Chem. Pharm. Bull.
24(10) 2556—2558 (1976)

UDC 547.586.2.08: 543.544.6.062

Quantitative Analysis of the Isomers of Hydroxyphenylalanine by Ion-exchange Chromatography[1]

Susumu Ishimitsu, Sadaki Fujimoto, and Akira Ohara

Kyoto College of Pharmacy[2]

(Received January 26, 1976)

A method for separatory determination of the isomers of hydroxyphenylalanine by liquid chromatography was established. The chromatographic conditions were as follows: Column, glass column (0.9×50 cm) packed with Hitachi #2612 ion-exchange resin; mobile phase, 0.2 M citrate buffer (pH 2.80) and 0.2 M citrate buffer (pH 3.90); flow rate, 1.0 ml/min; temperature change from 30° to 55° at 3.25 hr; buffer change at 1.5 hr; detector, ultraviolet and visible detector.

This procedure was found to be adapted for the analysis of *o*-tyrosine, *m*-tyrosine, *p*-tyrosine, 3,4-dihydroxyphenylalanine, and phenylalanine.

Metabolism of aromatic amino acids involves several hydroxylation reactions normally brought about by specific enzymes. However, if for some reason a normal specific hydroxylating system is blocked, as occurs for example in phenylketonuria, nonspecific hydroxylation might assume much greater importance. The object of our study is to elucidate the action of a nonspecific hydroxylating system on phenylalanine and some of its metabolites *in vivo*. In order to elucidate these phenomena quantitatively it is necessary to establish a systematic quantitative analysis of hydroxylated compounds of phenylalanine. Formerly, paper chromatography has been used for this purpose,[3] but it is not a reproducible and quantitative method. In recent years, several investigations have led to the development of ion-exchange chromatographic techniques for analysis of some amino acids.[4] Analysis of the isomers of hydroxyphenylalanine, however, has not been reported. As the first step of our study, a systematic quantitative analysis of hydroxylated compounds of phenylalanine was established. The present report describes chromatographic conditions for the analysis of phenylalanine and its hydroxylated derivatives by an ion-exchange chromatography.

Experimental

Apparatus——The quantitative determination for phenylalanine and its hydroxylated compounds was examined. A Hitachi Model 034 liquid chromatograph equipped with ultraviolet and visible detector was used. Color developed by ninhydrin reaction was determined at 440, 570, and 640 nm. Ultraviolet absorption was also determined at 250, 260, and 280 nm without ninhydrin reaction. A glass column (0.9×50 cm) packed with Hitachi #2612 ion-exchange resin was used. The buffers were prepared according to the method described by Spackman, *et al.*,[5] using caprylic acid in place of phenol.

Materials——β-(2-Hydroxyphenyl)alanine (*o*-tyrosine), β-(3-hydroxyphenyl)alanine (*m*-tyrosine), β-(4-hydroxyphenyl)alanine (*p*-tyrosine), β-phenylalanine, and β-(3,4-dihydroxyphenyl)alanine (3,4-DOPA) were purchased from Sigma Chemical Co., U.S.A. These compounds were of analytical grade and used without further purification. All other reagents used were of analytical grade.

1) A part of this research was presented at the 95th Annual Meeting of the Pharmaceutical Society of Japan in Nishinomiya, April, 1975.
2) Location: *5 Nakauchicho, Misasagi, Yamashina Higashiyama-ku, Kyoto, 607, Japan.*
3) T. Gerthsen, *Biochem. Z.*, **336**, 251 (1962).
4) P.B. Hamilton, "Advances in Chromatography," Vol. 2, ed. by J.C. Giddings and R.A. Keller, Marcel Dekker, Inc., New York, 1966, p. 3.
5) D.H. Spackman, W.H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

NII-Electronic Library Service

Results and Discussion

Separation of phenylalanine and its hydroxylated compounds was examined on the column packed with Hitachi #2612 ion-exchange resin and chromatographic conditions were as follows: A flow rate of 1.0 ml/min of the buffer was maintained throughout the analysis and buffer change from pH 2.80 to 3.90 was made after 1.5 hr. The column temperature was changed from 30° to 55° after 3.25 hr. The pressure was 10 kg/cm^2 at 30°. The recorder chart speed was 10 cm/hr with a printing speed of 1 dot/4 sec. The total analysis time was 5.5 hr.

Results of analysis of a synthetic mixture of phenylalanine and its hydroxylated compounds are shown in Fig. 1. Under this chromatographic condition, 3,4-DOPA, *m*-tyrosine, *p*-tyrosine, phenylalanine, and *o*-tyrosine were well separated from one another.

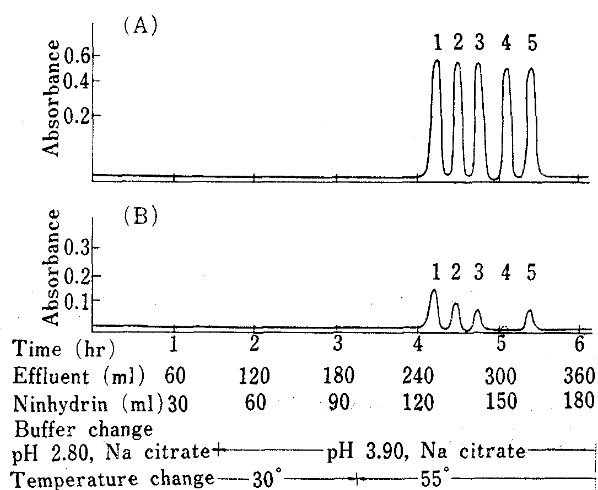

Fig. 1. Chromatograms of Phenylalanine and Its Hydroxylated Compounds

(A) absorbance determined at 570 nm after ninhydrin reaction
(B) absorbance determined at 250 (------) and 280 (——) nm without ninhydrin reaction
1: 3,4-DOPA, 2: *m*-tyrosine, 3: *p*-tyrosine, 4: phenylalanine, 5: *o*-tyrosine

