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FLUOROMETRIC DETERMINATION OF SECONDARY AMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN DERIVATIZATION

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SUMMARY

To obtain suitable conditions for the simultaneous determination of primary and secondary amines by high-performance liquid chromatography with post-column derivatization, the conversion of secondary amines into primary amines with sodium hypochlorite was reinvestigated by flow injection analysis. While NaOCl was required primarily for the conversion of secondary amines into primary amines, it also decreased the detectability of the latter with *o*-phthalaldehyde-2-mercaptoethanol reagent, depending on the reaction temperature and time. The conditions established for the post-column derivatization were applied to the determination of amino acids including L-proline, L-4-hydroxyproline and N-methyl amino acids, catecholamines and their 3-O-methyl derivatives.

INTRODUCTION

Various methods using high-performance liquid chromatography (HPLC) with fluorescence detection have been developed for the determination of secondary amines in biological materials. For this purpose, pre-column derivatization procedures with Dns chloride[1], 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole[2] or 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole[3] and a post-column procedure with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole[4] have been devised. These reagents derivatize both primary and secondary amines to fluorescent compounds. Alternatively, *o*-phthalaldehyde–2-mercapthoethanol (OPA ME) reagent which is selective to primary amines has been used in post-column derivatization after the conversion of secondary amines into primary amines with sodium hypochlorite (NaOCl)[5,6].

This NaOCl–OPA method has now almost replaced the conventional ninhydrin method for the determination of amino acids. However, there is a problem in that the sensitivities of primary amino acids are much lower compared with the case in the absence of NaOCl. These reduced sensitivities are supposed to be due to side reactions caused by the excess of NaOCl, such as the transformation of primary amino acids to corresponding N-chlorinated derivatives[7]. Böhlen and Mellet[5] reported a

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method in which NaOCl was added to the column eluate only when proline was eluted (switching flow method). Continuous addition of NaOCl resulted in a marked reduction in the sensitivities of primary amino acids, *i.e.*, to less than 10%. In contrast, Ishida *et al.*[8] recently developed the "non-switching flow method" in which NaOCl is always added to the column eluate. Contrary to the results of Böhlen and Mellet[5], they claimed that the sensitivity of the method was so high that a few picomoles of primary amino acids could be determined.

In a previous study[7] we succeeded in suppressing the effects of the excess of NaOCl by the addition of 2,2′-thiodiethanol (TDE) to NaOCl-treated secondary amines and established manual procedures for the fluorometric determination of secondary amines. As an extension of that work, we have reinvestigated the NaOCl–OPA fluorogenic reactions of typical secondary amines together with analogous primary amines by flow injection analysis (FIA) to find the optimum conditions for the simultaneous determination of primary and secondary amines. These conditions were then applied to the HPLC post-column derivatization of some biogenic primary and secondary amines.

EXPERIMENTAL

Materials

Packed columns of Partisil-10 SCX (25 cm × 4.6 mm I.D., 10 μm; strong cation exchanger bonded to silica gel) and TSK LS-410 (30 cm × 4.0 mm I.D., 5 μm; ODS-bonded silica gel) were purchased from Whatman (Clifton, NJ, U.S.A.) and Toyo Soda (Tokyo, Japan) respectively. OPA (Funaphthal) was purchased from Funakoshi (Tokyo, Japan), DL-metanephrine hydrochloride, DL-normetanephrine hydrochloride and N-methyl-L-leucine from Sigma (St. Louis, MO, U.S.A.), L-DOPA, dopamine hydrochloride, γ-aminobutyric acid (GABA), L-proline, L-4-hydroxyproline, DL-alanine and Brij-35 from Nakarai (Kyoto, Japan) and L-aspartic acid and L-lysine hydrochloride from Ajinomoto (Tokyo, Japan). Ethanol, antiformin (0.7 *M* NaOCl solution when assayed by iodometry) and reagents for preparing buffers were obtained from Kanto (Tokyo, Japan). L-Epinephrine bitartrate, L-norepinephrine bitartrate, methylamine hydrochloride and other chemicals were purchased from Tokyo Kasei Kogyo (Tokyo, Japan).

