Determination of Free and Total Catecholamines in Human Erythrocytes, Platelets and Plasma by High Performance Liquid Chromatography with Fluorescence Detection

Hitoshi NOHTA, Akane MITSUI, Yoshihiko UMEGAE and Yosuke OHKURA

Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi, Fukuoka 812

A simple and highly sensitive method for the determination of free and total (free+conjugated) catecholamines (norepinephrine, epinephrine and dopamine) in human erythrocytes, platelets and plasma is described which employs high performance liquid chromatography with fluorescence detection. Conjugated catecholamines are hydrolyzed by sulfatase-mediated reaction to the corresponding free amines. After cation exchange chromatography on a cartridge of a cation exchanger, Toyopak SP, catecholamines and isoproterenol (internal standard) in a sample are converted into the corresponding fluorescent compounds by reaction with 1,2-diphenylethylenediamine. These compounds are separated on a reversed-phase column, TSK-gel ODS-120T, with isocratic elution using a mixture of water, methanol and acetonitrile containing a Tris-hydrochloric acid buffer (pH 7.0). The detection limit for each catecholamine is ca. 2 fmol/100- μ l injection volume.

Keywords Catecholamine, high performance liquid chromatography, erythrocyte, platelet, 1,2-diphenylethylenediamine

Since the levels of catecholamines (CAs: norepinephrine, NE; epinephrine, E; dopamine, DA) in blood have been considered to be an index of sympathetic nerve functions, many studies of CA levels in various diseases have been performed using mainly plasma as sample. Erythrocytes and platelets also contain CAs, which can possibly be modulators of plasma CAs levels and stable indicator of sympathetic tone.^{1,2} Therefore, CAs levels in erythrocytes and platelets have recently been drawn much attention from biological and clinical investigators.^{1,2} Conjugated CAs also occur in blood, and their levels in plasma are not promptly affected by physiological and probably emotional stress.¹ Conjugated CAs can be hydrolyzed *in vivo* to free CAs³, and conjugated DA can be converted to free NE by dopamine β -hydroxylase.⁴ Therefore, the determination of conjugated CAs is also significant in clinical and biological investigations.

Many assay methods for free CAs in plasma have been proposed: radioenzymatic methods^{5,6}, radioimmunoassay⁷, gas chromatography⁸ and high performance liquid chromatography (HPLC) with fluorescence detection^{9,10} and electrochemical detection (ECD).^{11,12} However, both free and total CAs (the sum of individual free and conjugated CAs) in erythrocytes and plasma can be determined only by radioenzymatic methods^{1,13}, and free CAs in erythrocytes and platelets by gas chromatography¹⁴, HPLC-ECD¹⁵ and radioenzymatic methods.^{2,16-18} Although the radioenzymatic

methods are highly sensitive, they require complicated and time-consuming procedures. The two chromatographic methods are relatively simple, but they do not permit the determination of DA.

We have reported that a simple and sensitive HPLC method for the determination of CAs in plasma using precolumn fluorescence derivatization with 1,2-diphenylethylenediamine (DPE), a fluorogenic reagent for catechol compounds^{19,20}; CAs in plasma, after cation exchange chromatography on a sulfopropyl resin (Toyopak SP) cartridge, are converted into the corresponding fluorescent compounds by reaction with DPE and those compounds are separated on a reversed phase (TSK-gel ODS-120T) column with isocratic elution. The purpose of this study is to establish methods for the determination of free and total CAs in human erythrocytes and platelets, and also total CAs in human plasma, by applying the above principle. Isoproterenol (IP) is used as internal standard.

Experimental

Materials, apparatus and HPLC conditions

N-Ethylmaleimide (Wako, Osaka, Japan) was repeatedly recrystallized from hexane. Sulfatase (arylsulfatase, from *aerobacter aerogenes*, type VI) and β -glucuronidase (from *E. coli*, type VIII) were purchased from Sigma (St. Louis, U.S.A.). Other reagents and solutions were

prepared as described previously.²⁰

Apparatus and HPLC conditions were the same as described previously²⁰ except that a mixture of acetonitrile, methanol and 50 mM Tris-hydrochloric acid buffer (pH 7.0)(9:2:9, v/v) was used as a mobile phase.

