[Chem. Pharm. Bull. 34(11)4687—4693(1986)]

High-Performance Liquid Chromatographic Assay for Catechol-*O*-methyltransferase in Human and Rat Erythrocyte Membrane and Soluble Fractions, and Rat Tissues

HITOSHI NOHTA, SHUNYA NOMA and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812, Japan

(Received May 22, 1986)

The previously reported assay method for catechol-*O*-methyltransferase, which uses 2-(3,4-dihydroxyphenyl)naphtho[1,2-*d*]thiazole as a fluorogenic substrate, was applied to the assay of the enzyme in human and rat erythrocyte membrane and soluble fractions, and various rat tissue preparations, with some modifications. The *m*- and *p*-methylated products formed enzymatically were determined by normal-phase high-performance liquid chromatography with fluorescence detection. The detection limits for the *m*- and *p*-methylated products were 1 pmol per assay tube (50 fmol per injection volume of 50 μl) in each case. No difference among the preparations was observed in optimal reaction conditions for the enzyme, but the ratio of *m*- and *p*-methylated products and the Michaelis constant values for the substrate and *S*-adenosyl-L-methionine (methyl donor) varied from preparation to preparation.

Keywords——catechol-*O*-methyltransferase; fluorogenic substrate; high-performance liquid chromatography; erythrocyte membrane; rat tissue

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) catalyzes the transfer of the methyl group of *S*-adenosyl-L-methionine (SAM) to one of the phenolic groups of a catechol compound[1] and therefore COMT plays an important role in the metabolism of catecholamines. Many methods have been reported for the assay of COMT in biological materials; spectrophotometry,[2] fluorimetry,[3] high-performance liquid chromatography (HPLC)[4] and radioisotopic methods.[5] The enzyme occurs in various mammalian tissues and erythrocyte soluble fraction.[6] The enzyme has also been found in rat[7] and human[8] erythrocyte membrane, and the activity was increased in schizophrenic patients.[8] COMT activity in human erythrocyte, especially in its membrane fraction, is extremely low and can be assayed only by the radioisotopic methods. The methods, however, require a radiolabelled substrate and do not permit the determination of the ratio of the two *O*-methylated products.

Recently we have reported an assay method for human erythrocyte (mainly soluble) COMT using a synthetic catechol compound, 2-(3,4-dihydroxyphenyl)naphtho[1,2-*d*]thiazole (DNT) as a fluorogenic substrate[9]; 2-(4-hydroxy-3-methoxyphenyl)naphtho[1,2-*d*]thiazole (*m*-MNT) and 2-(3-hydroxy-4-methoxyphenyl)naphtho[1,2-*d*]thiazole (*p*-MNT) formed enzymatically are extracted into an organic solvent and separated by normal-phase HPLC. The purpose of the present study was to apply the HPLC method to the assay of human and rat erythrocyte membrane and soluble COMT and rat tissue COMT, and to examine comparatively some properties of the enzyme in these preparations.

Experimental

Materials, Apparatus and HPLC Conditions——Reagents, solutions, apparatus and HPLC conditions were the same as described previously.[9] Hypotonic phosphate buffer (pH 5.8) was prepared by mixing 10 mM sodium

dihydrogen phosphate and 7 mM disodium hydrogen phosphate, and isotonic phosphate buffer (pH 7.4) by mixing 0.16 M sodium dihydrogen phosphate and 0.10 M disodium hydrogen phosphate.

Enzyme Preparations——Human Erythrocyte Membrane and Soluble Fractions: The fractions were obtained according to the reported method[7,10] with minor modifications as follows. Venous blood (5 ml) obtained from a normal volunteer was collected into a 10-ml glass tube containing 100 μl of 0.1 M ethylenediaminetetraacetic acid disodium salt (EDTA·2Na). All further procedures were carried out at 0—5 °C. The blood was centrifuged for 10 min at 1500 *g* and the plasma was removed. The erythrocytes were washed twice with 5 ml of the isotonic phosphate buffer (pH 7.4). A portion (1 ml) of the erythrocytes was lysed with 29 ml of the hypotonic phosphate buffer containing EDTA·2Na and magnesium chloride (0.2 mM each; a stabilizer and an activator of COMT, respectively). The lysate was centrifuged at 1000 *g* for 10 min and the supernatant was again centrifuged at 20000 *g* for 20 min. The resulting supernatant was used as soluble COMT preparation. The precipitate (erythrocyte membrane) was washed twice with *ca.* 30 ml of the hypotonic phosphate buffer (pH 5.8) and then homogenized with 2 ml of 0.15 M potassium chloride containing EDTA·2Na and magnesium chloride (0.2 mM each) in a glass homogenizer. The homogenate was used as membrane COMT preparation. The soluble and membrane preparations were stored at −70 °C. The protein concentrations of the membrane and soluble preparations were approximately 6 and 10 mg/ml, respectively. Protein concentration was measured by the method of Lowry *et al.*[11] using bovine serum albumin as a standard protein.