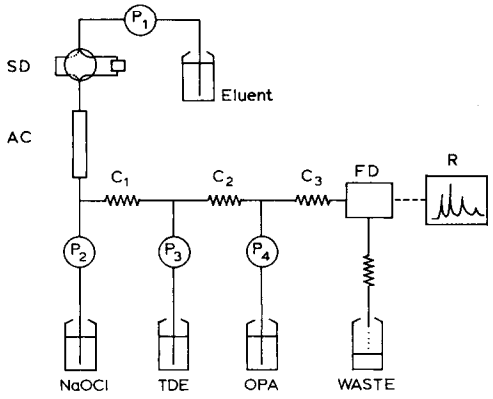

Fig. 1. Flow diagram of the present NaOCl–TDE–OPA post-column derivatization method. SD = Sampling device; FD = fluorescence detector; R = recorder; P_1–P_4 = pumps; C_1–C_3 = reaction coils.

Preparation of solutions

Ten millimolar solutions of amines were prepared with 20 m*M* citrate buffer (pH 3). The buffer was used to dilute the solutions. The NaOCl solution was prepared by diluting antiformin with buffers of various pH values. The TDE solution was prepared by dissolving TDE in phosphate buffer (pH 8) or in 5.7 % acetic acid.

The OPA–ME reagent was prepared by a modification of the method of Benson and Hare[9]: 1 ml of OPA solution (80 mg/ml in ethanol) and 0.2 ml of ME were mixed with 100 ml of 1 *M* borate buffer (pH 10.2).

Apparatus for HPLC of secondary amines

Fig. 1 shows the flow diagram of the present method. All tubings and coils were made of PTFE (0.5 mm I.D.). The eluent was delivered through a Mini-micro pump (Type KHD-26; Kyowa Seimitsu, Tokyo, Japan) to the analytical column (AC). The column temperature was ambient. The eluate was mixed in a three-way "tee" with the NaOCl solution delivered at a flow-rate of 0.55 ml/min through a Mini-micro pump (Type KSD-16; Kyowa Seimitsu). The mixture was incubated in the coil C_1 and mixed in the second three-way "tee" with TDE solution delivered at a flow-rate of 0.53 ml/min through a Mini-micro pump (Type KHD-16; Kyowa Seimitsu). The outlet of the second "tee" was connected through the coil C_2 to a third three-way "tee" and mixed with the OPA reagent delivered at a flow-rate of 0.60 ml/min through a Mini-micro pump (Type KSU-45H; Kyowa Seimitsu). Unless otherwise stated, the length of coils was 10 m and the reaction temperature was ambient, except

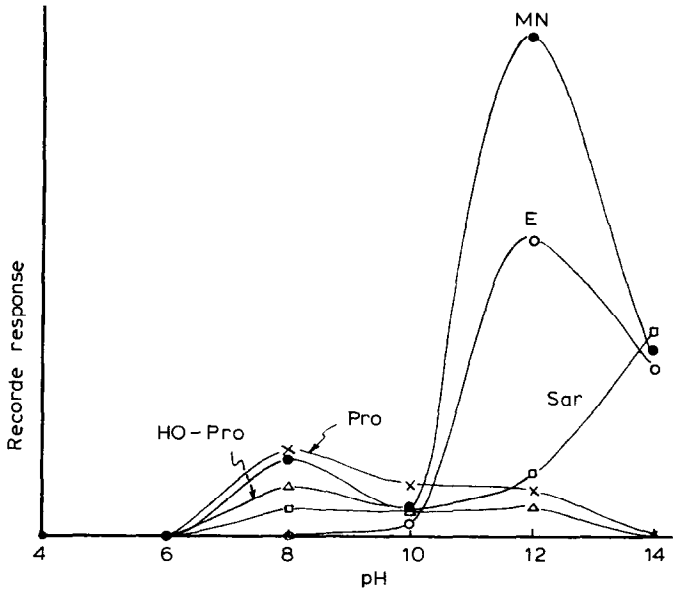

Fig. 2. Influence of pH on the conversion reaction. FIA was carried out as described in Experimental without delivering the TDE solution. The flow-rate of the carrier was 0.99 ml/min. NaOCl solutions (0.7 m*M*) were prepared as follows: 0.1 *M* citrate buffer (pH 4 and 6); 0.1 *M* phosphate buffer (pH 8 and 12); 0.1 *M* borate buffer (pH 10) and 1 *M* NaOH (pH 14). Injected amount of amines; 10 nmol. Abbreviations: Pro = proline; HO-Pro = 4-hydroxyproline; E = epinephrine; MN = metanephrine; Sar = sarcosine.

that the coil C_1 was set at 40°C. The outlet of the coil C_3 was introduced to a 14-μl quartz flow cell in a fluorescence detector (Type FLD-1; Shimadzu Seisakusho, Kyoto, Japan). It was equipped with a coated low-pressure mercury lamp which emits light of wavelength 300–400 nm (emission maximum at 360 nm) and a secondary filter (EM-3) which cuts off light of wavelength shorter than 405 nm. The fluorescence intensities were recorded with a recorder (Model EPR-100A; Toa Electronics, Tokyo, Japan).