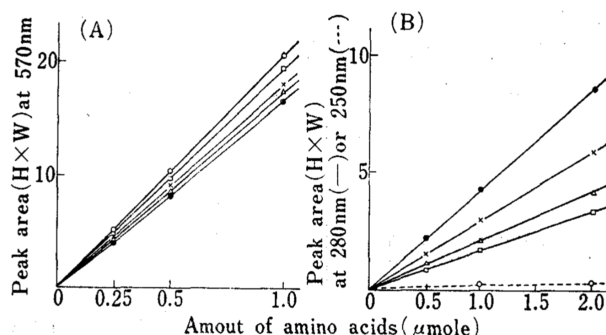

Fig. 2. Relationship of Peak Area (H×W) to Amount of Amino Acids

(A) peak area determined from absorbance at 570 nm after ninhydrin reaction
(B) peak area determined from absorbance at 250 (------) and 280 (——) nm without ninhydrin reaction
●: 3,4-DOPA, △: *m*-tyrosine, □: *p*-tyrosine, ○: phenylalanine, ×: *o*-tyrosine

The pH of the elution buffers and the column temperature were varied while maintaining the citrate concentration of 0.2M and the buffer flow rate of 1.0 ml/min. On raising the pH from 3.25 to 4.25 at 55° of column temperature, phenylalanine and *o*-tyrosine were not resolved, and the separation of *m*-tyrosine and *p*-tyrosine was poor. By changing the pH from 2.80 to 3.90 at 55°, *m*-tyrosine and *p*-tyrosine were well separated from each other but the separation of phenylalanine and *o*-tyrosine and *p*-tyrosine was still not good.

From these observations, the above-described conditions were found to be the optimal for the resolution of phenylalanine, *o*-, *m*-, and *p*-tyrosine, and 3,4-DOPA using Hitachi #2612 resin. Separation of a mixture including 2,3-, 2,5-, and 3,4-DOPA was, however, not successful in this system, because of the same retention time for dihydroxylated compounds.

Calibration curves for the amino acids from the chromatograms obtained in Fig. 1 are illustrated in Fig. 2. The reproducibility of this method was within ±2%, judging from the coefficient of variation of these amino acid equivalents.

An absorption ratio of the ninhydrin color of each amino acid at 440, 570, and 640 nm, and that of the amino acids in the ultraviolet region of 250, 260, and 280 nm without ninhydrin reaction are listed in Table I. These data indicate that ultraviolet absorption ratio is characteristic for each amino acid, though absorption ratio of ninhydrin color of each amino acid in the visible region is similar.

NII-Electronic Library Service

Table I. Absorption Ratio of Amino Acids

Amino acid	Ninhydrin color		Ultraviolet absorption	
	640/570 nm	440/570 nm	260/280 nm	250/280 nm
3,4-DOPA	0.389	0.243	0.246	0.095
m-Tyrosine	0.405	0.235	0.674	0.263
p-Tyrosine	0.356	0.205	0.535	0.174
Phenylalanine	0.404	0.208	0.266	0.278
o-Tyrosine	0.352	0.196	0.646	0.196

Therefore, it should be possible to identify these amino acids by checking not only the retention value but also the ratio of ultraviolet absorption. For the sample contaminated with ninhydrin-positive aliphatic compounds which have a similar retention time as those of hydroxylated phenylalanines, ultraviolet absorption measurement has been found to be very useful.

Analysis of hydroxylated metabolites of phenylalanine *in vivo* using the above-mentioned procedure will be reported elsewhere.

[Chem. Pharm. Bull. 24(10)2558—2563(1976)]

UDC 547.466.1.057 : 577.15.07

Studies on Peptides. LXI.[1,2] Alternate Synthesis of Human Corticotropin (ACTH)

Kaname Koyama, Hidehiko Watanabe, Hiroki Kawatani, Jun Iwai, and Haruaki Yajima

Faculty of Pharmaceutical Sciences, Kyoto University[3]

(Received January 26, 1976)

Succeeding to the syntheses of porcine and bovine corticotropin (ACTHs), human ACTH was alternatively synthesized by the conventional method. The synthetic peptide exhibited the *in vivo* steroidogenetic activity of 153.1 IU/mg.

In 1971, Riniker, *et al.*[4] revised the structure of human corticotropin (ACTH) proposed early by Lee, *et al.*[5] and the N-terminal portion (position 1 to 20) was confirmatively reexamined by Bennett, *et al.*[6] Leading from this firmly established formula of human ACTH (I), its total synthesis was accomplished by Sieber, *et al.*[7] using protecting groups removable by trifluoroacetic acid (TFA). Succeeding to this preliminary communication, alternate

1) Part LX: K. Koyama, H. Kawatani, H. Yajima, M. Fujino, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **24**, 2106 (1976).
2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission of Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Tos=*p*-toluenesulfonyl, OBzl=benzyl ester.
3) Location: *Sakyo-ku, Kyoto, 606, Japan.*
4) B. Riniker, P. Sieber, and W. Rittel, *Nature New Biol.*, **235**, 114 (1972).
5) T.H. Lee, A.B. Lerner, and V.B. Janush, *J. Am. Chem. Soc.*, **81**, 6084 (1959); *idem, J. Biol. Chem.*, **236**, 2970 (1961).
6) H.P.J. Bennett, P.J. Lowry, and C. McMartin, *Biochem. J.*, **131**, 11 (1973).
7) P. Sieber, W. Rittel, and B. Riniker, *Helv. Chim. Acta*, **55**, 1243 (1972).

NII-Electronic Library Service