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A Simple Determination of Catechol-O-Methyltransferase Activity

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^{3}H-Epinephrine is used as the substrate for the assay, and ^{3}H-metanephrine, the product, is efficiently separated by an adsorption column chromatography on Amberlite XAD-4. This radiochemical assay method is simple, highly sensitive, and quantitative, and put to practical use of the measurement of the activity of catechol-O-methyltransferase in red blood cells.

Keywords——catechol-O-methyltransferase; catecholamine; epinephrine; metanephrine; Amberlite XAD-4; red blood cell; MK-486; radiochemical assay

Catecholamines (CA) are inactivated *in vivo* by catechol-O-methyltransferase (COMT) (EC 2.1.1.6). COMT catalyzes the transfer of the methyl group of S-adenosyl-methionine (SAM) to the catechol substrates. Colorimetric,[2)] fluorometric,[3)] and radiochemical assay methods[4)] of its activity have been developed for biochemical and clinical researches. However the colorimetric assays were not so sensitive. In the fluorometric or radiochemical assays, O-methylated products are extracted from the reaction mixture with organic solvents such as toluene-isoamylalcohol[3b,4a)] or ethylacetate.[4b,c)] The efficiency of the extraction of the products is low and not reproducible. The blank value in the extract is fairly high because of the contamination with the substrates. Therefore model substrates are often used in place of CA themselves to improve the extraction procedure,[4b,c)] but the model substrates might elicit mischievous results due to the substrate specificity of the enzyme.

We developed a sensitive radiochemical assay method using ^{3}H-epinephrine (E), one of CA, as a substrate. This assay method is based on an adsorption column chromatography on Amberlite XAD-4, which adsorbs metanephrine (MN) but not E in a borate buffer. The validity of the method was confirmed with rat liver soluble COMT (sCOMT). The method was further applied to assay the activity of COMT in intact red blood cells (iRBC), which existence in RBC was thought to be in close connection with the metabolism of CA in blood.[5)]

Experimental and Results

Materials and Reagents——DL-E (Sigma) and DL-MN·HCl (Nakarai chemicals Co., LTD) were used. DL-E-[7-^{3}H]·L-bitartrate (11 Ci/mmol) and DL-MN-[7-^{3}H] (5.29 Ci/mmol) were purchased from New England Nuclear. SAM chloride (Tokyo Kasei Co., LTD), crystalline human serum albumin (HSA) (NBC) and crystalline bovine serum albumin (Pharmacia) were obtained. MK-486 (Carbidopa, L-α-methyl dopa-α-hydrazine) was gifted from Nippon Merck Banyu Co., LTD. The other reagents used were of reagent grade

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2) *a*) W.F. Herblin, *Anal. Biochem.*, **51**, 19 (1973); *b*) R.T. Borchardt, *ibid.*, **58**, 382 (1974).
3) *a*) J. Axelrod and R. Tomchick, *J. Biol. Chem.*, **233**, 702 (1958); *b*) J. Axelrod, *Methods Enzymol.*, **5**, 748 (1962).
4) *a*) J. Axelrod, W. Albers, and C.D. Clemente, *J. Neurochem.*, **5**, 68 (1959); *b*) M. Assicot and C. Bohuon, *Nature* (London), **212**, 861 (1966); *c*) R.E. McCaman, *Life Sci.*, **4**, 2353 (1965).
5) *a*) J. Axelrod and C.K. Cohn, *J. Pharmacol. Exp. Ther.*, **176**, 650 (1971); *b*) J-M. Frere, *Biochem. Pharmacol.*, **20**, 3353 (1971); *c*) M. Assicot and C. Bohuon, *Biochmie*, **53**, 871 (1971); *d*) A. Danon and J.D. Sapira, *Clin. Pharmacol. Ther.*, **13**, 916 (1972); *e*) H.F.K. Männl, K. Hempel, and W. Kübler, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **272**, 265 (1972); *f*) J. Griffiths and H. Linklater, *Clin. Chim. Acta*, **39**, 383 (1972); *g*) R.M. Weinshilboum, *Life Sci.*, **22**, 625 (1978).

purity. Polyamide Layer (Cheng Chin Trading Co., Taiwan), Autoradiograph films ^{3}H-type (Konishiroku), Sephadex G-200 (Pharmacia), Amberlite XAD-4 and Amberlite CG-120 (Rohm and Haas) were purchased.

