HMT activities in preparations obtained from the kidney, brain and stomach of a Sprague-Dawley male rat (6 weeks old) were 560, 83 and 2 μmol per min per mg protein, respectively, but no activity was detected in preparations from the lung, heart, liver, spleen and blood of the rat. The preparations from tissues other than kidney were obtained by a procedure similar to that used for the kidney.

This study provides the first method for the HPLC assay of HMT. The proposed method is sensitive, the entire procedure can be performed in about 2 h, and more than 20 samples can be assayed simultaneously. This method should be useful routinely in place of the radiochemical method.

References and Notes

1) R.W. Schayer, *Ann, Rev. Physiol.*, **39**, 116 (1959).
2) J.C. Schwartz, *Ann. Rev. Pharmacol. Toxicol.*, **17**, 325 (1977).
3) K.M. Lindahl, *Acta Physiol. Scand.*, **49**, 114 (1960).
4) D.D. Brown, J. Axelrod, and R. Tomchick, *Nature*, **183**, 680 (1959).
5) D.D. Brown, R. Tomchick, and J. Axelrod, *J. Biol. Chem.*, **243**, 2948 (1959).
6) S.H. Snyder and J. Axelrod, *Biochim. Biophys. Acta*, **111**, 416 (1965).
7) Y. Tsuruta, K. Kohashi, and Y. Ohkura, *J. Chromatogr.*, **224**, 105 (1981).
8) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9) Y. Tsuruta, K. Kohashi, and Y. Ohkura, *J. Chromatogr.*, **146**, 490 (1978).

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High Performance Liquid Chromatographic Separation and Detection of Methoxy Derivatives of 3,4-Dihydroxyphenylalanine

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A voltammetric detector and a fluorometric detector combined with a high performance liquid chromatograph were applied to the analysis of methoxy derivatives of 3,4-dihydroxyphenylalanine, and the results obtained were compared with those obtained with a 254 nm ultraviolet detector. Response was linear over a wide range of concentrations and detection was sensitive and selective.

The contents of 3-methoxy-4-hydroxyphenylalanine in the plasma of normal persons were measured.

Keywords——3,4-dihydroxyphenylalanine; 3,4-dihydroxyphenylalanine derivatives; high performance liquid chromatography; voltammetry; fluorimetry; metabolism

Catecholamines and their metabolites have been associated with a number of disease states.[1,2] Recently, various techniques have been developed to measure methoxy derivatives of catecholamines.[3–7] These methods have in general been proved to be applicable for the determination of only a few methoxy derivatives of 3,4-dihydroxyphenylalanine (DOPA).

Previously, we made a brief report on the detection of 3-methoxy-4-hydroxyphenylalanine(3-*O*-methyl-DOPA) and 3-hydroxy-4-methoxyphenylalanine(4-*O*-methyl-DOPA) by the use of rat liver homogenate.[8] In this paper, we describe chromatographic conditions for the analysis of methoxy derivatives of DOPA, including 3-*O*-methyl-DOPA, in plasma

by high performance liquid chromatography (HPLC). Further, the responses of DOPA and related methoxy derivatives to ultraviolet (UV) absorption, fluorescence and voltammetric detection (VMD) are compared.

Experimental

Materials——DOPA, dopamine (DA), 3,4-dihydroxyphenylacetic acid (DHPAC) and 3-methoxy-4-hydroxyphenylacetic acid (HVA) were purchased from Nakarai Chemical Co., Kyoto. 3-Methoxy-4-hydroxyphenethylamine (3-*O*-methyl-DA) was obtained from Calbiochem, U.S.A. 3-Methoxy-4-hydroxyphenyllactic acid (VLA) was purchased from Sigma Chemical Co. 3-Methoxy-4-hydroxyphenylpyruvic acid (VPA) was obtained from Tokyo Kasei Co. 3-Methoxy-4-hydroxyphenylalanine (3-O-methyl-DOPA) was purchased from Kyowa Hakko Co. These compounds were of analytical grade and were used without further purification. 3-Hydroxy-4-methoxyphenylalanine (4-O-methyl-DOPA) was synthesized by the method of Wilcox *et al.*[9] 3-Hydroxy-4-methoxyphenethylamine (4-O-methyl-DA) was synthesized by the method of Beke *et al.*[10] and N-methyl-3,4-dihydroxyphenylalanine (N-methyl-DOPA) and N-methyl-(3-methoxy-4-hydroxyphenyl)alanine (N-methyl-3-O-methyl-DOPA) were obtained by the method of Deulofeu *et al.*[11]

Sample Preparation——A 12 ml sample of blood was collected into a syringe with 0.1 ml of sodium heparin (1000 U/ml) and centrifuged immediately at $1000 \times g$ for 5 min at 4°C.