Optimization of post-column derivatization by FIA

All the experiments on the optimization of the post-column reactions were conducted by FIA. For these investigations the post-column derivatization system described above was modified by removing the analytical column and delivering distilled water as the carrier.

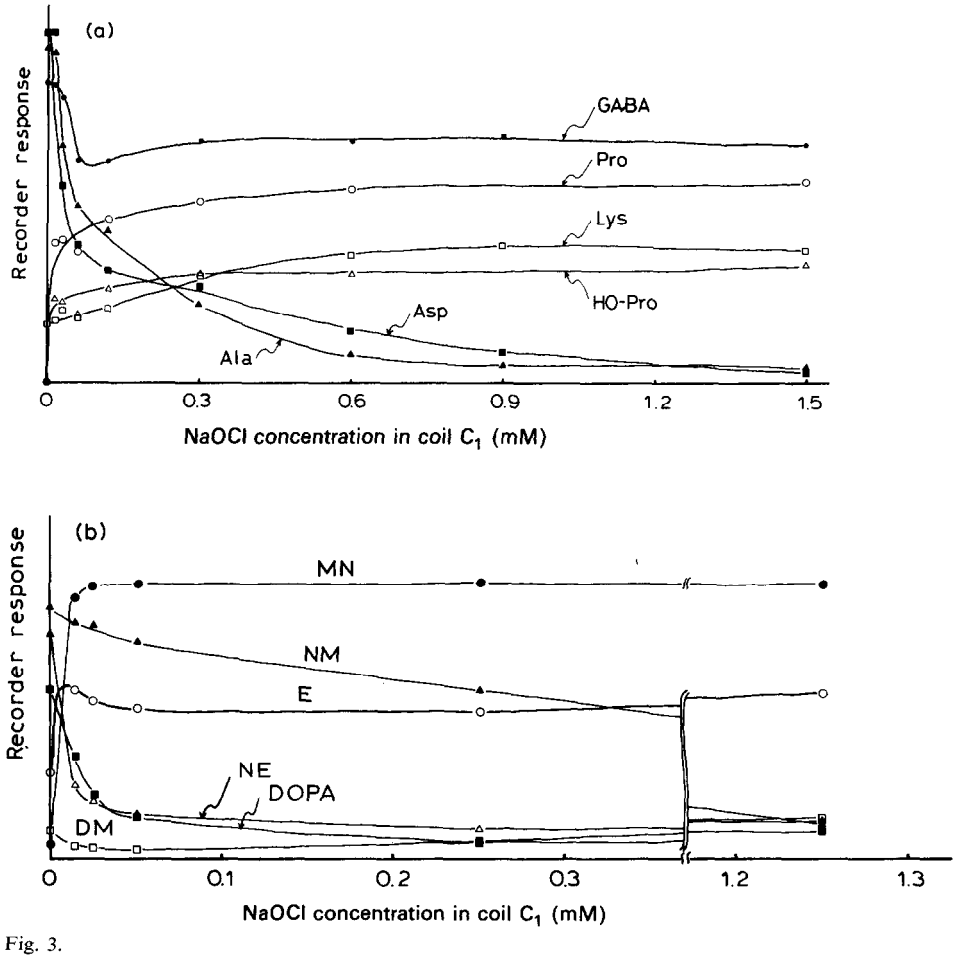

Fig. 3.

