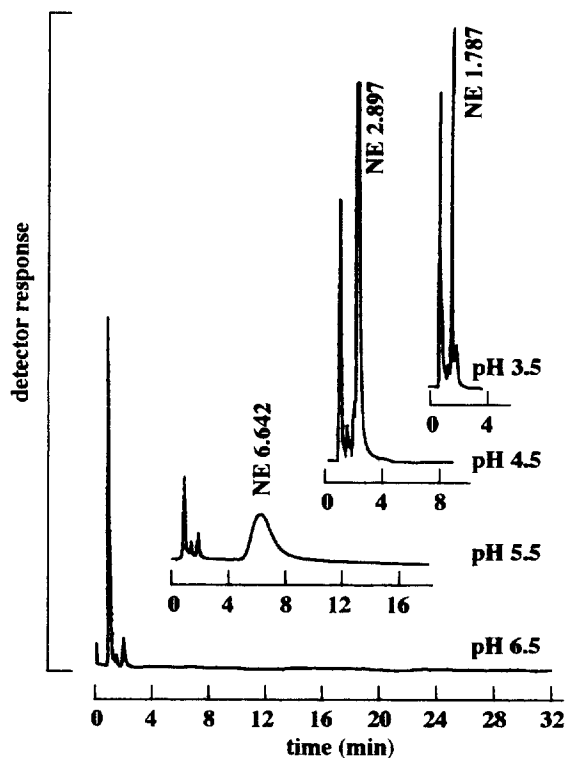

Fig. 4. PB-s column chromatograms of norepinephrine under various pH conditions. Column: SelectiSpher-10, 100×4.6 mm I.D. (packed by ourselves); mobile phase: 5% methanol, 40 mM trisodium citrate, pH was adjusted with HCl, 0.8 ml min^{-1}; UV detector: SPD-10AV (Shimadzu), 270 nm; sample: 1 μmol ml^{-1}; injection volume: 10 μl.

TSKgel Boronate-5PW), were examined using the above mobile phase for separation. CA was strongly retained on the alumina column, while the two PB columns eluted CA quickly at pH 3.5 and retained it completely at pH 6.5, as shown in Fig. 4. In order to establish the on-line system, the PB column was placed so that the concentrated CA can be back-flushed as a sharp band and introduced into column S. The pH of the desorbing mobile phase was fixed at 7.5 based on the above results.

Chromatograms obtained by the system and re-corded by the detector placed in front of the sepa-ration column are illustrated in Fig. 5, which shows that CA was eluted as a single, sharp peak. When both PB columns were tried in the final system with post-column fluorimetric detection, the PB-s column showed significant interference even after several cleaning processes, although it was successfully

utilized with electrochemical detection [23]. So we selected the PB-p column for our system, and the CA fraction was heart-cut and introduced into column S from 4–6 min after switching the valve for back-flush.

3.3. Separation and system sensitivity

An ODS column in ion-pair mode and a cation-exchange column have frequently been used for the separation of CA and both have been combined with the most frequently used post-column fluorimetric detection using DPE, reported by Jeon et al. [25]. We employed a cation-exchange column in our previous study [19] using the off-line alumina column method for preliminary screening of CA in saliva on the grounds that it may provide higher selectivity for CA
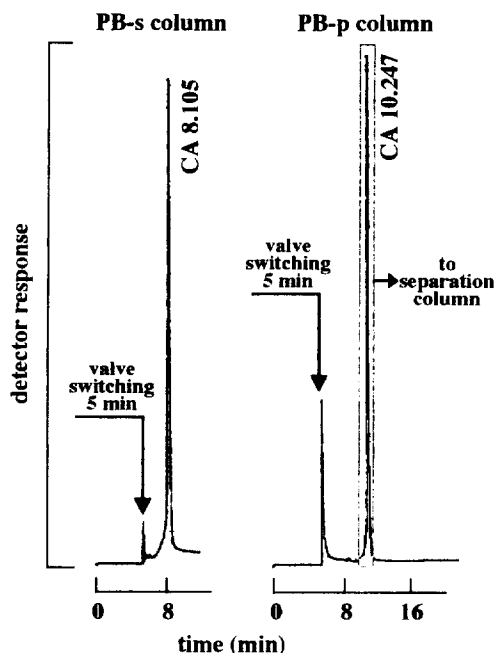
Fig. 5. PB-s and PB-p column chromatograms of catecholamines using the back-flush method. PB-s column: SelectiSpher-10, 100× 4.6 mm I.D. (packed by ourselves); PB-p column: TSK gel Boronate-5PW, 75×7.5 mm I.D.; mobile phase for adsorption: 5% methanol, 40 mM trisodium citrate, pH 7.5/HCl, 0.8 ml min^{-1}; mobile phase for desorption: 5% methanol, 40 mM trisodium citrate, pH 3.5/HCl, 0.8 ml min^{-1}; valve switching timing: 5 min; UV detector: SPD-10AV (Shimadzu), 270 nm; sample: 1 μmol each ml^{-1}; injection volume: 10 μl.

