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Analysis of plasma catecholamines by high-performance liquid chromatography with fluorescence detection: simple sample preparation for pre-column fluorescence derivatization

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ABSTRACT

Analysis of plasma catecholamines (norepinephrine, epinephrine and dopamine) by high-performance liquid chromatography using 1,2-diphenylethylenediamine as a fluorescent reagent is described. We have developed an automatic catecholamine analyser, based on pre-column fluorescence derivatization and column switching. The analysis time for one assay was 15 min. The correlation coefficients of the linear regression equations were greater than 0.9996 in the range 10–10 000 pg/ml. The detection limit, at a signal-to-noise ratio of 3, was 2 pg/ml for dopamine. A new method of sample preparation for the pre-column fluorescence derivatization of plasma catecholamines was used. In order to protect the catecholamines from decomposition, an ion-pair complex between boric acid and the diol group in the catecholamine was formed at a weakly alkaline pH. The stabilities of plasma catecholamines were evaluated at several temperatures. After complex formation, the catecholamines were very stable at 17°C for 8 h, and the coefficients of variation for norepinephrine, epinephrine and dopamine were 1.2, 4.2 and 9.3%, respectively.

INTRODUCTION

The radioenzymic method for the determination of the plasma catecholamines, norepinephrine (NE), epinephrine (E) and dopamine (DA), is highly sensitive, but it is expensive and complicated [1,2]. High-performance liquid chromatography (HPLC) has been used to measure plasma catecholamines, usually with electrochemical detection (ED) [3] or fluorescence detection. Although the sensitivity of ED is sufficient for accurate measurement of low concentrations of plasma catecholamines, interfering peaks from many other oxidizable compounds in

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plasma occasionally occur in the chromatogram, even after extensive and selective sample clean-up procedures. Routine measurements using ED thus seem out of the question at present. The fluorescence detection is of three types: native fluorescence, ethylenediamine condensation [4] and trihydroxyindole [5]. The native fluorescence method is neither sensitive nor selective for catecholamines. The ethylenediamine condensation method is fairly sensitive, but requires complicated preparation procedures. The trihydroxyindole method has been most widely used and has good sensitivity for NE and E. However, this method does not have sufficient sensitivity for DA at the plasma level.

Recently, a new more highly sensitive fluorescence detection method for plasma catecholamine determination with the fluorescent reagent, 1,2-diphenylethylenediamine (DPE), has been reported by Ohkura and co-workers [6–10]. Because of its high sensitivity, high selectivity and easy operation, this method has been increasingly used, with pre-column [7] or post-column derivatization [9]. By a combination of this fluorescence detection method with pre-column derivatization and a column-switching method, we have developed an automatic catecholamine analyser.

However, all these HPLC methods need an extensive clean-up procedure and sometimes a concentration step, even in the case of DPE [7]. Various methods have been used as the clean-up and concentration step: adsorption on alumina [11], isolation on boric acid gel [12], an ion-exchange column [7,8,10] and solvent extraction [13,14]. But there are several disadvantages, *e.g.* complicated and time-consuming tasks to activate the column in the cases of the alumina, the boric acid gel and the ion-exchange approaches. Solvent extraction is also complicated and includes time-consuming procedures. HPLC with a clean-up procedure cannot be easily automated when many samples have to be analysed. In addition to the disadvantages, improvement of the reproducibility is required of the deproteinization procedure with perchloric acid, which has been generally used. This introduces another problem, as it has been reported that free catecholamines, especially DA, are gradually liberated from sulphate-conjugated catecholamines by hydrolysis with perchloric acid [15]. It should be difficult to determine accurate values at the plasma level for DA. Though the reaction with DPE has been employed under neutral conditions (*ca.* pH 7.0) [6,14], catecholamines are very unstable at neutral and weakly alkaline pH [16]. An improved DPE method needs to take account of the stabilities of the catecholamines under such conditions.

This paper describes an assay in which derivatization with DPE follows complex formation between boric acid and the diol group in the catecholamines in plasma. An automatic catecholamine analyser using fluorescence detection is employed. The stabilities of the catecholamines at weakly alkaline pH are evaluated at several temperatures. This method is demonstrated as being simple and useful for the determination of plasma levels of catecholamines.

EXPERIMENTAL

Materials

Norepinephrine hydrogentartrate, epinephrine hydrogentartrate, dopamine hydrochloride and isoproterenol hydrogentartrate were obtained from Wako (Osaka, Japan). DPE was synthesized by the method of Irving and Parkins [17]. All other chemicals were of analytical-reagent grade, except ammonium molybdate tetrahydrate, which was of super special grade. Solvents acetonitrile and methanol were HPLC grade. Amicon Centricon 10 for ultrafiltration (molecular mass cut-off *ca.* 10 000 Da) was obtained from Amicon (Danvers, MA, U.S.A.) and was washed with water before use. For all experiments, water of HPLC grade from Wako was used. Human blood plasma was hemolytic and supplied by Mito Hospital (Ibaraki, Japan). It was stored at −40°C until use.

