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Note

Simple method for the determination of homovanillic acid and vanillylmandelic acid in urine by high-performance liquid chromatography

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The levels of homovanillic acid (HVA) and vanillylmandelic acid (VMA) in urine are important indices in the diagnosis of neuroblastoma and phaeochromocytoma [1, 2]. Various techniques have been used for their determination such as spectrophotometry [3, 4], paper chromatography [5] and thin-layer chromatography [6, 7]. These procedures are, however, time consuming and unreliable. Gas chromatography with packed columns [8—13] and glass capillary columns [14] and mass fragmentography [15, 16] have also been used. These methods are specific and sensitive, but complicated for routine use.

A practical method for the simultaneous determination of HVA and VMA in urine by high-performance liquid chromatography (HPLC) was first proposed by the authors of this paper [17, 18]. A column of porous poly-

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styrene was used in a gradient elution mode with increasing pH of the eluent. Absorbance at 280 nm was monitored for quantitative evaluation. Other workers used chemically bonded reversed-phase packings [19, 20]. However, these methods required gradient elution with an increasing ratio of acetonitrile in the eluent in order to obtain rapid elution. Methods based on HPLC with electrochemical detection have been tried, but they can not determine both acids simultaneously [21—24]. HPLC with post-column derivatization was successful in the analysis of VMA, but not of HVA [25, 26].

In this present work, a simplified HPLC method for the simultaneous determination of HVA and VMA in urine is presented. A column of hydroxymethylated porous polystyrene and isocratic elution are used. The reliability of the method was tested by gas chromatography—mass spectrometry (GC—MS).

EXPERIMENTAL

Materials

The column packing material was Hitachi Gel No. 3011-O (Tokyo, Japan) which consists of spherical porous particles of a hydroxymethylated styrene—divinylbenzene copolymer with an average particle diameter of 5 μm.

HVA, VMA, hippuric acid, vanillic acid, *p*-hydroxyphenylacetic acid, ferulic acid and *p-n*-amylbenzoic acid were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 3,4-Dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylacetic acid (DOPAC), vanillyllactic acid (VLA) and vanillylpyruvic acid (VPA) were from Sigma (St. Louis, MO, U.S.A.). Caffeic acid was from Nakarai Chemicals (Kyoto, Japan). All other chemicals were of reagent grade and were obtained from Wako Pure Chemical (Osaka, Japan).

High-performance liquid chromatography

A high-pressure liquid chromatograph Jasco Trirotor-II (Japan Spectroscopic Co., Tokyo, Japan) equipped with a variable-wavelength UV detector Uvidec-100-II (Japan Spectroscopic Co.) was used. Absorbance was continuously recorded at 280 nm. The packing materials, dispersed in water—acetonitrile (4:1, v/v), were packed into stainless-steel tubing at a pressure of about 150 kg/cm^2 by the slurry packing technique.

HVA and VMA were extracted from urine according to the method described previously [27]. To 5 ml of urine were added 1 ml of 1 *M* citric acid—hydrochloric acid buffer (pH 1.0) and 1.5 g of sodium chloride. After the resulting mixture had been extracted with 10 ml of ethyl acetate by shaking for 5 min, 8 ml of the organic phase were taken and evaporated to dryness under reduced pressure. The residue was dissolved in 0.4 ml of methanol and 4-μl aliquots of the solution were analyzed under the conditions shown in Fig. 1.

Gas chromatography—mass spectrometry [28]

Extraction and derivatization of urinary HVA and VMA for GC separation were performed by the methods described by Addanki et al. [12] and Muskiet et al. [13]. Five milliliters of urine were acidified to pH 1.0 with 6 *N* hydrochloric acid using a pH meter; 1.5 g of sodium chloride were added and the

solution was extracted three times with 5 ml of ethyl acetate by shaking for 3 min. To the combined ethyl acetate extract 30 μg of *p*-*n*-amylbenzoic acid were added as an internal standard (I.S.) and the solution was evaporated to dryness under reduced pressure. To obtain the trimethylsilyl derivatives, the dried residue was reacted at 70°C for 30 min with 100 μl of bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane. One microliter of the solution was injected into the gas chromatograph of the GC—MS—computer system and analyzed under the conditions described in the legend to Fig. 4.

