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Determination of Vanilpyruvic Acid in Urine by High-Speed Liquid Chromatography

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A determination method of urinary vanilpyruvic acid was developed by high-speed liquid chromatography. Sample urine was acidified and extracted with ethyl acetate. After evaporation of the organic solvent, the residue was left over phosphorous pentoxide to complete the conversion of keto form to enol form and dissolved in methanol. The methanol solution was injected into a high-speed liquid chromatograph equipped with a column of Hitachi Gel No. 3010 (spherical porous particles of styrene-divinylbenzene copolymer) and a ultraviolet (UV) monitor (280 nm). Vanilpyruvic acid was eluted with a mixture of 0.05 M tartrate buffer and methanol (85: 15, v/v) varying the pH of the buffer from 3.15 to 5.25. The quantity of vanilpyruvic acid was determined from the peak height.

By the present method, vanilpyruvic acid was found in urine of patients with neuroblastoma, a Parkinsonian patient treated with L-3,4-dihydroxyphenylalanine. However, this acid was not found in urines of normal subjects.

Keywords——high-speed liquid chromatography; L-DOPA; vanilpyruvic acid; Parkinsonian patient; neuroblastoma; gas chromatography; keto-enol tautomerism

Vanilpyruvic acid (3-methoxy-4-hydroxyphenylpyruvic acid, VPA) is an intermediate metabolite produced by 3-O-methylation and transamination of L-3,4-dihydroxyphenylalanine (L-DOPA).[2] The notable excretion of VPA in urine has been reported not only from patients with neuroblastoma[3] and melanoma,[4] but also from Parkinsonian patients treated with L-DOPA.[5] However, quantitative analysis of urinary VPA has not been reported except a semi-quantitative analysis using high-voltage electrophoresis.[2a] In a previous paper,[6] we reported the separation of VPA from catecholamine-originated aromatic acids by high-speed liquid chromatography (HSLC). During the further investigation, we found that VPA showed two peaks on the chromatogram. In the present paper, the peaks were identified with keto-enol tautomers. By the selection of condition to give the single peak of the enol form on the chromatogram, we established a quantitative determination method of VPA by HSLC and applied it to urine.

Experimental and Results

Reagents——VPA was obtained from Sigma Chemical Co. Tablets of L-DOPA and its decarboxylase inhibitor, N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)-hydrazine (Ro4-4602)[7] were from Nippon Roche K. K., Tokyo. All of the other chemicals used were of reagent grade purity.

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Sample Preparation of VPA from Aqueous Solution or Urine——To 5 ml of aqueous sample solution were added 1 ml of 1 M citrate-HCl buffer (pH 1.0), 1.5 g of NaCl and 10 ml of ethyl acetate. After shaking for 5 min, 8 ml of the organic phase was taken and evaporated to dryness under a stream of nitrogen. The residue was kept in a desiccator over phosphorous pentoxide for four days until gas chromatography or HSLC analysis. The residue was dissolved in 0.2 ml of methanol and 10 µl of the solution was analyzed by HSLC; alternatively the residue was reacted with 100 µl of N,O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) at 70° for 30 min and 1 µl of the solution was injected into a gas chromatograph.

Urine was collected in a bottle containing 30 ml of 6 N HCl during 24 hrs, stored in a freezer at −15°. Five ml of urine was analyzed by the same procedure.

HSLC of VPA and Its Tautomers——The conditions were same as those previously reported.[6] (*cf.* Fig. 1).

Gas Chromatography (GC)——The conditions are described in Fig. 6. The amount of VPA in urine was calculated from a linear working curve of peak height against concentration of VPA in urine (4—60 mg/l).

Gas Chromatography-Mass Spectrometry (GC-MS)——For the identification of the gas chromatographic peak corresponding to VPA, a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer equipped with a computer system (GC-MS PAC 300D Shimadzu Seisakusho Ltd.) was used. The conditions for the gas chromatographic separation were same as those in Fig. 6. The mass spectrum was measured under the condition of electron energy at 70 eV, with an accelerating voltage of 3.5 kV and an ionizing current of 60 µA. The ion-source temperature was 290°. Trimethylsilyl derivatives of degradation products from VPA in ethyl acetate were also analyzed by increasing GC column temperature from 160° to 220° at the rate of 10° per min.