Apparatus

A schematic diagram of the automatic catecholamine analyser is shown in Fig. 1. It consisted of a Gilson 231-401 auto-sampling injector, including a 500-μl sample loop and a water-bath with a temperature controller (Hitachi), Hitachi L-6000 and L-6200 pumps, a Rheodyne 7000 switching valve, a pre-column (10 mm × 4.0 mm I.D.) packed with 10 μm porous polymers with butyl residues (CQH-3BS; Mitsubishi Chemical Industries, Tokyo, Japan), a separation column (80 mm × 4.6 mm I.D.) packed with 3-μm ODS-modified silica (Hitachi 3057; Hitachi, Tokyo, Japan), column ovens, a Hitachi F-1050 fluorescence detector and a Hitachi D-2500 chromato-integrator. The system was controlled by a personal computer (NEC, PC9801VX).

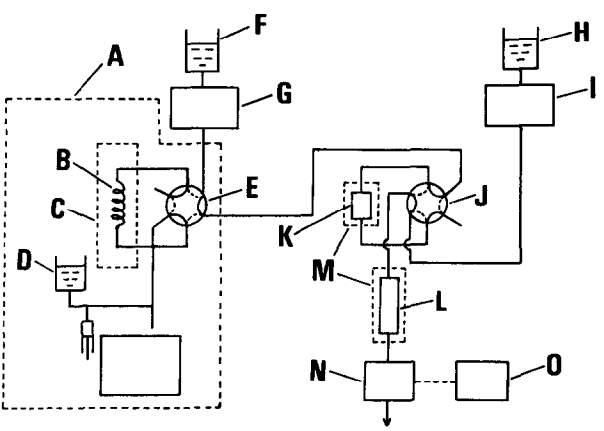


Fig. 1. Schematic diagram of the automatic catecholamine analyser: A = auto-sampling injector; B = sample loop; C = water bath; D = rinse solution; E and J = switching valves; F = eluent A; G and I = pumps; H = eluent B; K = pre-column; L = separation column; M = column oven; N = fluorescence detector; O = chromato-integrator.

Sample preparation

To a Centricon 10 tube were added 1.0 ml of plasma, 0.9 ml of 0.2 *M* sodium borate buffer (pH 7.3) containing 1 m*M* EDTA and 0.1 ml of 20 m*M* N-ethylmaleimide solution. The tube was shaken briefly, then the mixture was centrifuged (20 min, 25°C, 2000 *g*). The filtrate was used as the sample solution in the analyser.

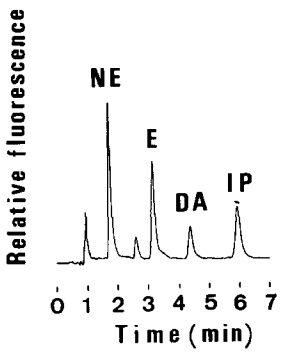
Derivatization and chromatography

To 0.4 ml of the prepared sample solution were added 0.2 ml of 50 m*M* DPE solution (in 80% acetonitrile, pH 7.0), 0.1 ml of 50 m*M* ammonium molybdate and 6 m*M* potassium ferricyanide solution (pH 7.3) and 0.1 ml of 0.5 *M* Tris–HCl buffer (pH 7.3). These additions were made using the auto-sampling injector. The flow was stopped after 0.4 ml of the mixture had been injected into the sample loop (reaction coil), and the derivatization reaction was carried out in the sample loop within the water-bath at 45°C for 3 min. After the reaction, 50 m*M* sodium borate buffer (pH 7.3) containing 0.1 *M* NaCl and 1 m*M* EDTA (eluent A) was passed through the sample loop to the pre-column at a flow-rate of 1.0 ml/min. The derivatives were adsorbed on the packing materials in the pre-column (25°C) and other components were eluted out for 3 min. The mobile phase (eluent B) was acetonitrile–methanol–50 m*M* sodium borate buffer (pH 7.3) containing 10 m*M* sodium dodecylsulphate (5:2:5, v/v), and its flow-rate was 1.0 ml/min. The mobile phase was passed from the pre-column through the separation column. The eluate from the pre-column was introduced into the separation column. When the derivatives from the pre-column were separated in the separation column (25°C), they were monitored by the fluorescence detector, with excitation at 350 nm and emission at 485 nm. The data from the detector were calculated by the recorder and were saved in the personal computer. The system was controlled by the personal computer so that its operation was very easy.