Rat Erythrocyte Membrane and Soluble Fractions, and Tissue Homogenates: Female WKA rats (5 weeks old, 180—200 g) were decapitated and the blood was collected in a 10-ml glass tube containing 100 μl of 0.1 M EDTA·2Na. From each rat, the liver, kidneys, brain and heart were removed and chilled on ice. All further procedures were conducted at 0—5 °C. Erythrocyte membrane and soluble COMT preparations were obtained in the same way as in the case of human erythrocyte. Tissues (1 g) were each homogenized with 4 volumes of 0.15 M potassium chloride containing EDTA·2Na and magnesium chloride (0.2 mM each) and the homogenate was centrifuged at 5500 *g* for 30 min. The supernatant was diluted with water to the protein concentrations (mg/ml) of approximately 0.2 (liver and kidney), 0.9 (brain) or 1.2 (heart) and used as enzyme preparations. All the COMT preparations were stored at −70 °C.

Procedure for COMT Assay (Procedure A)——To 100 μl of an enzyme preparation was added 100 μl of a mixture of 1.0 mM SAM, 0.2 M sodium phosphate buffer (pH 7.5) and 40 mM magnesium chloride (9:9:2, v/v). The mixture was preincubated at 37 °C for 10 min, then incubated again at 37 °C for 60 min (human erythrocyte membrane and soluble COMT) or 30 min (the others) after the addition of 20 μl of 0.2 mM DNT in aqueous 50% (v/v) isopropanol. The reaction was stopped by adding 1.0 ml of 0.2 M hydrochloric acid. To this mixture, 1.0 ml of a mixture of *n*-hexane and chloroform (4:1, v/v) was added and the resulting *m*- and *p*-MNT were extracted with mechanical shaking (*ca.* 300 rpm) for 10 min. A 50-μl aliquot of the upper organic layer was injected into the chromatograph. The organic layer could be used for more than a week when stored at room temperature (*ca.* 20—27 °C) in daylight. For the blank, the same procedure was carried out except that the DNT solution was added after the addition of 0.2 M hydrochloric acid. To prepare the calibration curves, 20 μl of the DNT solution was replaced with 20 μl of the DNT solution containing *m*- and *p*-MNT (each 5-1000 pmol per 20 μl).

Procedure for COMT Assay in the Presence of Pyrogallol (Procedure B)——To 100 μl of an enzyme preparation, 75 μl of a mixture of 0.2 M sodium phosphate buffer (pH 7.5), 40 mM magnesium chloride and 2.0 mM SAM (8:2:5, v/v) was added. The mixture was preincubated at 37 °C for 10 min, and then incubated again at 37 °C for 30 or 60 min (see procedure A) after the addition of 45 μl of a mixture of the DNT solution and 50 μM pyrogallol (4:5, v/v). Subsequent treatment was the same as in procedure A.

Results and Discussion

Human Erythrocyte Membrane and Soluble COMT

COMT in human erythrocyte soluble and membrane fractions is stable for at least 1 month when stored at −70 °C in the presence of EDTA·2Na and magnesium chloride. In the absence of the additives, the enzyme activity decreased by *ca.* 30% in a week in each preparation.

Figure 1 shows typical chromatograms obtained with human erythrocyte membrane and soluble fractions according to procedure A. No peak was seen in the chromatograms of the respective blanks. Peaks 1 and 2 had retention times of 2.8 and 3.5 min, respectively, and the eluates had fluorescence excitation and emission spectra identical with those for *m*- and *p*-MNT dissolved in the mobile phase, respectively (excitation and emission maxima (nm): 349 and 393 for *m*-MNT, 347 and 389 for *p*-MNT, respectively).[9]

Pyrogallol, a potent inhibitor of COMT,[12] has been reported to inhibit human erythrocyte COMT (mainly soluble) competitively with respect to DNT.[9] The same was true