Mass spectra were obtained at selected *m/z* and each substance was quantitated from its peak height ratio to *p*-*n*-amylbenzoic acid.

RESULTS

Using the present HPLC technique, HVA and VMA were well separated from catecholamine-related phenolic acids, hippuric acid, *p*-hydroxyphenylacetic acid, caffeic acid and vanillic acid as shown in Fig. 1. VPA was not eluted under the present conditions. The relationship between peak height and amount injected into the chromatograph was linear over the range of 10—300 ng for both HVA and VMA.

A typical chromatogram of urine from a normal subject is shown in Fig. 2. The recoveries of HVA and VMA were 72% and 68%, respectively. The coefficient of variation of the peak height was 2% for 10 mg of both acids per liter of urine. The minimum detectable concentration was 0.4 mg/l.

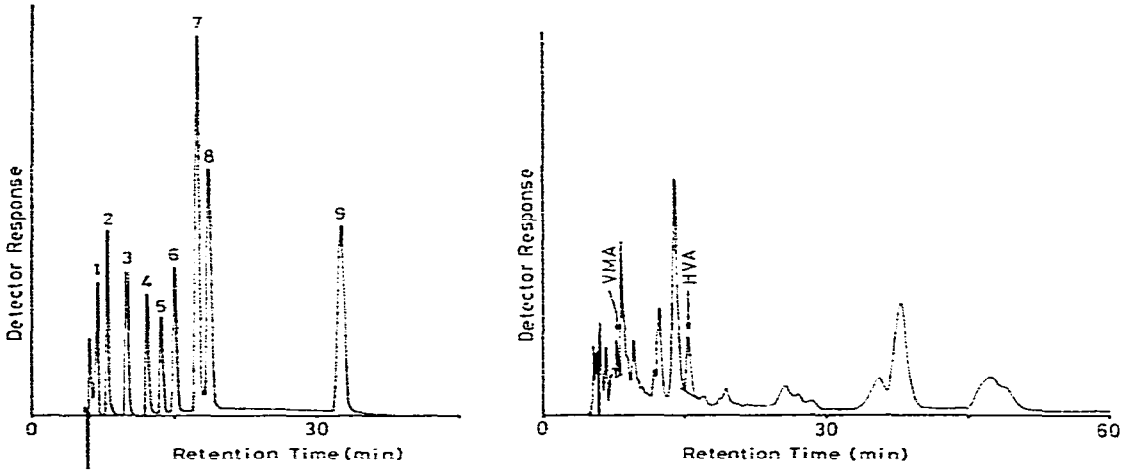

Fig. 1. Liquid chromatogram of a standard mixture of phenolic acids. Five microliters of a mixture (30—60 μg/ml) were applied to the chromatograph. Column: stainless-steel tubing (500 × 4.6 mm I.D.) packed with Hitachi Gel No. 3011-O (particle diameter about 5 μm) at 60°C. Eluent: 0.05 *M* tartaric acid—sodium hydroxide buffer (pH 3.20)—acetonitrile (4:1, v/v). Flow-rate: 1.0 ml/min. Detector: 280 nm, 0.04 absorbance units corresponded to full scale. Peaks: 1 = DOMA; 2 = VMA; 3 = DOPAC and VLA; 4 = hippuric acid; 5 = *p*-hydroxyphenylacetic acid; 6 = HVA; 7 = caffeic acid; 8 = vanillic acid; 9 = ferulic acid.

Fig. 2. Liquid chromatogram of a normal urine. For conditions see legend to Fig. 1.

The chromatogram shown in Fig. 3 was obtained from the urine of a patient with neuroblastoma. The concentrations of VMA and HVA in this sample were 7.4 ± 0.1 mg/l (8.4 ± 0.2 μg/mg of creatinine) and 15.5 ± 0.3 mg/l (17.7 ± 0.3 μg/mg of creatinine), respectively; these values represent the mean and range of duplicate determinations. Fig. 4 shows a mass chromatogram of the same urine. VMA and HVA were quantitated as 7.6 mg/l and 17.5 mg/l, respectively.