and can be operated without the ion pairing agent, which may cause a higher background on fluorimetric detection. Among six cation-exchange columns tested, Senshu Pak SCX (SCX) gave the best result here, as demonstrated by chromatograms obtained on the final system and shown in Fig. 6.

After optimization, the system sensitivity was determined to be 6, 5 and 12 fmol with the signal-to-noise (S/N) ratio of 3 for NE, E and DA, respectively, corresponding to 15, 12.5 and 30 fmol ml^{-1} saliva.

3.4. Stabilization of CA in saliva

Stabilization of CA in plasma and urine has been achieved by adding glutathione and EGTA [29,30] and sodium thiosulfate [20], but there has been no

report on the stability of CA in saliva, although its presence in saliva has been noted [5].

Saliva samples spiked with 1 pmol ml^{-1} of CA were stored at room temperature and 4°C, and the stability of CA was examined as shown in Fig. 7. CA in saliva decomposed quickly and even at 4°C, an assay should be done within 1 h to guarantee a recovery of over 90%. Stabilization by adding glutathione (reduced form)/EGTA as reported by Schwab [5] and storage at −85°C proved to be effective for at least three and half months.

3.5. Calibration, reproducibility and recovery

The calibration curves were linear from 0.05 to 1.00 pmol ml^{-1} for NE, E and DA ($y=8.5236\cdot10^3+1.9590\cdot10^6x$ ($r^2=1.000$), $y=6.9102\cdot10^3+3.4181\cdot10^6x$ ($r^2=1.000$) and $y=9.7791\cdot10^3+1.6270\cdot10^6x$ ($r^2=0.999$), respectively, where y and x are the detector response (peak area mV s^{-1}) and concentration of CA (pmol ml^{-1}), respectively).

Reproducibility and recovery of the developed system were examined using two 12-ml pooled saliva samples. Each pooled saliva sample was divided into two and one half was used as the spiked sample (0.5 pmol CA ml^{-1}) and the other as the intact sample. Determination was carried out 5 times for each sample according to the established procedure. Calibration curves were obtained over the range of 0.05–1.00 pmol ml^{-1}. The analytical results are summarized in Table 2. The recovery of 0.5 pmol ml^{-1} of CA, which is close to the reported level of salivary CA [1], was excellent, around 95% or more. The relative standard deviation was also excellent for CA at the 0.5 pmol ml^{-1} level. It became greater at the 0.1 pmol ml^{-1} level or less found in intact samples, probably due to the system sensitivity. Taking these results into account, the practical sensitivity was thought to be around 0.1 pmol ml^{-1} for NE and E and 0.5 pmol ml^{-1} for DA.

The values of intra-day precision ($n=4$) for a 0.5 pmol ml^{-1} standard sample, expressed as R.S.D., were 3.19% for NE, 1.97% for E and 1.97% for DA.