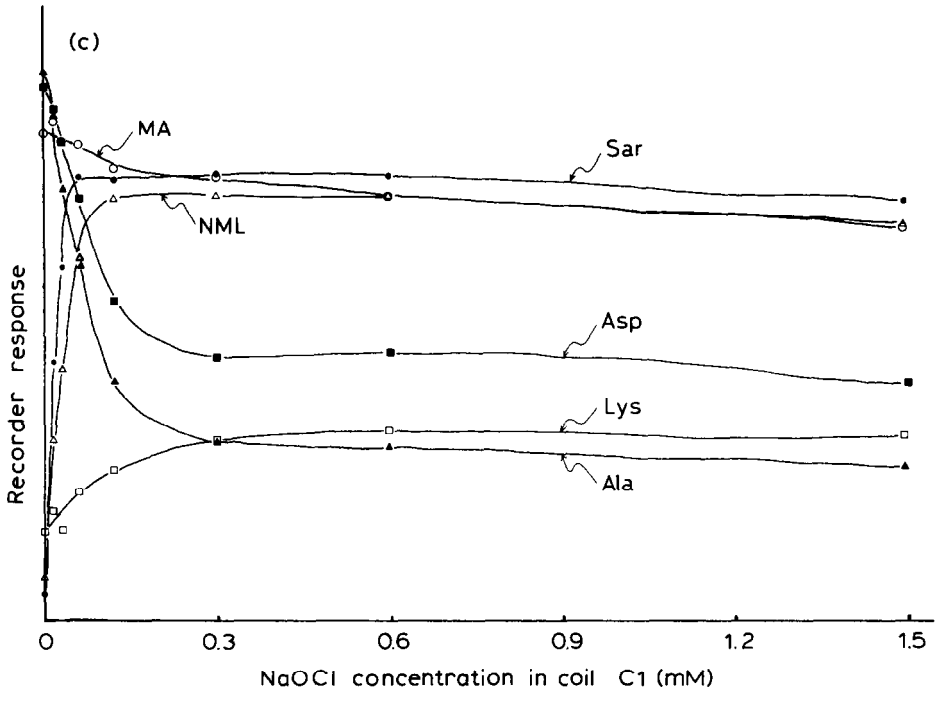

Fig. 3. Influence of NaOCl concentration on the conversion reaction. FIA was carried out as described in Experimental. The carrier flow-rates were 0.70 ml/min for group I(a) and group III(c), and 0.99 ml/min for group II(b). The NaOCl solution was prepared with 0.1 *M* phosphate buffer (pH 8) for group I, with 0.1 *M* phosphate buffer (pH 12) for group II and with 1 *M* NaOH for group III. For group II, 200 m*M* TDE solution was prepared with 0.5 *M* phosphate buffer (pH 8). Injected amounts of amines; 0.5 nmol (groups I and III) and 1.0 nmol (group I). Abbreviations: Asp = aspartic acid; Ala = alanine; GABA = γ-aminobutyric acid; Lys = lysine; NE = norepinephrine; NM = normetanephrine; DM = dopamine; NML = N-methylleucine; MA = methylamine.

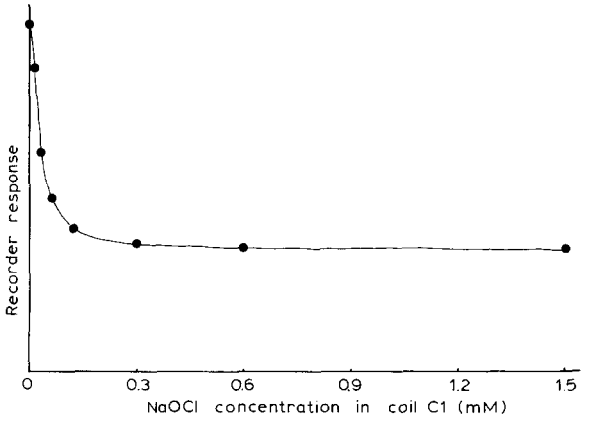

Fig. 4. Influence of NaOCl concentration on the fluorescence intensity of lysine with the addition of Brij-35 to the OPA–ME reagent at the concentration of 1.0 g/l. The conditions were the same as those for group III in Fig. 3. Injected amount of lysine: 0.3 nmol.