RESULTS AND DISCUSSION

Performance of automatic catecholamine analyser

Fig. 2 shows a typical chromatogram of the standard solution consisting of NE, E, DA and isoproterenol (IP), as internal standard, at 100 pg/ml each. The catecholamines and IP were rapidly separated within 7 min, and the total analysis time for one assay was 15 min. However, in the case of the measurement of the plasma sample, IP was not used because of some problems (described later). The amounts of catecholamines in the range 10–10 000 pg/ml were linearly related to the fluorescence intensities expressed as peak areas or peak heights. The correlation coefficients of the linear regression equations for NE, E and DA were 0.9999, 0.9998 and 0.9996, respectively, and their respective detection limits were 0.6, 0.9 and 2 pg/ml, at a signal-to-noise ratio of 3.



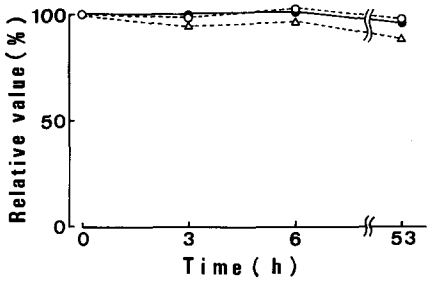


Fig. 2. Chromatogram of standard solution consisting of NE, E, DA and IP, at 100 pg/ml each.

Fig. 3. Stabilities of catecholamines in borate buffer. The catecholamines (1000 pg/ml each) in 0.2 *M* borate buffer (pH 7.3) were allowed to stand at 25°C. The stabilities were determined by the time-dependent decreases in their peak areas. Symbols: (○) NE; (●) E; (△) DA.

Stabilities of catecholamines

It is well known that an ion-pair complex is formed between phenylboric acid and the diol group in the catecholamines at alkaline pH [12,13]. Furthermore, this ion-pair complex formation has been used for the chromatography of catecholamines [12]. The stabilities of catecholamines in borate buffer are shown in Fig. 3. The catecholamines (1000 pg/ml each) in 0.2 *M* borate buffer (pH 7.3) were allowed to stand at 25°C. The decreases in the peak areas of the catecholamines were measured, and their stabilities were determined by the time-dependent decreases in their peak areas. Catecholamines have been reported as being very unstable in alkaline solution [16], but we observed no decrease in their original amounts after 6 h. Only 20% of the original amount of DA was decomposed even after 53 h. This result suggested that the ion-pair complex was formed between boric acid and the diol group in the catecholamines and a model of this is shown in Fig. 4. Hardly any of the diol group in the catecholamine was oxidized because it was protected from decomposition by the complex formation. Therefore, under these conditions catecholamines were very stable even at weakly alkaline pH.

There was interference with the fluorescence derivatization of NE and DA, and the recoveries of catecholamines added to plasma were less than 40%. We confirmed that the interfering compounds were substances containing an SH

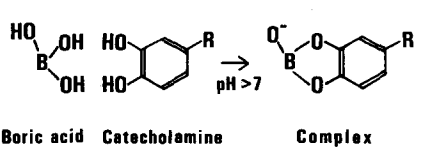


Fig. 4. Model of ion-pair complex formation between boric acid and catecholamine.

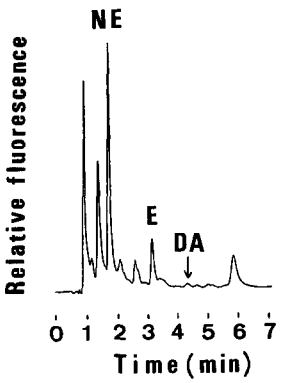


Fig. 5. Chromatogram of plasma sample: NE, 285 pg/ml; E, 94 pg/ml; DA, 23 pg/ml.

group, such as a glutathione, according to experiments in which several known materials were added to the standard solution before the reaction with DPE. In order to block the SH groups, N-ethylmaleimide, a typical and well known SH-blocking reagent, was added to the sample solution. Then the recoveries of catecholamines added to plasma were more than 80%.

Plasma has been reported to contain a substance that could be inactivated by N-ethylmaleimide [8,14], in the erythrocytes and platelets. In this experiment, there were many interfering compounds in the plasma because the sample was