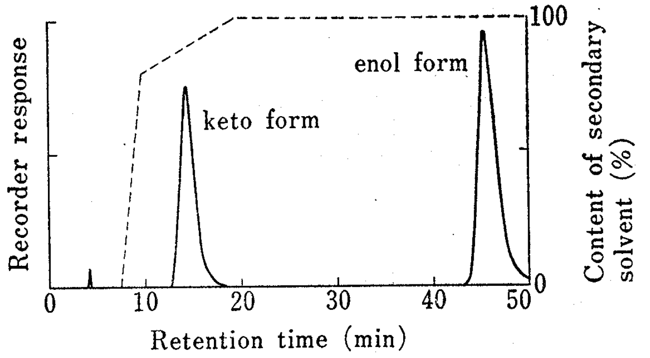

Fig. 1. Liquid Chromatogram of VPA

A VPA solution of 0.5 M phosphate buffer (pH 7.0)-methanol (11: 14, v/v) was analyzed under the following conditions. Column: Hitachi Gel No. 3010 (500 mm×2 mm I.D.) at 40°. Eluent: Primary solvent: 0.05 M tartrate buffer (pH 3.15)-methanol (85: 15, v/v), secondary solvent: 0.05 M tartrate buffer (pH 5.25)-methanol (85: 15, v/v). Flow rate: 0.6 ml/min. Detector: 280 nm, 0.01 a.u.f.s. A dotted line shows the content of secondary solvent. The peaks of keto and enol form of VPA were identified as described in the text.

Measurement of NMR Spectra——The proton nuclear magnetic resonance (NMR) spectra were measured with a NMR spectrometer JEOL-C-60HL. Tetramethylsilane was used as an internal standard.

Keto-Enol Tautomerism of VPA in Solvents——The liquid chromatogram of VPA in aqueous medium showed two peaks at 14 and 45 min as shown in Fig. 1. The eluate corresponding to the each liquid chromatographic peak was pooled and evaporated under a stream of nitrogen gas. The dried residue was dissolved in 5 ml of 0.1 N HCl and applied to a column of ground Amberlite XAD-4 (60 mm × 4 mm I.D.).[8] The column was washed with 15 ml of 0.1 N HCl and eluted with 10 ml of methanol. The eluate was evaporated

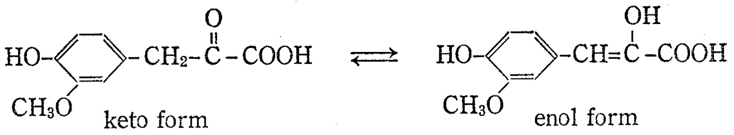

Chart 1

TABLE I. Content (%) of Enol Form of VPA determined by the Two Methods

Solvent	HSLC	NMR[a]
CD_3OD	100	100
CH_3OH	100	—
0.5 M phosphate (pH 7.0)/methanol (1: 4, v/v)	47±2	44±2
0.5 M phosphate (pH 7.0)/methanol (11: 14, v/v)	27±1	26±2

[a] Enol percent was determined from the areas of both types of aromatic proton resonance. NMR (CD_3OD) δ: 6.52 (1H, s), 6.82 (1H, d, *J*=8.0 Hz) 7.24 (1H, d of d, *J*=2.0, 1.8, 8.6 Hz), 7.61 (1H, d, *J*=1.5 Hz). NMR (0.5 M phosphate (pH 7.0)/methanol (1: 4, v/v)) δ: 6.55 (1H, s), 6.66—7.00 (3H, m), 6.90 (1H, d, *J*=7.9 Hz), 7.27 (1H, d of d, *J*=2.2, 2.0, 8.4 Hz), 7.60 (1H, d, *J*=2.0 Hz).

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under a stream of nitrogen, and the residue was derivatized and analyzed by GC described above. The each eluate showed a single GC peak, which retention time and mass spectrum were the same as the silylated derivative of authentic VPA (see Fig. 5 and 6). The NMR spectrum of VPA in 0.1 M perdeuterated acetic acid-deuterated sodium hydroxide buffer (pH 7) showed two singlets at 6.4 ppm (–CH=) and 4.0 ppm (–CH_2–). On the other hand, the NMR spectrum of crystalline VPA dissolved in perdeuterated methanol showed only the former singlet, and the liquid chromatogram of the solution showed the single peak at 45 min. Thus, the two peaks on the liquid chromatogram (Fig. 1) were identified as the keto and enol forms of VPA (Chart 1).

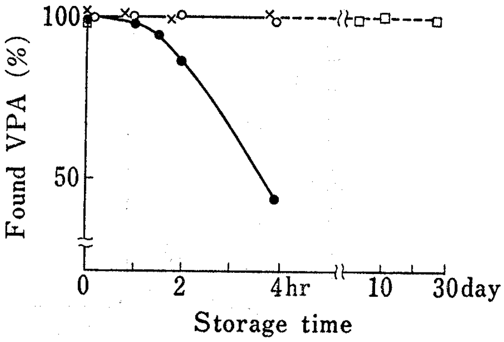

Fig. 2. Stability of VPA in Various Solutions

Solutions of VPA (1 mg/ml) in methanol (—○—), ethyl acetate (—●—) and diluted hydrochloric acid (pH 2, —×—) were left at room temperature. An acidified normal urine (see text) was added with 40 mg of VPA per liter and stored at −15° (—□—). The methanol solution was directly analyzed by the injection of 10 μl into the liquid chromatograph. As to ethyl acetate solution, the residue after evaporation to dryness was dissolved in methanol and analyzed. The acidic solution and the urine was analyzed by the method described in the text.