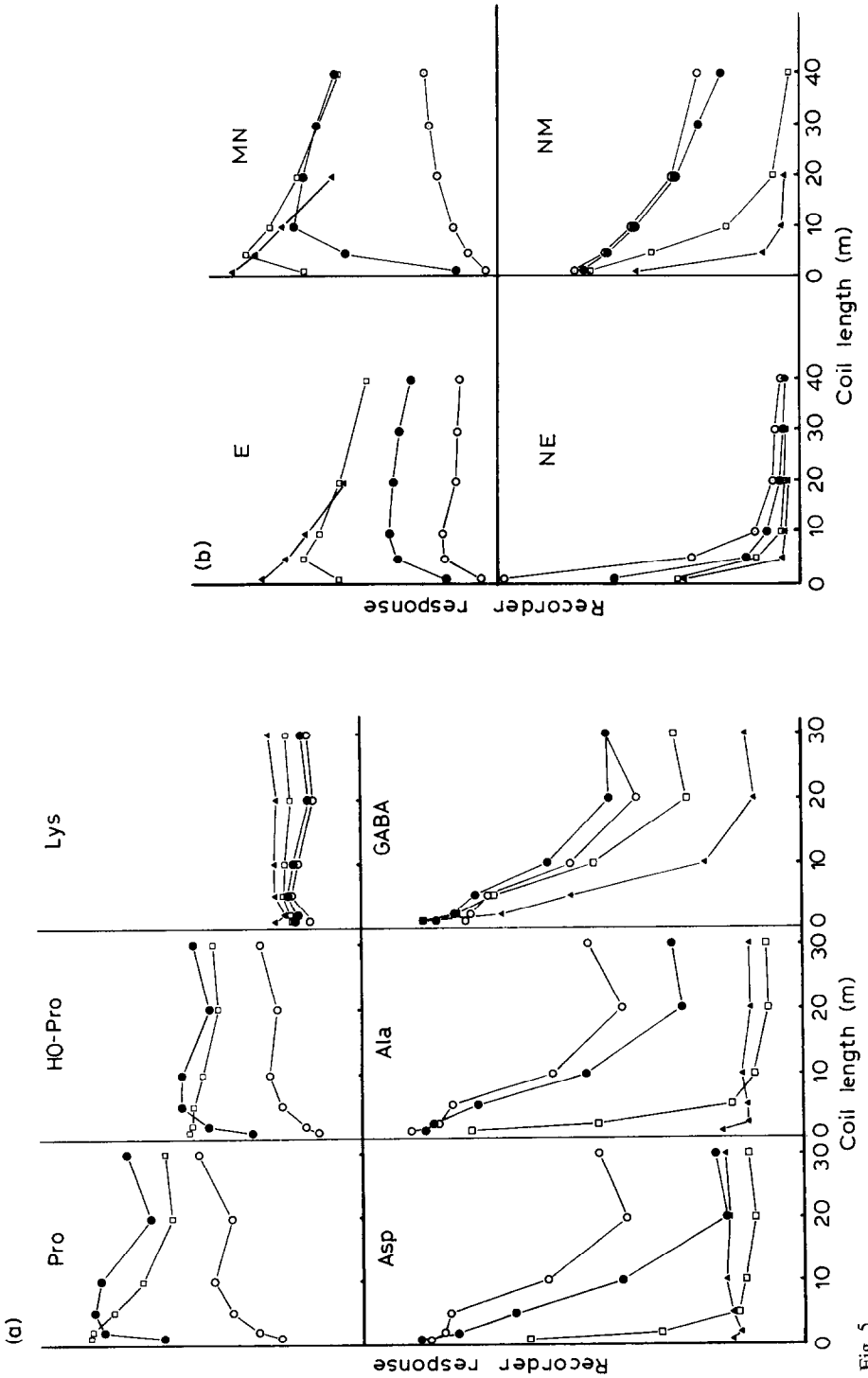
(a)
Pro
HO-Pro
Lys
Asp
Ala
GABA
Recorder response
Coil length (m)
0
10
20
30
(b)
E
MN
NE
NM
Recorder response
Coil length (m)
0
10
20
30
40

Fig. 5.