Preparation of erythrocytes, platelets and plasma samples

Heparinized blood (2 ml) was taken into a polyethylene tube containing 5 mg of reduced glutathione and centrifuged at 100g for 15 min at 10–15°C. The supernatant (platelets rich plasma, PRP; 0.5 ml) was transferred to a polycarbonate tube and centrifuged again at 10000g for 15 min at 10–15°C. The precipitates (platelets) were separated from the supernatant (plasma) by aspiration followed by inversion of the tube. The platelets were suspended in 0.55 ml of 13 mM dithiothreitol by sonication at 4°C for 5 min to extract CAs. The suspension was centrifuged at 1000g for 10 min and the supernatant (platelets CAs sample) was stored at –70°C until use. The lower layer (erythrocytes and plasma) obtained in the centrifugation at 100g was centrifuged again at 1500g for 15 min at 4°C, and the plasma and that obtained from PRP were combined and used as plasma sample. The plasma was stored at –70°C until use. The erythrocytes were washed twice with 5 ml of physiological saline containing 10 mM dithiothreitol. A 0.5-ml volume of the erythrocytes was lysed with 2.0 ml of 16 mM dithiothreitol containing 2.6 mM EGTA (pH 6.8, adjusted with 0.1 M sodium hydroxide) and centrifuged at 1500g for 10 min. The supernatant (erythrocytes CAs sample) was stored at –70°C until use.

The protein concentration of the platelets sample was determined by the method of Ross *et al.*²¹ using bovine serum albumin as a standard protein.

Clean-up and fluorescence derivatization

Free CAs in plasma were cleaned up and derivatized as described previously.²⁰

Free CAs in erythrocytes. To 2.0 ml of an erythrocytes CAs sample, 10 µl of 50 pmol/ml IP was added, and the mixture was poured into a Toyopak SP cartridge.²⁰ The cartridge was washed successively with 5 ml of water (twice) and 1 ml of aqueous 50%(v/v) acetonitrile (twice). The adsorbed amines were eluted with 300 µl of a mixture of 0.6 M potassium chloride and acetonitrile (1:1, v/v) containing 0.6 mM potassium hexacyanoferrate (III). To the eluate, 10 µl of 50 mM *N*-ethylmaleimide and 50 µl of 0.1 M DPE were added. The mixture was allowed to stand at 37°C for 40 min. A 100-µl aliquot of the mixture was injected into the chromatograph.

Free CAs in platelets. To 0.5 ml of a platelets CAs sample, 0.5 ml of 0.2 M lithium phosphate buffer (pH 5.8) and 10 µl of the IP solution were added. The mixture was poured into a Toyopak SP cartridge and then the same procedure was carried out as in the case

for erythrocytes. Total CAs in erythrocytes, platelets and plasma by sulfatase-mediated hydrolysis. To a sample (erythrocytes CAs sample, 500 µl; platelets CAs sample, 100 µl; plasma, 100 µl), 10 µl each of 0.13 M dithiothreitol and 27 mM EDTA·2Na (pH 6), 200 µl of 0.2 M Tris-hydrochloric acid buffer (pH 7.1) and 20 µl of 4 units/ml sulfatase solution were added. The mixture was incubated at 37°C for 25 min to hydrolyze the conjugated CAs. To the reaction mixture, 200 µl of the lithium phosphate buffer was added, and the resulting mixture was poured into a Toyopak SP cartridge, and the same procedure was carried out as in the case for free CAs.

Results and Discussion

Free CAs in erythrocytes and platelets

Figure 1 shows typical chromatograms obtained with human erythrocytes and platelets. The patterns of the chromatograms closely resemble each other. The peaks for CAs (peak 1–3)(retention times/min: NE, 3.5; E, 5.8; DA, 7.9) were identified on the basis of their retention times and fluorescence excitation and emission spectra (maxima/nm: 345 and 470 for NE, 350 and 480 for E, 345 and 485 for DA, respectively) of the eluates in comparison with the standard compounds and also by co-chromatography with standards. Peak 4 (IP, internal standard) has a retention time of 11.0 min, and its eluate shows fluorescence excitation and emission maxima at 365 and 480 nm, respectively. Peaks 6 and 7 have fluorescence excitation and emission maxima at *ca.* 350 and 460 nm, respectively, which are characteristic of catechol compounds.²⁰

Fig. 1 Chromatograms obtained with (a) erythrocytes and (b) platelets by clean-up procedure with Toyopak SP cartridge. Peaks: 1=NE; 2=E; 3=DA; 4=IP; 5=*N*-ethylmaleimide; 6 and 7=unidentified. Concentrations of CAs: erythrocytes (pmol/ml), NE=2.0, E=0.2, DA=0.09; platelets (pmol/mg protein), NE=1.0, E=0.09, DA=0.05.