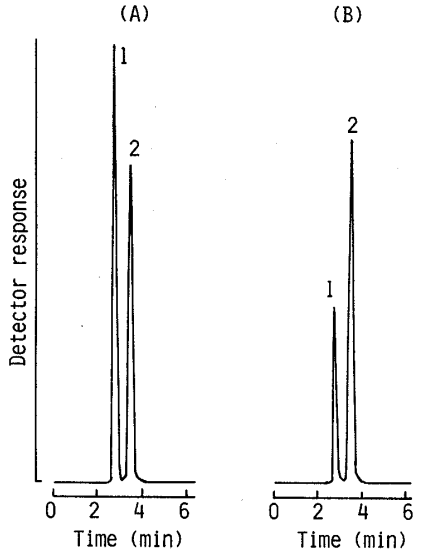

Fig. 1. Chromatograms Obtained from Human Erythrocyte (A) Membrane and (B) Soluble Fractions

Peaks: 1, *m*-MNT; *p*-MNT. COMT activities (pmol/min/mg protein): membrane, 1.04 *m*-MNT and 0.75 *p*-MNT; soluble, 0.25 *m*-MNT and 0.49 *p*-MNT.

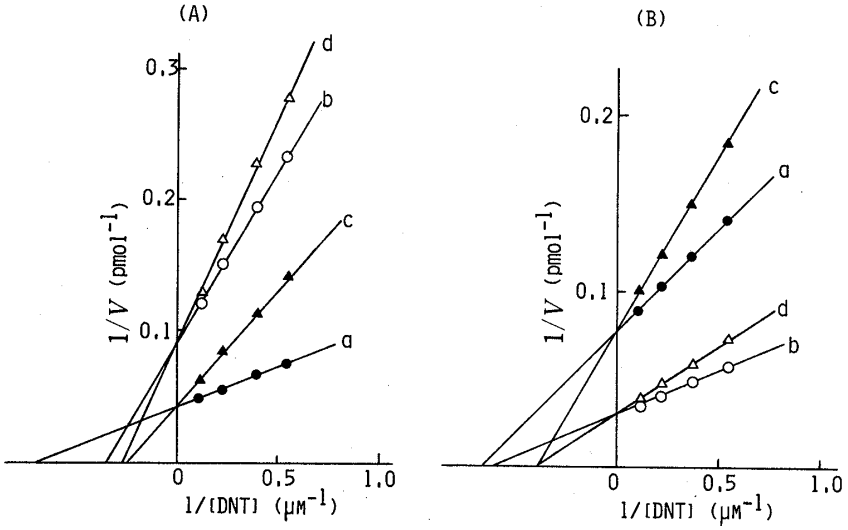

Fig. 2. Inhibition of COMT in Human Erythrocyte (A) Membrane and (B) Soluble Fractions by Pyrogallol

The data were plotted by linear regression analysis. Lines: a and c, *m*-MNT; b and d, *p*-MNT. Pyrogallol concentrations in the reaction mixtures: a and b, absent; c and d, 5 μM.

for both membrane and soluble COMT when the inhibition was examined according to procedure B (Fig. 2). The observed inhibitory constant (K_i) values calculated by the method of Dixon[13] are shown in Table I.

The optimum pH values for the enzymatic *m*- and *p*-methylation reactions were both 7.5 for membrane COMT (Fig. 3A), and 7.5—8.0 and 6.5, respectively, for soluble COMT (Fig. 3B); pH 7.5 was tentatively selected. DNT was stable in the pH range 5—10 for at least 2 h when incubated at 37 °C. Sodium phosphate buffer (pH 7.5) gave maximum and constant activities in the *m*- and *p*-methylation reactions for both the enzyme preparations at the concentrations of 0.1—0.5 M; 0.2 M was used. Although 0.1 M MOPS (3-*N*-morpholino)-propanesulfonic acid), 0.1 M TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) and 0.1 M Bicine (all Good's buffers) gave the same relationships between pH and the enzyme activity in the *m*- and *p*-methylation reactions with both enzyme preparations, the activity in each reaction at pH 7.5 did not exceed that obtained in the phosphate buffer. The pH affected the product ratio of *m*- and *p*-MNT (*m*/*p* ratio) in the case of soluble COMT and the ratio increased with increasing pH, particularly in the range of 6.0—8.0, whereas in the

TABLE I. Properties of COMT in Human and Rat Erythrocyte Membrane and Soluble Fractions, and Rat Tissues