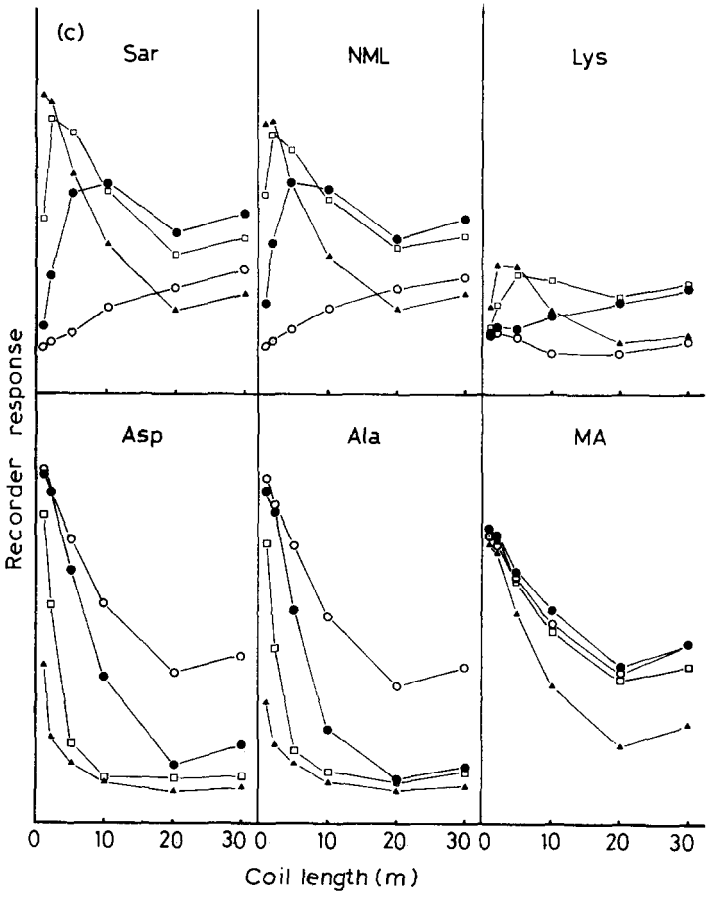

Fig. 5. Influence of reaction temperature and length of coil C_1 on the conversion reaction. The reaction temperature was changed from 20°C to 80°C and the coil length from 1 m to 40 m. Other conditions were the same as those of Fig. 3 except for the NaOCl concentration: a, 0.07 m*M* for group I; b, 0.14 m*M* for group II and c, 0.35 m*M* for group III. Injected amounts of amines: 0.5 nmol for groups I and III except prolines (2.5 nmol), and 1.0 nmol for group II. ○, 20°C; ●, 40°C; □, 60°C; ▲, 80°C.

RESULTS AND DISCUSSION

Optimization of the conversion reaction

pH. The influence of pH on the conversion reaction with NaOCl was investigated using FIA without delivering the TDE solution. Solutions of NaOCl were prepared with buffers of various pH values. As shown in Fig. 2, the optimum pH for the conversion reaction was about 8 for prolines, about 12 for epinephrine and metanephrine and about 14 for sarcosine. Therefore, in the following investigations, the model secondary amines were divided into three groups, *i.e.*, group I (prolines), group II (epinephrine and metanephrine) and group III (N-methyl amino acids) and reacted with NaOCl at their respective optimum pH values. Within each group, related primary amines were treated in the same manner. Based on our previous finding[7], the reaction with TDE was carried out at about pH 8.