The robustness of the developed system depends on the SCX column, which had a lifetime of about three weeks (about 600 samples). The other columns, PW and PB-p columns, were stable for more than two months.
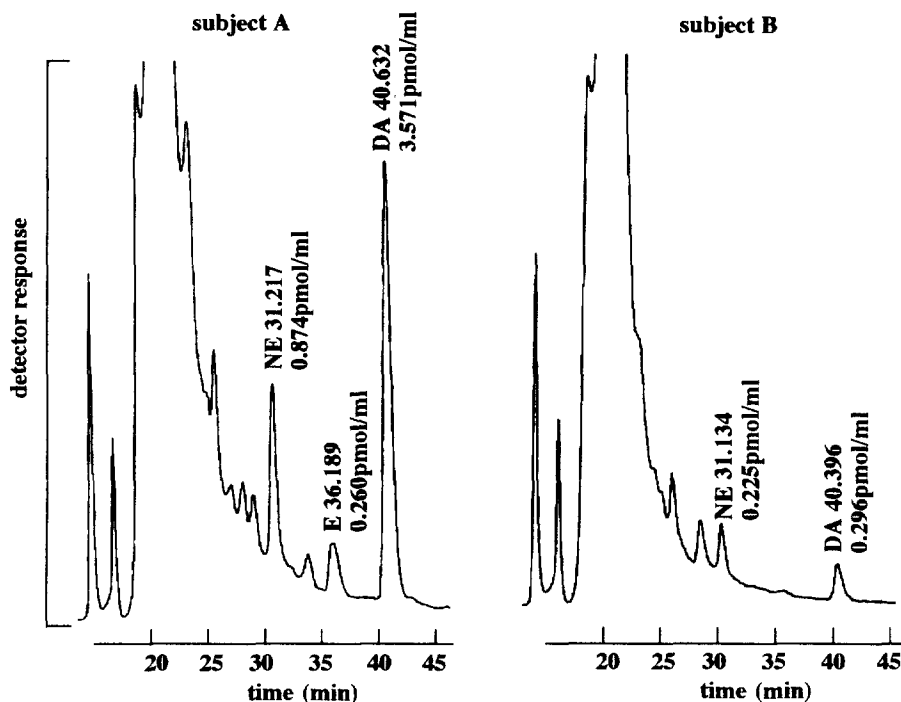
Fig. 6. Chromatograms of saliva taken by the developed system under the final condition. Analytical conditions were the same as shown in Table 1; sample: 0.5 pmol each ml^{-1}; injection volume: 800 μl.
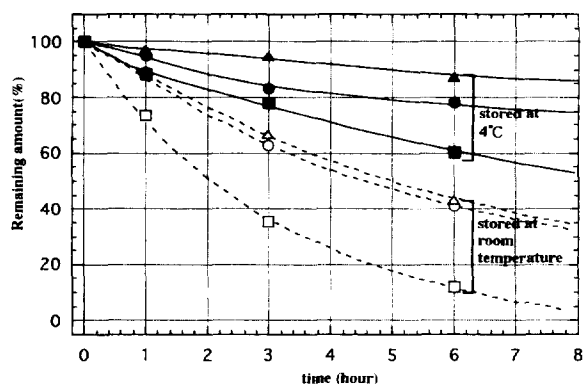

Fig. 7. Stabilities of catecholamines in saliva stored at room temperature and 4°C. Analytical conditions were the same as shown in Table 1; sample: 2-fold-diluted saliva spiked with 1.0 pmol ml^{-1} of catecholamines; injection volume: 800 μl; ○=NE, □=E and △=DA stored at room temperature. ●=NE, ■=E and ▲=DA stored at 4°C.

3.6. Behavior of CA in saliva

3.6.1. Normal salivary CA

To our knowledge, a normal level of CA (normal value) in saliva has never been established in a large-scale study. McClelland et al. reported the presence of NE at about 1.5 pmol ml^{-1} in 35 of 46 college students and the level increased to about 2.5 pmol ml^{-1} immediately after an examination, but there was no information on the analytical method except a simple statement that analyses were conducted by HPLC [4]. Schwab et al. [5] reported a level of 0.2–2.0 pmol ml^{-1} in 12 healthy volunteers by using the modified radioenzymatic assay originally reported by Peuler and Johnson [31], but again there was no mention of the selectivity of the method.