Eighty mg of VPA was dissolved in 1 ml of various solvents and kept for 4 hr at 25°. Ten μl of the solution was diluted with 500 μl of methanol and 10 μl of it was analyzed by HSLC (Fig. 1). Furthermore, the NMR spectra of the original solution were measured. The contents of enol form of VPA in the various solvents determined by HSLC were consistent with those by NMR as shown in Table I. From this table, it seemed that the tautomerization in the column of the high-speed liquid chromatograph was negligibly slow. When dried over phosphorous pentoxide, the keto-enol mixture in the residue converted to the enol form. Therefore, it was possible to determine VPA in aqueous solutions or urine by using the enol peak.

Stability of VPA——VPA was unstable in an alkaline solution and its instantaneous degradation was

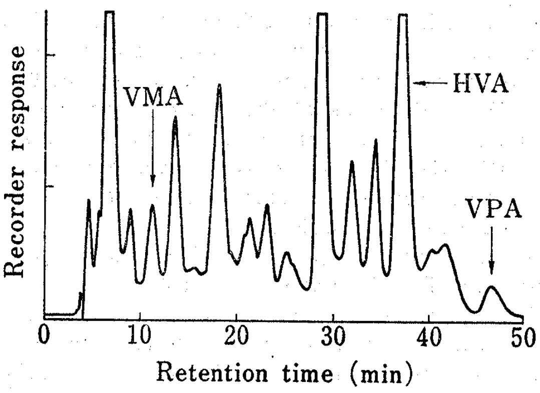

Fig. 3. Liquid Chromatogram of Urine from a Patient with Neuroblastoma

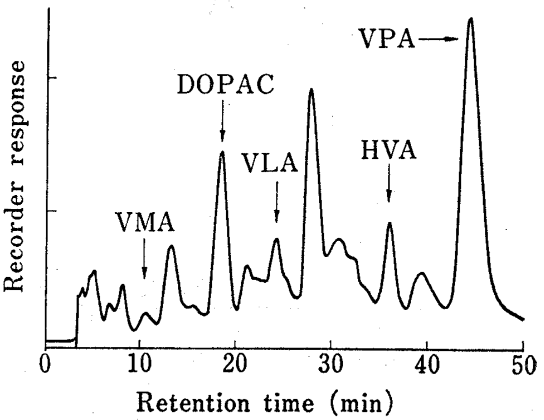

Fig. 4. Liquid Chromatogram of Urine from a Parkinsonian

Patient treated with L-DOPA and Ro4-4602

Table II. Urinary Excretion of VPA determined by the Two Methods

Patient				Related drug daily administered	VPA (mg/day) by	
Number	Age	Sex	Diagnosis		HSLC	GC
1	3	M	Neuroblastoma		3.1	2.8
2	7	F	Neuroblastoma		1.1	1.2
3	3	M	Neuroblastoma		Not detected	
4	7	M	Neuroblastoma		Not detected	
5	8	M	Neuroblastoma		Not detected	
6	61	M	Parkinsonism	0.5 g L-DOPA and 0.125 g Ro 4—4602	47	46
6	61	M	Parkinsonism	2 g L-DOPA	11	11

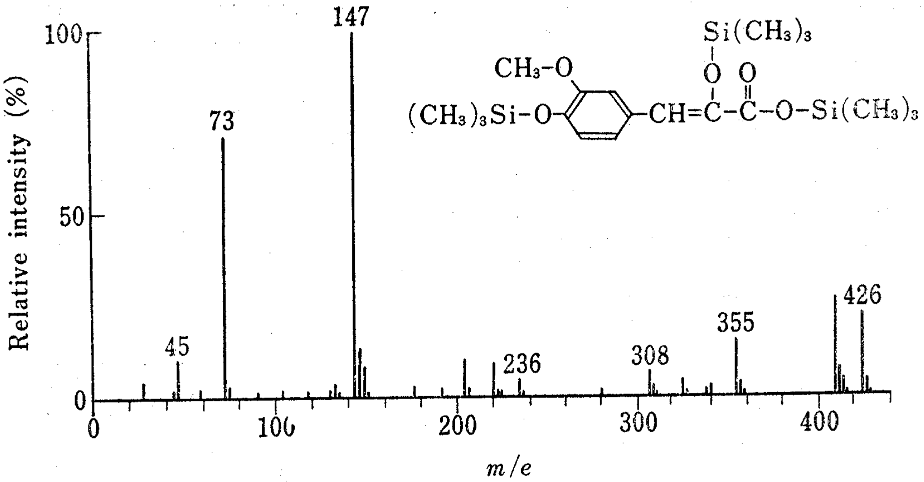

Fig. 5. EI Mass Spectrum of Silylated VPA

The GC peak of Fig. 6 was analyzed.