Free 3-O-Methyl-DOPA: A 2 ml sample of plasma was deproteinized by the addition of 0.45 ml of 1.0 M trichloroacetic acid. The plasma was centrifuged at $10000 \times g$ for 10 min. The supernatant was decanted, and the precipitated protein was treated with 0.05 ml of 1.0 M trichloroacetic acid and centrifuged again. The supernatants were combined.

Total 3-O-Methyl-DOPA: A mixture of 2 ml of deproteinized plasma and 0.3 ml of 9 N $HClO_4$ was heated in a boiling water bath for 20 min. Further, the supernatant was treated with 0.2 ml of 20% KOH and centrifuged again.

Apparatus——A Yanagimoto L-2000 high speed liquid chromatogroph equipped with a Yanagimoto VMD 101 voltammeter and a Hitachi 650-10S fluorescence spectrophotometer was used for analysis of methoxy derivatives of DOPA.

Chromatographic Conditions——Yanapak ODS was packed in a 4.0×250 mm i.d. stainless steel column; column temperature, 25 ± 0.1°C; mobile phase, (1) 0.05 M phosphate buffer (pH 3.1) and (2) 15% methanol in 0.05 M phosphate buffer (pH 3.1); flow rate, 0.56 ml/min; applied potential, 0.9 V *vs.* Ag/AgCl; fluorimeter sensitivity, 1.0; recorder range, 0.2 V; chart speed, 5 mm/min. The fluorescence was monitored with excitation at 282 nm and emission at 322 nm, using a fluorescence spectrophotometer equipped with an 18 μl quartz flow cell. For the measurements of retention time and peak area, a Shimadzu C-RIA Chromatopac was used.

Results and Discussion

The HPLC separation of standard methoxy derivatives of DOPA is shown in Fig. 1. The effect of various pH values on the resolution were studied and the mobile phase at pH 3.1 gave complete separation of DOPA derivatives. On raising the pH above 4.0, the sensitivity become poor and on lowering the pH from 3.1, the retention times become longer and

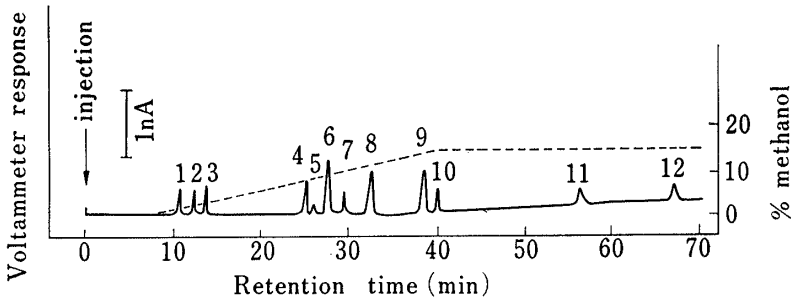

Fig. 1. Liquid Chromatogram of Standard Methoxy Derivatives of DOPA.

Injection sample: 10 μl of solution containing 1 ng each of methoxy derivatives of DOPA. For HPLC conditions, see the text.
Peaks: 1, DOPA; 2, N-methyl-DOPA; 3, DA; 4, 3-O-methyl-DOPA; 5, N-methyl-3-O-methyl-DOPA; 6, 3-O-methyl-DA; 7, 4-O-methyl-DOPA; 8, 4-O-methyl-DA; 9, DHPAC; 10, VPA; 11, VLA; 12, HVA.

the peaks of 3-O-methyl-DA, N-methyl-3-O-methyl-DOPA, *etc.* began to overlap (not shown in the figure).