In order to establish a normal level of CA using our developed and verified system, saliva samples were collected from 30 healthy volunteers (17 males and 13 females, average age 30 years old) at 10 am, 1 pm and 4 pm. Further, another 8 volunteers (4

Table 2
Results of recovery studies of salivary CA

| | | CA found ($n=5$) (pmol ml^{-1} saliva) | | | | | |
| | | Subject F | | | Subject G | | |
		NE	E	DA	NE	E	DA
(1) Saliva	Average	0.100	n.d.	n.d.	0.071	n.d.	0.143
	R.S.D. (%)	6.77	–	–	11.07	–	23.65
(2) Saliva spiked[a]	Average	0.583	0.517	0.491	0.568	0.515	0.643
	R.S.D. (%)	1.72	1.94	1.93	1.52	1.22	2.90
(2)−(1)		0.483	0.517	0.491	0.497	0.515	0.500
Recovery (%)		96.6	103.5	98.2	99.4	103.0	100.0

[a] 6 ml of saliva was spiked with 3 pmol of each CA (0.5 pmol ml^{-1} saliva).

males and 4 females, average age 30 years old) were traced for five days at 7 am and midnight (0 am) in addition to the above three points. The results are summarized in Fig. 8 for each time point (40 measurements for 7 and 0 am, and 70 for 10 am, 1 pm and 4 pm). There was no apparent time dependency (circadian rhythm), in contrast to the case of salivary CS. E was detected at a fairly low level, almost at the detection limit of the system and only in about 50% of the samples at 7 and 0 am, and 10–20% of the samples at other time points. DA was found at the highest level of about 0.3 pmol ml^{-1} and NE at a little less than 0.1 pmol ml^{-1}, which are not in agreement with any of the above reported values [4,5]. However, since we employed our verified analytical system, we believe that our data provide a valid baseline for future studies. Data listed at the top of each section in Fig. 8 were considered as an 'abnormal values' which far exceeded +3 S.D. and were 10 or more times higher than normal values. Since all the volunteers had been asked to take saliva samples on an ordinary day when there were no special events on that day or the day before, these abnormal values may reflect sudden emotional or psychological changes, which are the prime target of our study to measure human stress. The sensitivity of our system was proved to be sufficient for the putative normal values of NE and DA, and is more than enough for higher, abnormal values. In Table 3, averages and S.D. values of NE and DA are shown for all subjects and for the 8 individuals involved in the 5-day experiment to see if establishment of individual normal values is necessary to evaluate more accurately the

stress status. Averages were markedly different from each other, and the standard deviations of individuals were apparently smaller than that of all data. However, abnormal values are so much higher that the establishment of individual normal values is thought to be unnecessary.

There was no statistically significant difference between males and females, and the normal values calculated after excluding the putative abnormal values were less than 0.17, 0.01 and 0.45 pmol ml^{-1} for NE, E and DA, respectively.

3.6.2. Correlation between salivary and plasma CA

In order to investigate the correlation between salivary and plasma CA, 4 young male volunteers (average age: 27 years) were assigned hard exercise using an ergometer, which is known to increase plasma CA level. The level of exercise was set based on preliminary experiments using plasma lactic acid as a threshold marker and was classified as easy or hard. The experiment was repeated for two days, but the order of the easy and hard exercises was reversed on day 2. The results are summarized in Fig. 9 with normalized values based on the value of the first measurement taken as 1.0. The plasma CA showed a clear elevation during hard exercise and this was reproduced on day 2, and there was a slight elevation during the easy exercise conducted before the hard one, which indicated that the experimental design is appropriate. However, there was no correlation of plasma CA with salivary CA, even when the possibility of lag time was taken into consideration. Increases of NE and DA were observed during
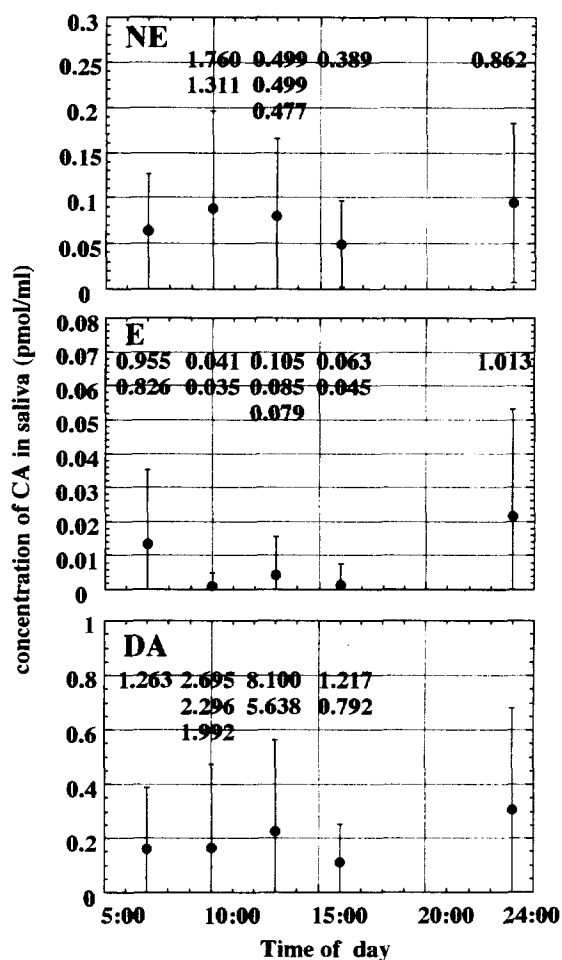
Fig. 8. Intra-day changes of catecholamines in saliva. Analytical conditions were the same as shown in Table 1; sample: 2-fold-diluted saliva; injection volume: 800 μl; ●=average of 40 measurements from 8 subjects at 7 am and 0 am, and 70 measurements from 38 subjects at 10 am, 1 pm and 4 pm; error bar=1 S.D.; the numbers indicated on the upper area of the graphs are the abnormal values (pmol ml⁻¹) (see the text).