An electrolyte solution (ES) composed of main human plasma ingredients, 5 mM KCl, 97 mM NaCl, 29 mM $NaHCO_3$, 2 mM Na_2HPO_4, 4.5 mM lactic acid, 0.5 mM H_2SO_4, 1 mM Na_2SO_3, 4 mM dextrose, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ was prepared and the pH was adjusted to 7.4 with 0.1 M NaOH.

Assay Method of COMT Activity——Amberlite XAD-4 was ground and washed according to the method of Wang *et al.*[6] Particles of 60—100 mesh were poured into a glass tube (consisting of a 10-ml cylindrical reservoir attached to a tube of 5 mm diameter) until the packed adsorbent was 2 cm high. The column was washed with 5 ml of 0.4 M borate buffer, pH 8.0 (BB), before use.

A reaction mixture in a siliconized 10-ml test tube was contained 1 mM $MgCl_2$, SAM (100 μM for sCOMT, 40 μM for iRBC), 1 mM Na_2SO_3, 25 nM—200 μM DL-E-[7-^{3}H] (^{3}H–E was diluted with cold E) and 250 μl of an enzyme sample or iRBC suspension, and was made up to 500 μl by the addition of 0.05 M phosphate buffer, pH 8.0, or ES. For the inhibition test, MK-486 (1 mM),[7] L-dopa (1 mM),[7,8] EDTA (5—20 mM) to mask divalent cations,[3] or 40 mg/ml of HSA was added to the reaction mixture.

After shaking the mixture at 37° for 60 min, 2 ml of chilled BB were added to stop the reaction. The ghosts of RBC were discarded after centrifugation. Two milliliters of the stopped solution or the supernatant were passed through the Amberlite XAD-4 column at a flow rate of 0.6 ml/min. The column was washed with 10 ml of BB and eluated with 10 ml of 0.1 M acetate buffer, pH 4.5 in 50% methanol. A 1-ml aliquot of the eluate was mixed with 10 ml of Bray's scintillation solution and counted for radioactivity. The mixture without enzyme sample was treated simultaneously as the blank.

Characteristics of the Method——Though E and MN in a phosphate buffer were similarly adsorbed on Amberlite XAD-4, adsorption of MN was increased at the higher concentration of borate while that of E was much decreased. The elution profile of E and MN was shown in Fig. 1. From counts of ^{3}H–E or ^{3}H–MN, more than 99% of E was found to pass through the column by the washing. The recovery of MN after the elution was found to be 94±1% (n=7) and not influenced by the presence of proteins and inhibitors of COMT. When XAD-4 was not ground, the recovery of MN was 60±2% (n=4).

The calibration curve by the present method was compared with that by the solvent extraction method[3b] as shown in Fig. 2. The extraction method recovered only 46% of added ^{3}H–MN and gave a high blank

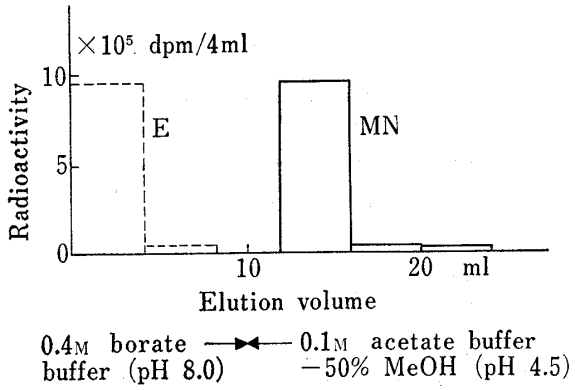

Fig. 1. Elution Profile of E and MN from the Column of Amberlite XAD-4

A model solution of the reaction mixture containing 100 μM E, 10 μM MN, 0.5 μCi of ^{3}H–E or ^{3}H–MN and rat liver sCOMT inactivated at 100° for 1 min was treated and separated as described in the text.