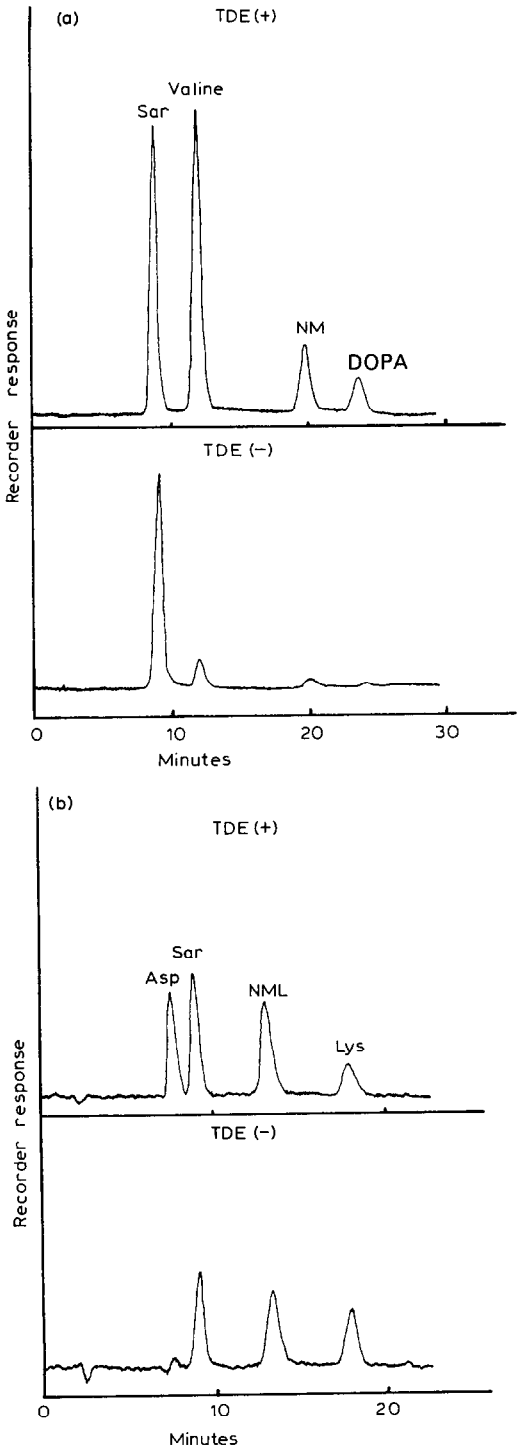

Fig. 6. Detection of amines with or without the addition of TDE. a, Method II was used except that reaction temperature was set at 60°C; injected amount of each amine was 4 nmol. b, Method III was used; injected amount of each amine was 0.2 nmol except lysine (0.3 nmol).

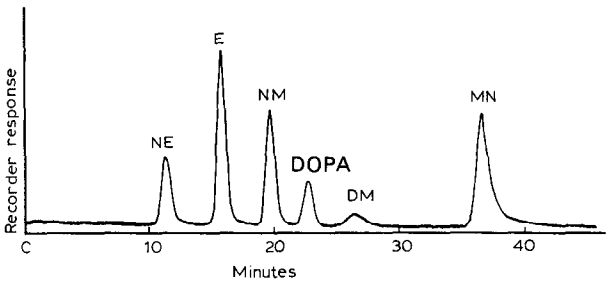

Fig. 7. Separation of catecholamines and their 3-O-methyl derivatives. The conditions were as in Fig. 6a. Injected amount of each amine: 2.7 nmol.

NaOCl concentration. The influence of NaOCl concentration on the conversion reaction was next investigated by FIA. As shown in Fig. 3a (pH 8), the fluorescence intensities of prolines and GABA reached plateaus at NaOCl concentrations over 0.3 m*M* in coil C_1, while those of aspartic acid and alanine decreased rapidly with increasing NaOCl concentration. Unlike other α-amino acids, lysine showed a similar profile to those of prolines and GABA. When the conversion reaction was performed at pH 12 (Fig. 3b), epinephrine and metanephrine fluoresced intensely even with higher concentrations of NaOCl, while the fluorescence intensities of norepinephrine, normetanephrine, DOPA and dopamine decreased with increasing NaOCl concentration. As shown in Fig. 3c (pH 14), the fluorescence intensities of sarcosine and N-methylleucine reached plateaus at >0.2 m*M* NaOCl. Similar to the case in Fig. 3a, the fluorescence intensities of aspartic acid and alanine decreased rapidly and that of lysine increased gradually with increasing NaOCl concentration, while that of meth-

TABLE I

CONDITIONS FOR HPLC WITH NaOCl–TDE–OPA POST-COLUMN DERIVATIZATION

	Method I	*Method II*	*Method III*
Column	Partisil-10 SCX	TSK LS-410	Partisil-10 SCX
Eluent	0.1 *M* citrate buffer (pH 3)	phosphate buffer (pH 3)*	0.1 *M* citrate buffer (pH 3)
Flow-rate	0.70 ml/min	0.48 ml/min	0.70 ml/min
Length of coil C_1	3.0 m	1.0 m	2.0 m
Temperature	40°C	80°C	60°C
NaOCl concentration in coil C_1	0.03 m*M*	0.07 m*M*	0.15 m*M*
Solvent for NaOCl solution	0.5 *M* phosphate buffer (pH 8)	0.1 *M* phosphate buffer (pH 12)	1 *M* NaOH
TDE concentration in coil C_2	6.0 m*M*	68 m*M*	6.0 m*M*
Solvent for TDE solution	0.5 *M* phosphate buffer (pH 8)	0.5 *M* phosphate buffer (pH 8)	5.7% acetic acid

* Prepared by titrating 50 m*M* NaH_2PO_4 with H_3PO_4.