exercise, independently of the level of exercise, but were not reproducible. These results indicated that the source of salivary CA is not permeation of plasma CA through the salivary gland, in contrast to salivary CS, which was reported to correlate well with free plasma CS [32]. The results were in agreement with the report by Schwab et al. [5], who conducted a similar experiment involving E infusion. In addition, total CA including conjugated CA was examined in 5 volunteers. The rates of conjugation

for NE and DA were close to those reported in plasma (data not shown), but this dose not necessarily imply the dependence of salivary CA on plasma CA.

Since salivary CA may reflect diffusion of transmitter CA from the synaptic cleft into saliva, stimulation of the salivary gland by citric acid was attempted in two volunteers, one male and one female. Saliva samples were taken 4–5 times at 15-min intervals, but only one was taken using a citric acid-impregnated Salivette, without prior notice to the volunteers, and this schedule was repeated for two days. The rate of saliva secretion was more than doubled by citric acid stimulation, but the CA concentration was not much affected and stayed within the normal range, as shown in Fig. 10, though total CA secreted showed a sharp increase because of the increased rate of saliva secretion per se. Thus, salivary CA could be a marker of the activity of the sympathetic nerve system, which is also affected by stress.

3.6.3. Preliminary experiment on stress detection

The sympathetic nerve system is stimulated in various emergency situations, but such situations are difficult to create experimentally for humans. Therefore, a lecture or presentation before a big audience was again chosen, as was done in our previous study on salivary CS [1], which showed a sharp increase right after the presentation in many cases. Eleven subjects were examined at three time points, 10 min before the start of the presentation, right after, and at least 1 h after the presentation. Along with the sample collection, a questionnaire was given at each time point to evaluate emotional, psychological and physical status. Emotional and psychological status was classified into 9 categories, i.e., anger, fear and anxiety, joy, dislike and hate, sadness, energetic excitation, spiritless and inactive stagnation, tension and comfort. The results are shown in Fig. 11. Four out of eleven cases showed increases of NE and DA to the abnormal level defined in Section 3.6.1. before the presentation and elevation of E after it. No changes were observed in the other cases. Analysis of the questionnaire revealed that all 4 cases had reported strong fear or anxiety. This finding may support the usefulness of the developed CA system,

Table 3
Averages and S.D. values of salivary NE and DA of 8 individuals for 5 days (pmol ml^{-1})