These peaks were not observed when the DPE solution was omitted from the derivatization reaction. Therefore, these peaks are due to endogenous catechol compounds that can not be removed by the cation exchange chromatography, though the compounds could not be identified.

Free CAs in plasma can be simply and effectively separated and concentrated by the cation exchange chromatography on Toyopak SP cartridge.²⁰ This technique was successfully applied to erythrocytes and platelets CAs samples. The recoveries (%; mean $\pm$ S.D., $n=10$ each) of NE, E, DA and IP added to the samples (0.5 pmol each) were 77.0 $\pm$ 2.0, 79.5 $\pm$ 1.8, 82.5 $\pm$ 1.3 and 80.6 $\pm$ 1.6 (erythrocytes), and 82.3 $\pm$ 1.2, 86.6 $\pm$ 0.9, 92.2 $\pm$ 1.8 and 86.9 $\pm$ 1.0 (platelets). Widely used clean-up by alumina treatment^{20,22} also permitted the determination of erythrocytes and platelets CAs (Fig. 2), but the recoveries of CAs and IP added to the samples (0.5 pmol) were less than 30% in erythrocytes and 50–60% in platelets. Peaks 6–14 in Fig. 2 were found to be catechol compounds by the same techniques as in peaks 6 and 7 in Fig. 1, but they were not identified. These peaks with exception of peak 7 were not observed in the chromatogram of plasma sample.²⁰

Erythrocytes and platelets samples contain compound(s) which interferes with the fluorescence derivatization reaction of NE and DA, and could not be removed by the chromatography on Toyopak SP cartridge. The interference can be eliminated by adding *N*-ethylmaleimide, a SH-blocking reagent, to the derivatization reaction mixture; peak heights from NE and DA added to the eluate from the Toyopak SP cartridge were decreased by 30–70% provided that *N*-

ethylmaleimide was absent and they recovered completely in the presence of 0.8–5 mM *N*-ethylmaleimide: 1.4 mM was selected in the derivatization. Thus, the interfering compound(s) is probably sulfhydryl compound(s). Actually, 0.5 μ M reduced glutathione added in the derivatization reaction reduced the heights of the peaks for standard NE and DA to 50 and 15%, respectively, but did not affect those of E and IP. This inhibition was not due to the consumption of potassium hexacyanoferrate (III); the reason remains

Fig. 2 Chromatograms obtained with (a) erythrocytes and (b) platelets by clean-up procedure with alumina. Samples: the same as in Fig. 1. Peaks: 1–7=as in Fig. 1, 8–14=unidentified.

Fig. 3 Chromatograms obtained with hydrolyzed samples from (a) erythrocytes, (b) platelets and (c) plasma. Peaks: as in Fig. 1. Concentrations of NE, E and DA: erythrocytes (pmol/ml), 3.9, 1.3 and 7.9; platelets (pmol/mg protein), 1.3, 0.20 and 0.30; plasma (pmol/ml), 5.1, 0.8 and 18, respectively.

unknown.

N-Ethylmaleimide gave a peak (peak 5; retention time, 3 min) and its eluate showed fluorescence excitation and emission maxima at 310 and 440 nm, respectively. This peak was observed even when extremely purified *N*-ethylmaleimide alone was injected into the chromatograph and disappeared by the addition of equimolar reduced glutathione. Since this peak could not be separated from NE peak under the previous HPLC conditions for plasma CAs²⁰, a mobile phase with weak elution power, which resulted in

satisfactory separation of these peaks, was employed (see the experimental). The other derivatization and HPLC conditions were the same as for plasma CAs.

Platelets CAs could be completely extracted by suspending in 13 mM dithiothreitol (recoveries, greater than 98%, respectively). Platelets could not be washed with saline or isotonic phosphate buffer (pH 6.8), because approximately a half of CAs (40–60%) in platelets were washed out at a time when the platelets corresponding to 1.0 ml of PRP were washed with 1.0 ml of saline or the phosphate buffer.