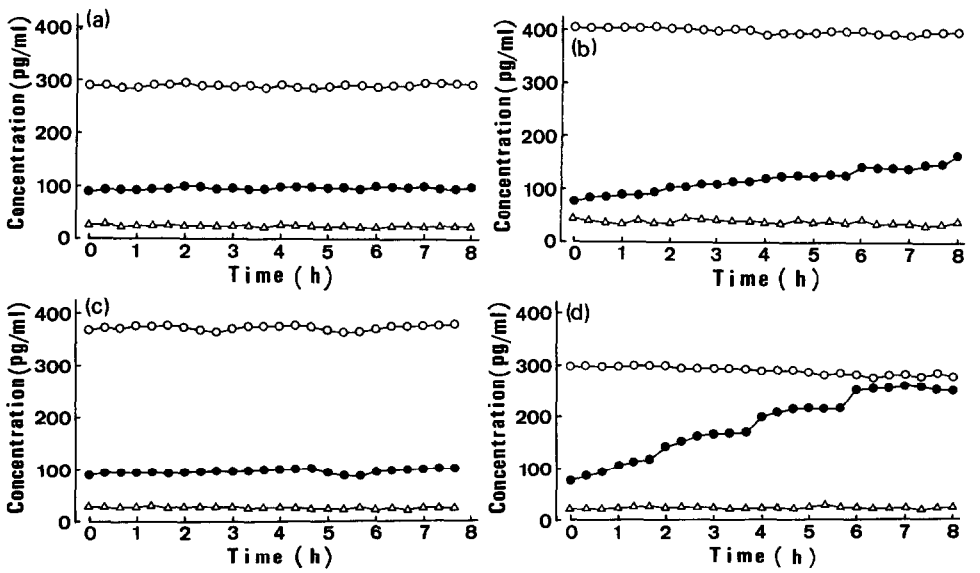


Fig. 6. Stabilities of plasma catecholamines after ion-pair complex formation under the conditions described in *Sample preparation*. The plasma catecholamines were allowed to stand at various temperatures: (a) 7°C; (b) 17°C; (c) 27°C; (d) 37°C. Symbols: (○) NE; (●) E; (△) DA.

TABLE I

REPRODUCIBILITIES OF SAMPLE STABILITIES AT VARIOUS TEMPERATURES

Temperature (°C)	Coefficient of variation (%)		
	NE	E	DA
7	1.1	2.8	6.8
17	1.2	4.2	9.3
27	1.1	20.3	9.9
37	3.3	34.1	10.2

abnormal, *i.e.* hemolytic plasma. Although the interfering compounds were thiols, a problem arose in that N-ethylmaleimide addition resulted in a large front peak that greatly increased the analysis time. A typical chromatogram obtained with plasma is shown in Fig. 5. An internal standard method was used at first, but unknown peaks overlapped with the IP peak and it was difficult to use this method for the analysis of plasma catecholamines. If necessary, an internal standard can be used with a more efficient column (150 mm × 4.6 mm I.D.). However, this will lead to increased analysis time. Finally, an external standard method was used in the analyser for a rapid analysis. The separation time of the catecholamines was 7 min because of the large front and the unknown peaks. The total analysis time was 15 min, the same as for the standard solution.

The stabilities of plasma catecholamines allowed to stand for 8 h at 7, 17, 27 and 37°C are shown in Fig. 6a–d, respectively. Coefficients of variation (C.V.) for NE, E and DA are shown in Table I. The catecholamines complexed with boric acid, and the catecholamine obtained by simple sample preparation, were very stable at 17°C for 8 h, and the C.V. for NE, E and DA were 1.2, 4.2 and 9.3%, respectively, depending on the plasma levels of the catecholamines. In particular, NE was stable even at 37°C for 8 h, and its C.V. was 3.3%. However, the peak for E gradually increased at temperatures above 27°C. In order to confirm whether this was due to an increase in the level of E or production of other unknown substances, we measured plasma catecholamines allowed to stand at 37°C using a more efficient column (150 mm × 4.6 mm I.D.). These findings indicated that the level of E gradually increased and that no other peaks appeared around the E peak. The reason why the level of E increased at temperatures above 27°C is unknown. However, it is no problem to determine the plasma catecholamines using this sample preparation when samples have been allowed to stand for less than 8 h at temperatures below 17°C.

CONCLUSION

We have developed an automatic catecholamine analyser, which includes pre-

column fluorescence derivatization and a column-switching method. Rapid analysis of the catecholamines was achieved within 15 min. The correlation coefficients of the linear regression equations for catecholamines were over 0.9996 in the range 10–10 000 pg/ml. The detection limits (signal-to-noise ratio of 3) for norepinephrine (NE), epinephrine (E) and dopamine (DA) were 0.6, 0.9 and 2 pg/ml, respectively. The operation of the analyser is very easy.

A new method of sample preparation for the pre-column fluorescence derivatization of plasma catecholamines was used. In order to protect the catecholamines from decomposition, an ion-pair complex between boric acid and the diol group in the catecholamines was formed at weakly alkaline pH. The complexed catecholamines were very stable for times up to 8 h at 17°C. The C.V. for NE, E and DA were 1.2, 4.2 and 9.3%, respectively, even for an abnormal sample such as haemolytic plasma. In particular, NE was stable even at 37°C for 8 h, and its C.V. was 3.3%. However, the level of E gradually increased at temperatures above 27°C.

The sample preparation for this analyser is simple. The method seems to be appropriate for routine measurements of plasma catecholamines.

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