Enzyme preparation	Methylation	Optimal pH	*m*/*p* ratio at pH 7.5	K_m value for DNT (μM)	K_m value for SAM (μM)	K_i value for pyrogallol (μM)	Heat inactivation (%) (50 °C, 10 min)
Human							
Erythrocyte membrane	*m*	7.5	1.54	1.4	3.4	1.1	28
	p	7.5—8.0		2.1	4.2	1.5	29
Erythrocyte soluble	*m*	7.5—8.0	0.60	1.6	5.4	3.8	20
	p	6.5		1.7	4.5	4.5	22
Rat							
Erythrocyte membrane	*m*	7.0	1.02	2.7	2.6	0.8	49
	p	8.0		7.0	3.7	2.9	45
Erythrocyte soluble	*m*	8.0	0.61	17	4.2	15	21
	p	6.5		11	4.5	16	20
Liver	*m*	7.5	0.59	—	37	—	31
	p	6.5		—	44	—	34
Kidney	*m*	7.5	0.59	2.9	16	4.0	38
	p	6.5		2.6	21	3.6	33
Brain	*m*	7.5	0.77	5.1	6.7	32	72
	p	6.5		6.8	3.8	39	75
Heart	*m*	7.5	0.61	6.8	5.3	1.1	72
	p	6.5		6.0	6.0	1.1	70

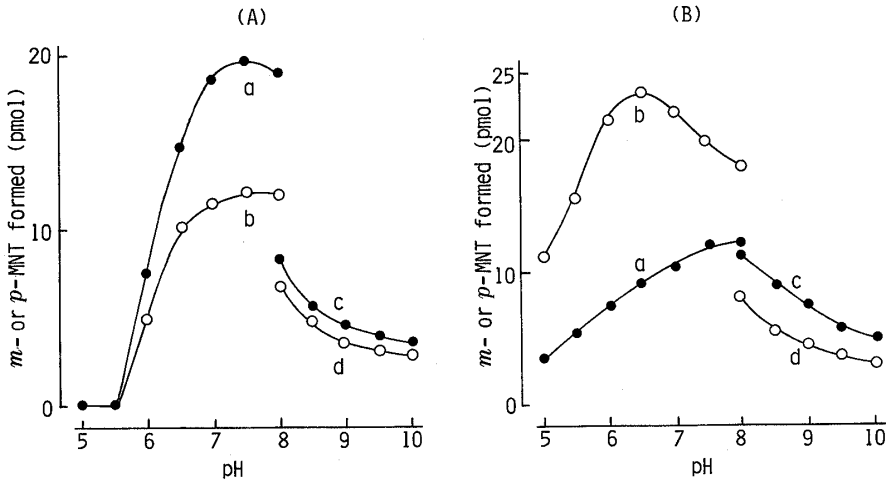

Fig. 3. Effect of pH on the Amounts of *m*- and *p*-MNT Formed in the Reactions of Human Erythrocyte (A) Membrane and (B) Soluble COMT

Curves: a and c, *m*-MNT; b and d, *p*-MNT. Buffers: pH 5.0—8.0, 0.1 M sodium phosphate buffer; pH 8.0—10.0, 0.1 M Tris–hydrochloric acid buffer.

case of membrane COMT the ratio was almost constant in the same pH range. The ratios for membrane and soluble COMT at pH 7.5 are shown in Table I.

Maximum and constant velocity of the *m*- and *p*-methylation reactions of membrane and soluble COMT was attained in the presence of 15—50 μM DNT in the incubation mixture. The Michaelis constant (K_m) values for DNT, which did not exceed 2.1 μM in each reaction (Table I), suggest that the affinity of each COMT for DNT is very high; 20 μM was used as a saturating concentration. DNT was dissolved in aqueous 50% (v/v) isopropanol as described previously.[9]

Maximum and constant velocity of the *m*- and *p*-methylation reactions of both enzyme

preparations was achieved in SAM concentrations of 100—500 μM and the K_m value for SAM did not exceed 6 μM in each reaction (Table I); 200 μM was selected. Magnesium chloride gave almost maximum and constant activities in the range of 0.45—4.5 mM, and higher concentrations caused inhibition of the enzyme; 1.8 mM was used in the procedure. Membrane COMT was more susceptible to heat inactivation than soluble COMT (Table I). The resulting enzyme reaction conditions are similar to those described in the previous paper except for the pH of the buffer.[9]

Rat Tissue COMT and Comparative Study

All the enzyme preparations from rat were stable for at least a month at -70 °C.