Table 1 Concentrations of free and total catecholamines in erythrocytes, platelets and plasma from healthy men^a

Age		Erythrocytes/pmol ml ⁻¹			Platelets/pmol mg ⁻¹ protein			Plasma/pmol ml ⁻¹		
		NE	E	DA	NE	E	DA	NE	E	DA
54	Free(F)	3.2	0.6	0.09	1.5	0.06	0.03	3.0	0.5	0.05
	Total(T)	5.7	1.0	14.9	1.8	0.18	0.49	9.6	1.4	40
	F/T(%)	57	54	0.6	84	38	6.3	31	39	0.1
39	Free(F)	2.5	0.5	0.14	1.0	0.09	0.05	2.5	0.4	0.07
	Total(T)	3.9	1.3	8.0	1.3	0.20	0.30	5.1	0.8	19
	F/T(%)	63	42	1.8	82	46	15.9	49	45	0.4
34	Free(F)	2.2	0.4	0.12	0.4	0.04	0.02	3.6	0.2	0.14
	Total(T)	3.5	1.0	7.8	0.5	0.20	0.20	6.4	0.8	15
	F/T(%)	64	40	1.6	73	21	7.7	57	27	0.9
31	Free(F)	2.2	0.2	0.09	1.5	0.09	0.05	1.2	0.2	0.07
	Total(T)	4.0	0.7	7.7	2.3	0.19	0.58	6.3	0.4	20
	F/T(%)	54	30	1.2	64	47	8.3	31	47	0.3
28	Free(F)	2.1	0.3	0.10	0.6	0.04	0.01	3.9	0.2	0.08
	Total(T)	4.1	0.8	11.6	0.9	0.11	0.19	7.8	0.7	20
	F/T(%)	52	34	0.8	72	32	6.9	50	34	0.4
26	Free(F)	1.8	0.2	0.13	0.8	0.06	0.03	2.4	0.4	0.10
	Total(T)	3.1	0.5	7.3	1.7	0.26	0.39	6.1	0.7	26
	F/T(%)	59	48	1.8	47	21	7.7	39	62	0.4
26	Free(F)	2.2	0.4	0.16	0.8	0.07	0.04	2.5	0.2	0.10
	Total(T)	3.7	1.2	14.3	1.0	0.19	0.49	5.4	0.8	34
	F/T(%)	59	33	0.4	73	37	7.8	46	30	0.3
24	Free(F)	1.8	0.3	0.09	0.6	0.04	0.01	1.3	0.2	0.03
	Total(T)	3.9	0.7	9.7	0.9	0.07	0.22	8.2	1.0	14
	F/T(%)	45	42	1.0	67	57	5.2	16	19	1.2
23	Free(F)	1.3	0.5	0.06	0.8	0.07	0.03	1.3	0.4	0.05
	Total(T)	5.0	1.4	16.7	1.1	0.18	0.25	5.0	0.8	14
	F/T(%)	26	37	0.4	75	39	12.9	27	48	0.3
23	Free(F)	1.9	0.4	0.10	0.7	0.06	0.02	2.4	0.2	0.06
	Total(T)	4.7	1.1	10.2	1.7	0.30	0.28	5.0	0.6	10
	F/T(%)	40	32	0.9	41	21	5.3	47	39	0.6
mean ±S.D.	Free	2.1 ±0.5	0.4 ±0.1	0.11 ±0.03	0.9 ±0.4	0.06 ±0.02	0.03 ±0.01	2.5 ±0.8	0.3 ±0.1	0.08 ±0.03
	Total	4.2 ±0.7	1.0 ±0.3	10.8 ±3.2	1.3 ±0.5	0.19 ±0.06	0.34 ±0.13	6.5 ±1.5	0.8 ±0.2	21 ±9
	F/T(%)	52 ±11	39 ±7	1.0 ±0.5	68 ±13	36 ±11	8.4 ±3.2	39 ±12	39 ±12	0.4 ±0.2

a. Blood samples were collected at 9:30–10:00 AM from healthy volunteers who had a light breakfast at ca. 7:30 AM.

Total CAs in erythrocytes, platelets and plasma

Figure 3 shows typical chromatograms obtained with human erythrocytes, platelets and plasma according to the procedure for total CAs. The peaks for CAs were identified by the same techniques as for free CAs.

Maximum amounts of CAs were liberated in the presence of 0.04–0.4 units/tube sulfatase for the incubation time of 20–45 min at 37°C (widely used temperature^{1,13}) in each sample: 0.16 units/tube sulfatase and 25-min incubation time were selected.