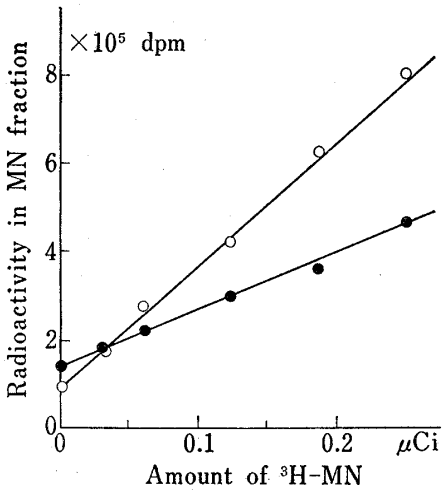

Fig. 2. Calibration Curves obtained by the Present Method and the Extraction Method[3b]

A 500-μl model reaction mixture containing 2.5 μCi of ^{3}H–E, 0.03—0.25 μCi of ^{3}H–MN and the inactivated rat liver sCOMT was measured as shown in Fig. 1 (—○—). Another 100-μl model solution containing the same amounts of ^{3}H–E, ^{3}H–MN and 20 μl of the inactivated enzyme solution was treated and extracted with toluene-isoamylalcohol according to Axelrod (—●—).[3b]

6) M-T. Wang, M. Yoshioka, K. Imai, and Z. Tamura, *Clin. Chim. Acta*, **63**, 21 (1975).
7) R.J. Baldessarini, *J. Pharm. Pharmacol.*, **24**, 78 (1972).
8) *a*) R.J. Baldessarini, *Biochem. Pharmacol.*, **22**, 247 (1973); *b*) H.C. Guldberg and C.A. Marsden, *Pharmacol. Rev.*, **27**, 135 (1975).

value of ^{3}H-E as described by McCaman,[4c] Griffiths and Linklater.[5f] The operation time and labor by the present method were as much as those by the convenient extraction method.

Assay of Rat Liver sCOMT——The livers of Donryu rats (150—200 g) were used. All operations were carried out at 4° according to the method of Axelrod.[3b] But the centrifugation (Step 1) was done at 15000×*g* and gel filtration through a Sephadex G-200 column was carried out instead of the calcium phosphate adsorption and elution (Step 3). The fractions equal to molecular weight 24000[9] were pooled, and diluted to 2 mg/ml with 0.05 M phosphate buffer, pH 8.0, prior to use. The protein concentration was determined by the method of Lowry *et al.*[10] Effect of E on the activity of rat sCOMT was measured by the present method. From the reciprocal plot of velocity *vs.* E concentration (Fig. 3), the apparent *Km* value for E was estimated 2×10^{-4} M. This value is close to the values reported by Axelrod, Tomchick[3a] and Creveling.[11] The activity was inhibited noncompetitively by MK-486,[7] competitively by L-dopa,[7,8] and completely by 20 mM EDTA.[3] Na_2SO_3 (1 mM) as an antioxidant did not influence on the activity of COMT. The product (eluate from the XAD-4 column) was identified as MN by an ion exchange chromatography with Amberlite CG-120[12] and the autoradiography of thin-layer chromatogram on the polyamide layer developed with a mixture of isobutanol, acetic acid, and cyclohexane (80: 7: 10).[13]