All the tissue COMT preparations could catalyze the O-methylation of DNT. Optimal pH, *m*/*p* ratios at pH 7.5 and kinetic data for the enzyme, and its susceptibility to heat inactivation are shown in Table I. The optimal pH values for the *m*- and *p*-methylation reactions with rat erythrocyte membrane COMT were fairly similar to those with human erythrocyte membrane COMT, and the values in the cases of rat erythrocyte soluble COMT and tissue COMT were similar to those for human erythrocyte soluble COMT. Other optimal conditions for the enzyme reactions with preparations from rat were the same as those for human erythrocyte soluble and membrane COMT. The *m*/*p* ratio with erythrocyte membrane COMT was higher than those with erythrocyte soluble COMT and tissue COMT.

The K_m values for DNT and SAM, and the K_i value for pyrogallol differed depending on the COMT preparation used (Table I). It was reported that, when epinephrine or dopamine was used as a substrate, the K_m values of rat erythrocyte membrane COMT were 60—70 times lower than those of soluble COMT.[7] In this study, however, DNT did not show very great differences in K_m value among the preparations. The K_m values for DNT in the case of liver COMT could not be calculated by linear regression analysis (Lineweaver–Burk plots) because the enzyme was strongly inhibited at low DNT concentrations (0.1—5 μM) for an unknown reason, and thus K_i values for pyrogallol could not be obtained. In the brain and heart preparations, weak COMT activities, which corresponded to 11% (brain) and 23% (heart) of those in the presence of 200 μM SAM in each methylation reaction, occurred even when SAM was not added to the enzyme reaction mixtures. The K_m values of these COMT preparations for SAM (Table I) were thus obtained by using the preparations dialyzed against 0.15 M potassium chloride containing 0.2 mM EDTA·2Na and 0.2 mM magnesium chloride (both additives were required to prevent inactivation of the enzyme) at 4 °C for 1 h. This dialysis did not cause any loss of the enzyme activity.

Rat erythrocyte membrane COMT was more susceptible to heat inactivation than the soluble COMT (Table I), as reported previously.[7] In the case of the other tissue COMT, on the other hand, the susceptibility varied greatly depending on the preparation (Table I).

Although the *m*/*p* ratio, the K_m values for DNT and SAM and the susceptibility to heat inactivation vary from preparation to preparation as mentioned above, these observations are not enough to allow us to decide whether the COMT activities in the preparations are essentially different.

COMT Activities in Human and Rat Erythrocyte Fractions and Rat Tissues

The recoveries of *m*- and *p*-MNT added to the incubated blank in amounts of 0.1 nmol were 90% or greater with standard deviations of 2% or less in all cases. The amounts of *m*- and *p*-MNT formed enzymatically were proportional to the amounts of protein in the enzyme preparations up to at least 1200 (human erythrocyte membrane), 2000 (human erythrocyte soluble), 450 (rat erythrocyte membrane), 800 (rat erythrocyte soluble), 100 (liver), 50 (kidney), 180 (brain) or 450 (heart) μg. The COMT activities for the *m*- and *p*-methylation reactions in all the enzyme preparations were linear with incubation time at 37 °C up to at least 120 min. The within-day precision of the method was established by using human erythrocyte

membrane and rat kidney preparations. The coefficients of variation ($n=12$) were 1.3% and 1.9% for mean activities of 1.59 pmol of *m*-MNT and 0.95 pmol of *p*-MNT per min per mg of protein, respectively (erythrocyte membranes), and 2.3% and 1.5% for those of 0.37 and 0.56 nmol per min per mg of protein, respectively (kidney). Linear relationships were observed between the peak heights and the amounts of *m*- and *p*-MNT added in the range of 5-1000 pmol to the incubated blank reaction mixtures with all the enzyme preparations. The detection limits ($S/N=2$) for *m*- and *p*-MNT were both 1 pmol per assay tube (50 fmol per injection volume of 20 μl) in each sample.