Standard curves obtained by plotting the peak area against the amount of substance injected were linear in the range of 50—500 pg with VMD or with the fluorometric detector, and 0.1—1.0 ng with the UV detector. The minimum detectable quantities were approximately 5 pg with VMD and approximately 15 pg with the fluorometric detector. VMD and fluorometric detectors are more sensitive than the UV detector. However, DHPAC, VPA, VLA, and HVA do not fluoresce under the conditions used, and UV monitoring is essential for their detection.

A chromatogram of the plasma from normal persons is shown in Fig. 2. The recovery of 3-O-methyl-DOPA through the sample preparation procedures was 98.4±1.2% in the concentration range tested (0.1 ng—1.0 ng). The coefficients of variation determined at two different concentrations, 0.25 ng and 0.75 ng, were 1.8% ($n=5$) and 2.1% ($n=5$), respectively. However, 4-O-methyl-DOPA was not detected in the plasma from normal persons.

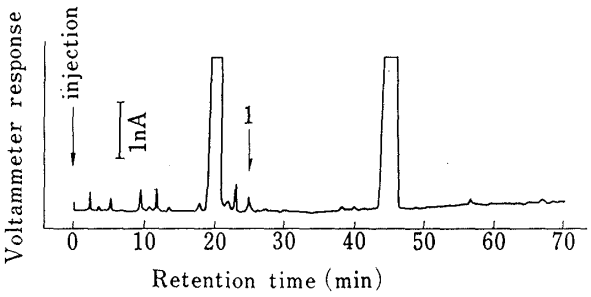

Fig. 2. Liquid Chromatogram of Plasma from a Normal Person.

Peak 1, 3-O-methyl-DOPA.

Table I. Contents of Free and Total 3-O-methyl-DOPA in the Plasma (Voltammetric Detector)

Subject		3-O-Methyl-DOPA	
Age	Sex[a]	Free (ng/ml)	Total (ng/ml)
34	M	27.5	53.8
34	M	26.3	61.5
28	M	54.0	98.3
21	M	35.3	71.9
19	M	55.0	80.0
18	M	43.8	63.8
23	F	18.8	46.3
22	F	35.4	57.5
21	F	41.5	78.8

a) M, male; F, female.

The amounts of free and total 3-O-methyl-DOPA in the plasma are listed in Table I. The values are not very different from those obtained by another method.[12] The values for normal persons obtained here are in the same range as those found with the fluorometric detector. The regression equation for free 3-O-methyl-DOPA was $y=1.068x-3.159$, where y is the result from VMD data and x is the result from fluorometric data; the coefficient of correlation was 0.991. The regression equation for total 3-O-methyl-DOPA was $y=1.001x+0.4325$ with $r=0.990$.

References and Notes

1) G.H. Wada and J.H. Fellman, *Biochem.*, **12**, 5212 (1973).
2) B.C. Barras, D.B. Coult, and R.M. Pinder, *J. Pharm. Pharmac.*, **24**, 499 (1972).
3) V.K. Werse and I.J. Kopin, *Life Sci.*, **19**, 1673 (1976).
4) J.H. Knox and J. Jurand, *J. Chromatogr.*, **125**, 89 (1976).
5) S. Takahashi, M. Yoshioka, S. Yoshiue, and Z. Tamura, *J. Chromatogr.*, **145**, 1 (1978).
6) J.P. Crombeen, J.C. Kraak, and H. Poppe, *J. Chromatogr.*, **167**, 219 (1978).
7) I. Molnar and C. Horvath, *Clin. Chem.*, **22**, 1497 (1976).
8) T. Ishimitsu and S. Hirose, *Chem. Pharm. Bull.*, **28**, 2272 (1980).
9) M.E. Wilcox, H. Wyler, T.J. Mabry, and A.S. Dreiding, *Helv. Chim. Acta*, **24**, 252 (1965).
10) D. Beke and Cs. Szantay, *Acta Chim. Acad. Sci. Hund.*, **14**, 325 (1958).
11) V. Deulofeu and T.J. Guerrero, "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc, New York, 1955, p. 586.
12) N.S. Sharpless, M.D. Muenter, G.M. Tyce, and C.A. Owen, Jr. *Clin. Chim. Acta*, **37**, 359 (1972).