The treatment of the individual samples with sulfatase did not affect the clean-up using Toyopak SP cartridge, and the recoveries (%), mean±S.D., $n=10$ each) of free NE, E, DA and IP (0.5 pmol each) added to the samples were 76.4±2.4, 73.0±2.5, 87.0±2.1 and 75.5±2.5 (erythrocytes), 82.4±2.2, 80.3±2.3, 83.7±2.0 and 80.0±2.3 (platelets) and 86.7±2.0, 84.8±2.1, 92.5±1.5 and 86.7±1.9 (plasma), respectively.

Although most of the conjugated CAs in plasma and erythrocytes are in the sulfate form^{4,23}, the glucuronides also occur in plasma in only a minor part.¹³ No data for total CAs in platelets have been reported so far. In the present study, no liberation of the three CAs from the individual samples was observed when β -glucuronidase (100–1000 units/tube) was used in place of sulfatase. The concentrations of total CAs (pmol/ml) in platelets and plasma determined by the sulfatase-mediated hydrolysis(x, Table 1) were compared with those obtained by hydrolysis with perchloric acid (0.5 M in the reaction mixture, 100°C, 20 min)(y). The data showed a good correlation: the linear regression equations (correlation coefficient in parentheses) were, in platelets, $y=1.01x+0.01(1.00)$ for NE, $y=1.04x-0.01(0.99)$ for E and $y=1.01x-0.01(1.00)$ for DA; in plasma, $y=1.04x+0.21(0.99)$ for NE, $y=1.04x-0.02(0.99)$ for E and $y=0.98x+1.5(0.99)$. The above results suggest that conjugated CAs in blood are in the sulfate form. The acid hydrolysis, however, could not be applied to erythrocytes total CAs, because both free and conjugated CAs completely coprecipitated with the protein denatured by perchloric acid. Thus, hydrolysis by sulfatase is selected in the present procedure.

Calibration curves, detection limits and precision

Linear relationships were obtained between the ratios of the peak heights of CAs to that of IP and the amounts of CAs added in the range 0.02–10 pmol each to erythrocytes CAs sample, platelets CAs sample or plasma sample. The limits of detection for NE, E and DA were 1, 1 and 2 fmol per 100- μ l of injection volume in each sample, respectively, at a signal-to-noise ratio of 2. The coefficients of variation (%) for NE, E and DA in the within-day precision ($n=10$) obtained using samples from a normal volunteer (28 years of age, Table 1) were 2.0, 2.1 and 4.4 (free, erythrocytes), 2.3, 3.4 and 4.1 (free, platelets), 2.0, 2.5 and 1.9 (total, erythrocytes), 1.9, 2.5 and 1.8 (total, platelets) and 1.9, 1.8 and 1.9 (total, plasma), respectively.

Determined values of free and total CAs in erythrocytes, platelets and plasma

The concentrations of free and total CAs in erythrocytes, platelets and plasma from 10 healthy men measured by the present method are summarized in Table 1. The values for free NE and E in all the samples agree approximately with the data obtained by other workers.^{1,2,16–18}

However, free DA concentrations in erythrocytes and platelets measured by the present method were lower than those obtained by the radioenzymatic methods.^{1,2} The same was also true for DA concentration in plasma, as described previously.²⁰ The radioenzymatic methods usually use catechol-*O*-methyltransferase for the isotope-labelling of free CAs, but the enzyme also acts on DA sulfate to form the same products (3-*O*- and 4-*O*-methyl DA) as those formed from DA.²⁴ Therefore, the radioenzymatic methods may overestimate free DA. The concentration ratios of free to total CA (*F/T*) in plasma and erythrocytes are in fairly good agreement with the data obtained by other workers.¹³ The ratios for NE and DA in platelets are higher than those in the other samples for unknown reason.