Subject	Time	NE			DA		
		10:00	13:00	16:00	10:00	13:00	16:00
C	Average	0.0064	0.0219	0.0050	n.d.	0.0510	n.d.
	S.D.	0.0143	0.0207	0.0112	–	0.0947	–
D	Average	0.0564	0.0912	0.0316	0.0448	0.2055	0.1263
	S.D.	0.0519	0.0082	0.0447	0.0474	0.0809	0.0269
E	Average	0.0576	0.0845	0.0419	0.0793	0.1894	0.0703
	S.D.	0.0275	0.0259	0.0290	0.0543	0.0781	0.0693
F	Average	0.0797	0.0657	0.0298	0.2977	0.3632	0.0762
	S.D.	0.0460	0.0274	0.0256	0.1125	0.1891	0.0715
G	Average	0.0634	0.0862	0.0367	0.0654	0.1902	0.0900
	S.D.	0.0881	0.0289	0.0519	0.0563	0.1413	0.0923
H	Average	0.0377	0.0117	0.0139	0.0781	0.1396	0.1196
	S.D.	0.0471	0.0110	0.0278	0.0745	0.2185	0.0379
I	Average	0.1048	0.0861	0.0744	0.9190	0.4268	0.1121
	S.D.	n.a.[a]	n.a.[a]	n.a.[a]	n.a.[a]	0.1486	0.0445
J	Average	0.0146	n.a.[b]	0.0083	0.0199	0.0166	0.0332
	S.D.	0.0208	–	0.0186	0.0446	0.0332	0.0383
Normal value	Average	0.0888	0.0797	0.0497	0.1630	0.2236	0.1105
(38 subjects)	S.D.	0.1077	0.0860	0.0472	0.3116	0.3385	0.1444

All the values shown here are those calculated after exclusion of data considered (see text).
n.d.; not detected in all samples.
[a] Only one value available.
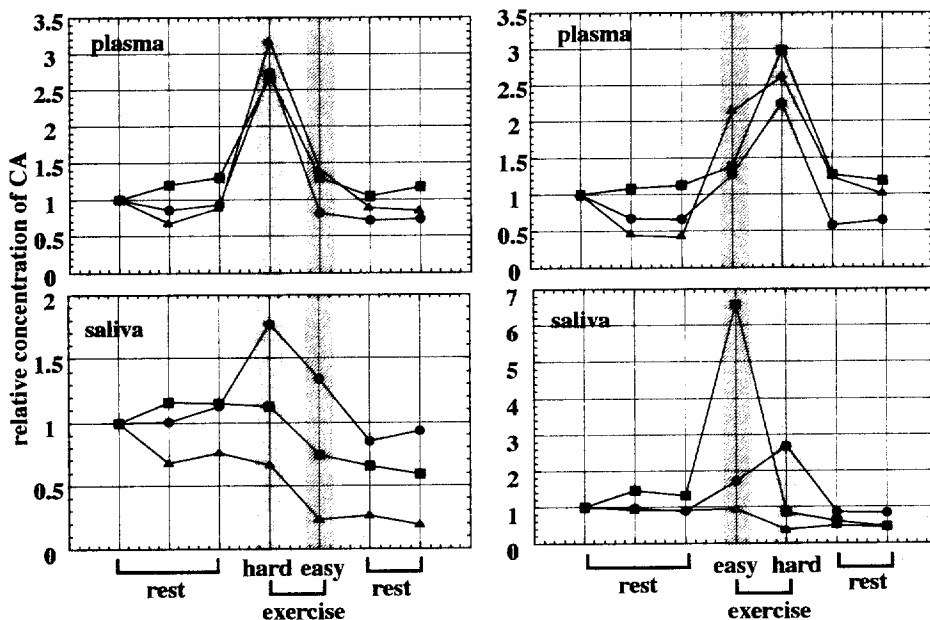[b] Sample was not available in all cases.

Fig. 9. Comparison of catecholamines in plasma and saliva under physical stress. Analytical conditions were the same as shown in Table 1; sample: 2-fold-diluted saliva; injection volume: 800 μl; ●=NE, ■=E and ▲=DA (average of 4 subjects).
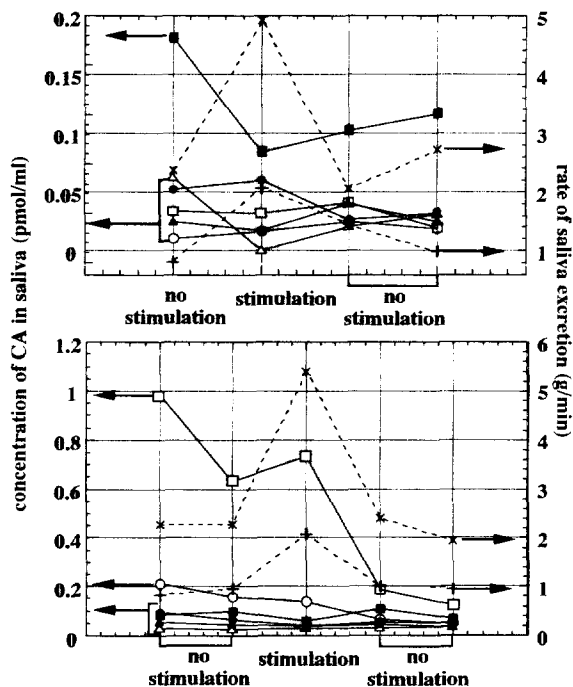
Fig. 10. Concentrations of catecholamines in saliva and rate of saliva excretion under citric acid stimulation. Analytical conditions were the same as shown in Table 1; sample: 2-fold-diluted saliva; injection volume: 800 μl; O=NE, □=E, △=DA and x=excretion rate of female; ●=NE, ■=E, ▲=DA and +=excretion rate of male.