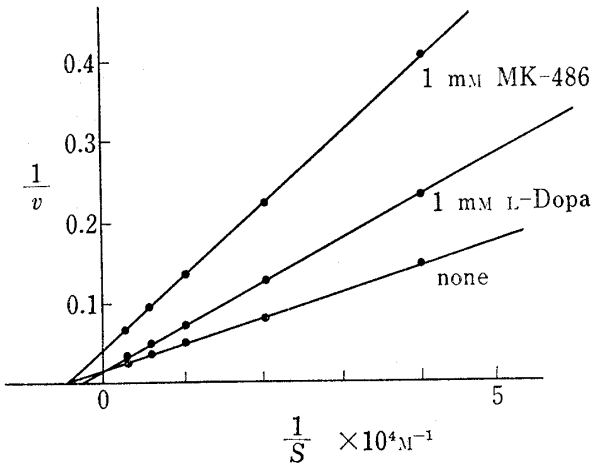

Fig. 3. Lineweaver-Burk Plots of Effect of E on the Activity of Rat Liver sCOMT in the Presence and Absence of Inhibitors

v: nmol of metanephrine formed/hr/mg of protein.
S: concentration of E, in 10^{-4} M.

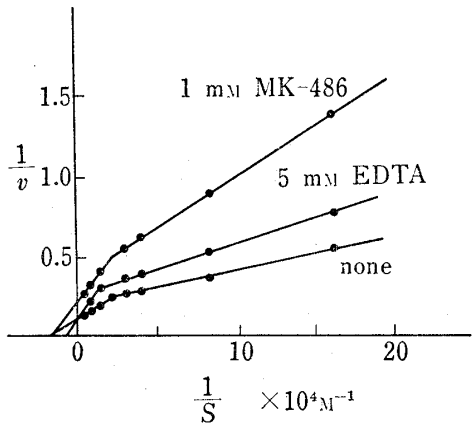

Fig. 4. Lineweaver-Burk Plots of Effect of E on the Activity of Rat iRBC COMT in the Presence and Absence of Inhibitors

v: nmol of metanephrine formed/hr/ml of blood.
S: concentration of E, in 10^{-4} M.

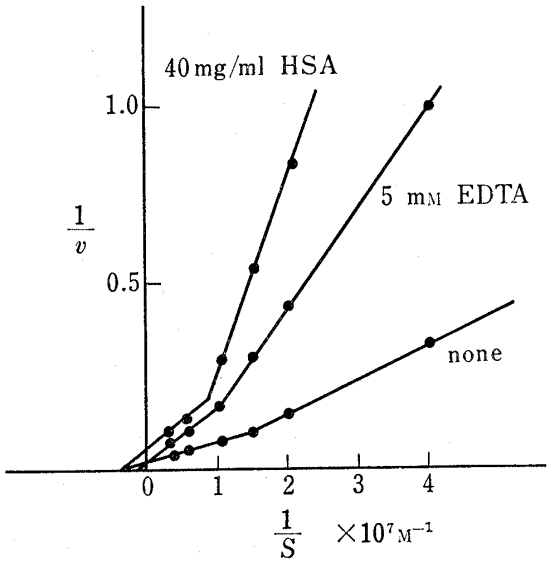

Fig. 5. Double Reciplocal Plots of Effect of E on the Activity of Human iRBC COMT in the Presence of HSA and EDTA

v: pmol of metanephrine formed/hr/ml of blood.
S: concentration of E, in 10^{-7} M.

9) M. Assicot and C. Bohuon, *Eur. J. Biochem.*, **12**, 490 (1970).
10) O.H. Lowry and N.J. Rosenbrough, *J. Biol. Chem.*, **193**, 265 (1951).
11) C.R. Creveling, N. Dalgard, H. Shimizu, and J.W. Daly, *Mol. Pharmacol.*, **6**, 691 (1970).
12) J. Häggendal, *Scandinav. J. Clin. Lab. Invest.*, **14**, 537 (1962).
13) K. Randeratb, "Thin-Layer Chromatography," Academic Press, New York, 1966, p. 108.

Assay of COMT Activity in RBC——Human blood was drawn from the branchial vein. Rat blood was drawn from the carotid artery. The following operations were carried out at 4°. One milliliter of acid citrate dextrose solution (0.07 M dextrose–0.12 M citrate, pH 5.0) was added to 10 ml of blood and centrifuged at $1000 \times g$ for 15 min. The supernatant and the buffy coat containing white blood cells were removed by aspiration and the pellet was washed three times with ES. Finally the pellet was suspended in 5 ml of ES, and used as the enzyme sample of iRBC. The sample was further frozen and thawed, and the supernatant was obtained by centrifugation as the enzyme sample of soluble COMT of RBC (RBCsCOMT).