TABLE II

DETERMINATION RANGES OF THE PRESENT METHOD

Compound	*Determination range (nmol)*		
	Method I	*Method II*	*Method III*
Proline	0.1–2.0		
Hydroxyproline	0.1–2.0		
Sarcosine			0.02–2.0
N-Methylleucine			0.02–2.0
Aspartic acid	0.02–10.0		0.02–10.0
Lysine	0.02–0.2		0.075–15.0
γ-Aminobutyric acid	0.02–5.0		
Epinephrine		0.5–10.0	
Metanephrine		0.5–10.0	

ylamine was hardly affected. The unexpected result obtained with lysine is thought to be due to the quenching property of the intramolecular interaction between the two isoindole fluorophores derived from the two amino groups of lysine[10]. The apparent increase in the fluorescence intensity induced by lysine with increasing NaOCl concentration is due to the decrease in this interaction caused by the loss of the α-amino group sensitive to NaOCl, but not obviously due to the increase in the number of primary amino groups. In fact, when a surfactant Brij-35 was added to the OPA–ME reagent to suppress the interaction, the fluorescence intensity obtained from lysine decreased with increasing NaOCl concentration (Fig. 4), showing a similar profile to those of other primary aminon acids. On the basis of the above results, the NaOCl concentration in coil C_1 was set at 0.03 m*M* for group I, 0.07 m*M* for group II and 0.15 m*M* for group III.

Reaction temperature and time. Fig. 5 shows the dependence of the conversion reaction on the reaction temperature and the length of coil C_1. The fluorescence intensities of secondary amines increased with increasing reaction temperature. However, at elevated temperatures, they decreased as the length of coil C_1 increased. The

TABLE III

REPRODUCIBILITIES OF THE PRESENT METHOD

Compound	*Coefficient of variation (%)*	
	Method I (n = 10)	*Method III (n = 8)*
Proline	2.7 (1.0 nmol*)	
Hydroxyproline	2.2 (1.0 nmol)	
Sarcosine		2.5 (0.2 nmol)
N-Methylleucine		1.8 (0.2 nmol)
Aspartic acid	2.3 (0.1 nmol)	6.3 (0.2 nmol)
Lysine		6.6 (0.3 nmol)
γ-Aminobutyric acid	1.7 (0.1 nmol)	

* Injected amount of the compound is given in parentheses.

fluorescence intensities of primary amines except lysine decreased rapidly as the reaction temperature or time increased. The adopted conditions for the NaOCl–TDE–OPA post-column derivatization are summarized in Table I. Methods I, II and III were for the determination of groups I, II and III respectively.

Application to the HPLC of biogenic amines

The present NaOCl–TDE–OPA post-column derivatization was applied to the HPLC of some standard compounds. Fig. 6 shows the chromatograms (upper) obtained with the present method and the corresponding chromatograms (lower) obtained by delivering the solvent in place of the TDE solution. Comparison of the two sets of chromatograms revealed that TDE suppressed the effects of the excess of NaOCl except in the case of lysine. Fig. 7 shows a chromatogram of catecholamines and their 3-O-methyl derivatives obtained by Method II. Tables II and III show the determination ranges and reproducibilities, obtained by a peak height method, of the present method.

We were not able to obtain any condition under which all secondary amines were sensitively detected. This is thought to be due mainly to differences either in the optimum conditions for the conversion reaction of secondary amines or in the stability to NaOCl of primary amines converted from secondary amines. However, as can be seen from the profile of fluorescence intensity and NaOCl concentration (Fig. 3), the primary amine compounds produced from the secondary amines tested seem to be relatively stable to NaOCl. This suggests that the differences in the optimum conditions for the conversion reaction dominate the overall determination of secondary amines. The identification of the reaction products of the conversion reaction is now in progress.

REFERENCES

1 T. Yamabe, N. Takai and H. Nakamura, *J. Chromatogr.*, 104 (1975) 359.
2 J. H. Wolfram, J. I. Feinberg, R. C. Doerr and W. Fiddler, *J. Chromatogr.*, 132 (1977) 37.
3 Y. Watanabe and K. Imai, *Anal. Biochem.*, 116 (1981) 471.
4 M. Roth, *Clin. Chim. Acta*, 83 (1978) 273.
5 P. Böhlen and M. Mellet, *Anal. Biochem.*, 94 (1979) 313.
6 H. H. Myers and J. V. Rindler, *J. Chromatogr.*, 176 (1979) 103.
7 A. Himuro, H. nakamura and Z. Tamura, *Anal. Chim. Acta*, 147 (1983) 317.
8 Y. Ishida, T. Fujita and K. Asai, *J. Chromatogr.*, 204 (1981) 143.
9 J. R. Benson and P. E. Hare, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 619.
10 R. F. Chen, C. Scott and E. Trepman, *Biochim. Biophys. Acta*, 576 (1979) 440.