observed at pH 12, however, it was stable in acidic water as well as in methanol as shown in Fig. 2. The degradation in ethyl acetate was slight for 60 min and accelerated afterward. After 4 hr's standing in the solvent, a large amount of vanillin and small amounts of vanillic acid and 3-methoxy-4-hydroxyphenylethanol (homovanillyl alcohol) were identified by GC–MS. VPA was stable enough to be stored in the dried extract from urine. Practically, we could carry out the whole procedure of extraction and injection of VPA within 20 min except a storage period.

Determination of VPA in Urine by HSLC——The working curve of peak height against concentration of VPA in urine was linear from 4 to 80 mg/l. The coefficient of variation of the peak height was about 5% for 20 mg of VPA in one liter of urine. The recovery of VPA throughout the whole procedure was about 61%.

The chromatograms of urines from a patient with neuroblastoma and a Parkinsonian patient, who was daily treated with L-DOPA and Ro4-4602, are shown in Fig. 3 and 4, respectively. Urinary excretion of VPA by patients was shown in Table II.

The same urine samples were also analyzed by GC. The trimethylsilyl derivative of VPA gave often two peaks.[5] Since these peaks gave rise to the identical mass spectrum (Fig. 5), they seemed to be *cis-trans* isomers. Only the latter peak was obtained when the solution of the derivative was injected into the gas chromatograph just after trimethylsilylation (Fig. 6). The values obtained by GC (Table II) confirmed the reliability of the present HSLC.

In urines of normal subjects, VPA was not found by the present method.

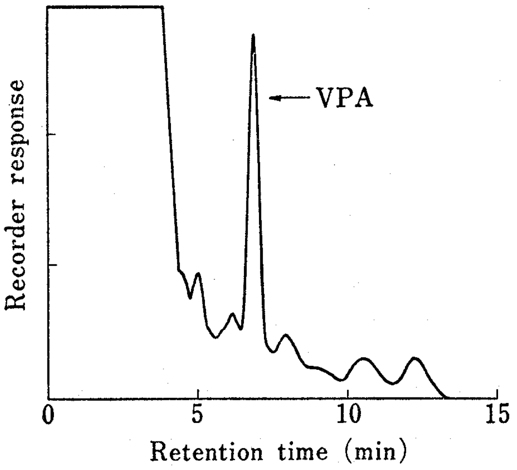

Fig. 6. Gas Chromatogram of Urine

The urine of Fig. 4 was analyzed under the following conditions. A gas chromatograph Hitachi 063 with a glass column of chromosorb W-AW-DMCS, 80—100 mesh (1000 mm × 3 mm I.D.) coated with 3% OV-61 was equipped with a flame ionization detector. The injector and the detector were kept at 260°. The column oven was kept at 200°. The recorder response was 1×10^{-10} A per full scale.

Discussion

Because of lack of a suitable method for determination, the urinary excretion of VPA has not been measured or discussed. The present method confirms that the determination of the excretion of VPA will be useful for the investigation of unnoticed metabolisms of L-DOPA or catecholamines. The effect of co-administration of Ro4–4602 to the Parkinsonian patient treated with L-DOPA on the urinary excretion of VPA (47 mg/day) was distinct, and only 11 mg per day of VPA was excreted from the same patient treated solely with L-DOPA. Urinary level of VPA seems to be of significance also in the diagnosis of neuroblastoma.

Furthermore, by the present HSLC, the other constituents such as vanilmandelic acid (VMA), 3,4-dihydroxyphenylacetic acid (DOPAC), vanillactic acid (VLA) and homovanillic acid HVA) can be simultaneously determined together with VPA (see Fig. 3 and 4).

The similar values of VPA were obtained by GC method developed in this paper (Table II) demonstrating that the both methods were reliable.

The keto and enol forms of VPA were separated on the column, and since the former was contaminated with urinary constituents, it had to be converted to the enol form. The time consuming conversion is the defect of the present method. Conversely, the HSLC, in the place of NMR,[9] will be useful for the determination of the keto-enol ratio of VPA and other α-keto acids in various solvents.

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