along with the CS system, for the measurement of the human stress.

4. Conclusion

The automated method we have established is suitable for routine analyses of CA in saliva and has enough sensitivity to measure the normal values at the 0.1 pmol ml^{-1} level established in a fairly large-scale experiment. NE and DA were always detected in more than 300 samples, but the frequency of E detection was 10–50%, independently of the time of day, and it was almost at the detection limit of the system, 0.01 pmol ml^{-1}. The method requires only an 800-μl aliquot of 2-fold diluted saliva and the analysis time is about 45 min. We speculate that salivary CA originates from diffusion of the transmitter CA secreted by the sympathetic nerve system
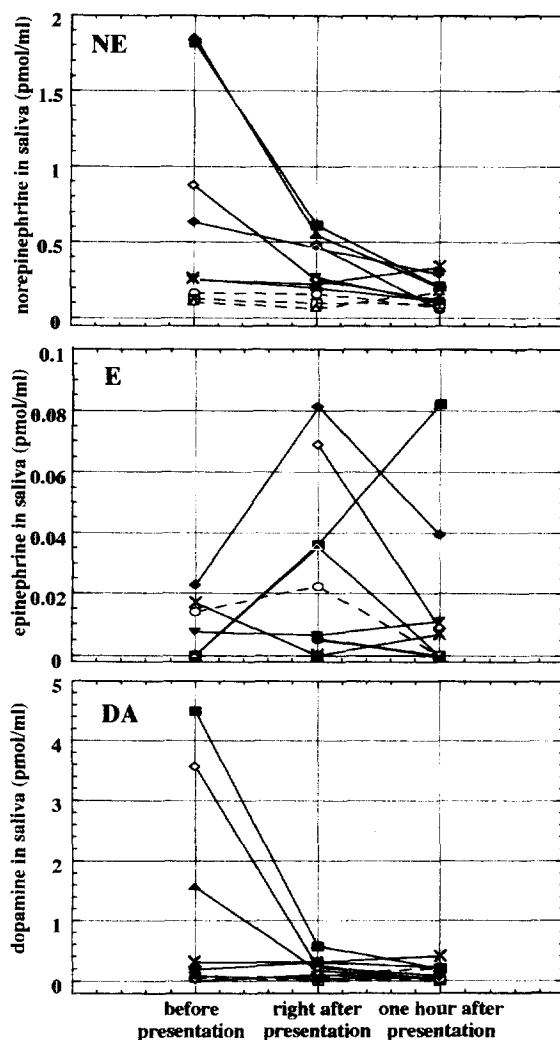

Fig. 11. Behavior of catecholamines in saliva of subjects under the stress of giving a presentation to a large audience. Analytical conditions were the same as shown in Table 1; sample: 2-fold-diluted saliva; injection volume: 800 μl; ●=subject K (volume of saliva sample acquired before presentation was not sufficient for determination), ■=subject L, ▲=subject M, O=subject N, □=subject O, △=subject P, ▽=subject Q, ◇=subject R (value before presentation could not be determined because of an interfering peak), x=subject S, ◆=subject T, ▼=subject U.

regulating the salivary gland, not from the permeation of plasma CA. Findings of abnormal values (more than 10 times higher than normal) and the elevation of CA to this level in subjects reporting strong anxiety or intense nervousness suggested that the system is useful for measurement of human

stress. Although the deproteinization column was changed from that used in the automated CS system, the CA and CS system could, in principle, be combined for simultaneous monitoring of these two major stress markers.

Acknowledgments

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