The concentrations of the individual free CAs did not correlate well with those of the respective total CAs: the correlation coefficients for NE, E and DA were 0.31, 0.82 and -0.38 (erythrocytes), 0.82, 0.38 and 0.71 (platelets) and 0.30, 0.58 and 0.12 (plasma), respectively. On the other hand, it has been reported that, in tumor-bearing rat, free NE and DA concentrations in erythrocytes correlate well with those of the respective amines in plasma.²⁵ However, in the samples from healthy men, such good correlations were not observed in any combinations of the two of the three samples (Table 2). Plasma CAs concentrations have mostly been determined in clinical investigation. However, in order to obtain more elaborate correlations between concentrations in the samples for clinical investigation, it seems significant to determine free and total CAs in all the three samples prepared from

Table 2 Correlation coefficients of free and total CAs concentrations between the two of erythrocytes, platelets and plasma^a

	Correlation coefficient					
	Free CAs			Total CAs		
	NE	E	DA	NE	E	DA
Erythrocytes+Platelets ^b	0.55	0.27	0.29	0.34	0.09	0.06
Erythrocytes+Plasma ^b	0.53	0.56	0.64	0.32	0.18	0.35
Platelets+Plasma ^b	-0.19	0.20	-0.03	0.02	-0.37	0.68

a. Calculated from the data given in Table 1.

b. Combination of the samples.

individual bloods.

This study provides the first HPLC with fluorescence detection for the determination of free and total CAs in erythrocytes and platelets. This method permits the determination of both forms of CAs in the three samples prepared from only 2 ml of a whole blood, and should be useful for clinical investigations of CAs.

References

1. J. de Champlain, M. Bouvier, J. Clroux and L. Farley, *Clinical and Experimental Hypertension*, **A6**, 523 (1984).
2. A. J. Zweifler and S. Julius, *N. Eng. J. Med.*, **306**, 890 (1982).
3. J. Clroux, F. Peronnet and J. de Champlain, *Med. Sci. Sports Exerc.*, **15**, 95 (1983).
4. N. T. Buu and O. Kuuchel, *Life Sci.*, **24**, 783 (1979).
5. K. Engelman, B. Portnoy and W. Lovenberg, *Am. J. Med. Sci.*, **255**, 259 (1968).
6. D. C. Siggers, C. Salter and P. A. Toseland, *Clin. Chim. Acta*, **30**, 373 (1970).
7. W. J. Rau and R. S. Swerdloff, *Life Sci.*, **28**, 2819 (1981).
8. K. Imai, M.-T. Wang, S. Yoshiue and Z. Tamura, *Clin. Chim. Acta*, **43**, 145 (1973).
9. A. Yamatodani and H. Wada, *Clin. Chem.*, **27**, 1983 (1981).
10. K. Mori, *J. Chromatogr.*, **218**, 631 (1981).
11. H. Hallmann, L.-O. Farnebo, B. Hamberger and G. Jonnson, *Life Sci.*, **23**, 1049 (1978).
12. C. L. Davis and S. G. Molyneux, *J. Chromatogr.*, **231**, 41 (1982).
13. S. Yoneda, M. Alexander and N. D. Vlachakis, *Life Sci.*, **33**, 935 (1983).
14. H. G. Lovelady and L. L. Foster, *J. Chromatogr.*, **108**, 43 (1975).
15. C. C. T. Smith and J. Betteridge, *Clin. Chem.*, **30**, 1432 (1984).
16. N. Alexander, M. Velasquez and N. D. Vlachakis, *Life Sci.*, **29**, 477 (1981).
17. M. da Prada and G. B. Picotti, *Br. J. Pharmacol.*, **65**, 653 (1979).
18. G. B. Picotti, B. P. Bondiolotti, A. M. Cesura, C. Ravazzani, M. D. Galva and P. Montegazza, *Adv. Exp. Med. Biol.*, **164**, 257 (1984).
19. H. Nohta, A. Mitsui and Y. Ohkura, *Anal. Chim. Acta*, **165**, 171 (1984).
20. A. Mitsui, H. Nohta and Y. Ohkura, *J. Chromatogr.*, **344**, 61 (1985).
21. E. Ross and G. Schatz, *Anal. Biochem.*, **54**, 304 (1973).
22. A. H. Anton and D. F. Sayre, *J. Pharmacol. Exp. Ther.*, **138**, 360 (1962).
23. K. Imai, M. Sugiura and Z. Tamura, *Chem. Pharm. Bull.*, **18**, 2134 (1970).
24. Y.-Q. Qu, K. Imai, Z. Tamura, Y. Hashimoto and H. Miyazaki, *Life Sci.*, **32**, 1811 (1983).
25. N. Alexander, S. Yoneda and N. D. Vlachakis, *Life Sci.*, **36**, 571 (1985).

(Received March 28, 1986)

(Accepted April 30, 1986)