The activity of COMT of rat iRBC was measured. The reciprocal plot of velocity *vs.* E concentration is shown in Fig. 4. This suggests that two different enzymes are present in iRBC. The estimated *Km* of the high affinity enzyme was 4.5×10^{-6} M. On the other hand the reciprocal plot of rat RBCsCOMT similarly measured gave a linear line and a *Km* of 2.5×10^{-4} M. These figures were in good agreement with those described by Assicot and Bohuon.[4b] The inhibition pattern was the same as that of rat liver sCOMT. The activity of COMT of human iRBC was measured and the reciprocal plot gave a bent line similar to Fig. 4 at higher concentrations of E, while at the lower concentrations it gave a adverse bent line as shown in Fig. 5. Furthermore the activity was inhibited markedly by HSA.

Discussion

We are able to separate MN from E completely by the use of XAD-4 in the borate buffer. The reason why the more MN was adsorbed with XAD-4 in the presence of borate was unknown, while E was probably not adsorbed by the complex formation with borate.[14] From this and previous experiments described by us,[6,15] the chromatography with XAD-4 is a useful technique for separation and purification of CA metabolites. The other O-methyl CA, namely normetanephrine and 3-methoxytyramine, were adsorbed and eluted on the column of XAD-4 efficiently[6] so that norepinephrine or dopamine used as a substrate was separable. The column as well adsorbs further metabolites of O-methyl CA such as vanilmandelic and homovanillic acids[15a] and another metabolite of L-dopa as a CA precursor, vanilpyruvic acid.[15b]

In this assay about 1% of the radioactivity of the substrate was detected in the eluent; the rate was higher as the isotope older. On the other hand it was recognized that the older isotope contained a more degradated product by the ion exchange chromatography of Amberlite CG-120.[12] So it was thought that the degradation product of ^{3}H-E was eluated in the fraction of MN. This fact, however, did not interfere with the assay because the blank value was constant and independent of concentration of cold E.

The present method is sensitive because the substrate is tritiated. The recovery of MN is much higher than the ones by the other extraction methods.[3b,4] Even 1 nM MN is reproducibly detected by this assay method. Another good feature of this method is that E itself is used as the substrate. This is especially fit for the measurement of the activity of COMT of iRBC, where CA are uptaken into the cells by active transport and metabolized.[5c–d] But to get more real data L form should be used as the substrate instead of DL form, although no stereospecificity of COMT, that is, almost the same magnitude of affinity to epinephrine optical isomers, was described.[3a,11]

The enzyme activities measured by the present method in Fig. 3, 4 were reasonably corresponded to the ones obtained by the other methods as described in Results. However MK-486, a potent dopa decarboxylase inhibitor, was found to be noncompetitive to COMT, while Baldessarini mentioned it competitive.[7] From the plot in Fig. 5, apparently two types of COMT in human iRBC as well as rat seemed to be present but was difficult to be kinetically explained. Although Griffiths and Linklater determined COMT in hemolysate without considering membrane COMT[5f] and Danon and Sapira did whole blood COMT without supple-

14) E.M. Trautner and M. Messer, *Nature* (London), **169**, 31 (1952).
15) *a*) S. Takahashi, M. Yoshioka, S. Yoshiue, and Z. Tamura, *J. Chromatogr.*, **145**, 1 (1978); *b*) A. Yoshida, M. Yoshioka, T. Sakai, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **26**, 1177 (1978).

mentation of SAM,[5d] these data were not comparable. Inhibition of HSA at the physiological concentration was referred to high affinity binding (10^{+7} M^{-1}) of HSA to CA.[16]

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16) A. Danon and J.D. Sapira, *J. Pharmacol. Exp. Ther.*, **182**, 295 (1972).