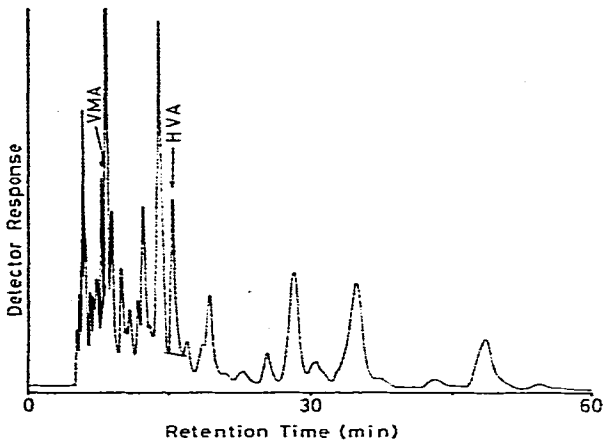

Fig. 3. Liquid chromatogram of urine from a patient with neuroblastoma. For conditions see legend to Fig. 1.

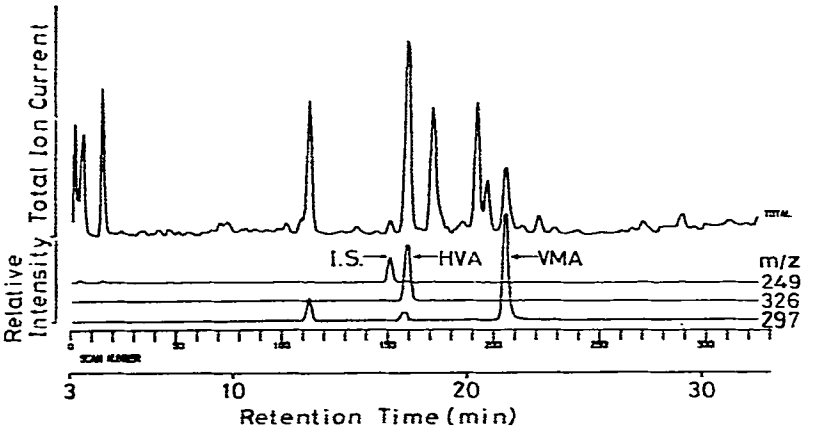

Fig. 4. Total ion current and mass chromatogram at selected *m/z* of the urine of a neuroblastoma patient (see Fig. 3). A Shimadzu—LKB 9000 GC—MS combination with a glass column of Gas-Chrom Q, 100—120 mesh (3 m × 3 mm I.D.) coated with 1% OV-101 was equipped with a computer system (GC—MS PAC 300D, Shimadzu Seisakusho). The column temperature was programmed from 130°C to 220°C at 3°C/min. The injector was kept at 250°C. The helium flow-rate was 30 ml/min. The mass spectrum in the range of *m/z* 150—430 was taken by continuous scanning as an electron energy at 20 eV, accelerating voltage 3.5 kV and ionizing current of 60 μA. The ion source and the slit separator were kept at 270°C and 250°C, respectively. The interval time of the continuous scanning was 5 sec.

DISCUSSION

The affinity of HVA for porous polystyrene such as Hitachi Gel No. 3010 is much stronger than that of VMA. Therefore, the HPLC spearation method reported previously [17, 18] required gradient elution. The difference in affinity between HVA and VMA is smaller for porous polystyrene with hydrophilic hydroxymethyl groups such as Hitachi Gel No. 3011-O. HVA and VMA are eluted, therefore, from such a gel column with a single eluent. In order to obtain satisfactory resolution, the particle diameter of No. 3011-O has to be 5 μm.

Under the conditions described in this work, HVA was not separated adequately from 5-hydroxyindole-3-acetic acid (5-HIAA). However, since 5-HIAA is not well extracted by ethyl acetate at pH 1.0 [8], the normal amount of 5-HIAA excreted in urine (2—5 mg/day) does not interfere with the determination of HVA. Vanillic acid, a metabolite of dietary vanillin, caused high apparent HVA values in the colorimetric assay of Ruthven and Sandler [29]. It did not interfere with HVA in the present method. Caffeic acid, a metabolite of chlorogenic acid contained in coffee, which affected the determination of HVA in the previously described method [18], was completely separated from HVA.

Due to the simplicity of the procedure, the present HPLC method seems promising for routine analysis of HVA and VMA in urine.

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