COMT activities in erythrocyte soluble and membrane fractions from eleven healthy persons differed from individual to individual, but the *m*/*p* ratios were almost constant (Table II). The activities of membrane and soluble COMT in the individual samples did not correlate well; the correlation coefficients were 0.70 and 0.63 for the *m*- and *p*-methylation reactions, respectively. Although further investigations are required to clarify the relationship between erythrocyte membrane and soluble COMT, it may be necessary for clinical investigation to

Table II. COMT Activities and *m*/*p* Ratios in Human Erythrocyte Membrane and Soluble Fractions

Age	Sex[a]	Membrane fraction			Soluble fraction		
		COMT activity (pmol/min/mg protein)		*m*/*p* ratio	COMT activity (pmol/min/mg protein)		*m*/*p* ratio
		m-MNT	*p*-MNT		*m*-MNT	*p*-MNT	
54	m	0.95	0.67	1.42	0.40	0.68	0.60
36	m	1.59	0.95	1.68	0.35	0.64	0.55
30	m	1.29	0.87	1.47	0.53	0.93	0.57
27	m	1.12	0.79	1.43	0.37	0.55	0.66
26	m	1.33	0.91	1.47	0.49	0.40	0.64
25	m	1.04	0.75	1.38	0.25	0.49	0.51
24	m	1.23	0.75	1.43	0.37	0.57	0.65
24	m	1.58	0.93	1.71	0.40	0.71	0.57
23	m	1.70	1.04	1.64	0.50	0.81	0.61
25	f	0.42	0.29	1.49	0.22	0.36	0.60
23	f	0.67	0.42	1.59	0.27	0.43	0.64
Mean±S.D.		1.18±0.37	0.68±0.22	1.54±0.12	0.38±0.10	0.63±0.17	0.60±0.04

a) m, male; f, female.

Table III. COMT Activities and *m*/*p* Ratios in Rat Erythrocyte Fractions and Tissues[a]

Erythrocyte fraction or tissue	COMT activity (pmol/min/mg protein)		*m*/*p* ratio
	m-MNT	*p*-MNT	
Erythrocyte membrane	4.1±0.7	4.1±0.7	1.02±0.07
Erythrocyte soluble	7.1±2.9	11.7±4.7	0.60±0.01
Liver	739±54	1250±90	0.59±0.01
Kidney	284±17	479±31	0.59±0.05
Brain	102±9	132±10	0.77±0.01
Heart	9.0±1.0	14.8±1.6	0.61±0.01

a) Each value is the mean±S.D. obtained from 10 rats.

assay both COMT activities.

COMT activities in the enzyme preparations from rats (WSK, female, 5 weeks old, 180—200 g) assayed by the present method are shown in Table III. The erythrocyte COMT activities in 10 rats were higher than those in human.

COMT activities in human and rat erythrocyte membrane and soluble fractions, and various rat tissue preparations were found to differ in m/p ratio, K_m values for DNT and SAM, and susceptibility to heat inactivation when DNT was used as substrate. The present assay method may be useful for the investigation of isoenzymes of COMT.

Acknowledgement A Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan is gratefully acknowledged.

References

1) J. Axelrod and R. Tomchick, *J. Biol. Chem.*, **233**, 702 (1958).
2) P. J. Anderson and A. D'Iorio, *Can. J. Biochem.*, **44**, 347 (1966); R. T. Borchardt, *Anal. Biochem.*, **58**, 382 (1974).
3) M. Assicot and C. Bohuon, *Life Sci.*, **8**, 93 (1969); Y. Okada, K. Zaitsu, K. Ohtsubo, H. Nohta and Y. Ohkura, *Chem. Pharm. Bull.*, **29**, 1670 (1981).
4) R. T. Borchardt, M. F. Hegazi and R. L. Schowen, *J. Chromatogr.*, **152**, 255 (1978); E. J. M. Pennings and G. M. J. Van Kempen, *Anal. Biochem.*, **452**, 98 (1979); K. Zaitsu, Y. Okada, H. Nohta, K. Kohashi and Y. Ohkura, *J. Chromatogr.*, **211**, 129 (1981).
5) J. Axelrod, W. Albers and C. D. Clemente, *J. Neurochem.*, **5**, 68 (1959); M. Assicot and C. Bohuon, *Nature* (London), **212**, 861 (1966); H. Masayasu, M. Yoshioka and Z. Tamura, *Chem. Pharm. Bull.*, **27**, 633 (1979).
6) H. C. Guldberg and C. A. Marsden, *Pharmacol. Rev.*, **27**, 135 (1975).
7) M. Assicot and C. Bohuon, *Biochimie*, **53**, 871 (1971).
8) P. Poitou, M. Assicot and C. Bohuon, *Biomedicine*, **21**, 91 (1974).
9) H. Nohta, S. Noma, Y. Ohkura and B. Yoo, *J. Chromatogr.*, **308**, 93 (1984).
10) J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem.*, **100**, 119 (1963).
11) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
12) J. Axelrod and M. J. LaRoche, *Science*, **130**, 800 (1959).
13) M. Dixon, *Biochem. J.*, **55**, 170 (1953).