SIMULTANEOUS DETERMINATION OF HOMOVANILLIC ACID, VANILLYLMANDELIC ACID AND 5-HYDROXYINDOLE-3-ACETIC ACID IN URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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A simple and reliable method is described for the simultaneous determination of the biogenic amine metabolites homovanillic acid, vanillylmandelic acid and 5-hydroxyindole-3-acetic acid in human urine. The metabolites in urine were cleaned up by chromatography on a column of QAE-Sephadex A-25 and organic extraction. They were separated by isocratic elution high performance liquid chromatography on a reversed phase column. Detection and quantification were achieved with both ultraviolet and electrochemical detection. The correlation between the detection methods was high. The coefficients of variation of the determination in both within-day and between-day were 5-10 % for about 1.5 mg of each acid per liter urine.

Homovanillic acid (HVA) and vanillylmandelic acid (VMA) are the main metabolic end-products of dopamine and norepinephrine or epinephrine, respectively. 5-Hydroxyindole-3-acetic acid (5-HIAA) is a main metabolite of serotonin. These urinary levels are of great importance in the diagnosis of neuroblastoma, pheochromocytoma or carcinoid syndrome¹⁾⁻³⁾ and of interest in the fields of neurology and psychiatry⁴⁾.

We have proposed practical high performance liquid chromatography (HPLC) for the determination of urinary HVA and VMA⁵⁾⁻⁷⁾, and urinary 5-HIAA⁸⁾. Many analytical procedures, which are summarized in our previous papers⁵⁾⁷⁾⁸⁾, have been devised for HVA, VMA and 5-HIAA in urine. All the methods, however, did not intend to determine three acids simultaneously.

Joseph et al.⁹⁾ developed HPLC for the simultaneous determination of urinary levels of five amine metabolites including HVA, VMA and 5-HIAA by using electro-

chemical (EC) detection and single step gradient elution with an increasing ratio of methanol in the eluent. The EC detection, however, is inconvenient compared with the ultraviolet (UV) detection for routine use in clinical laboratory because the sensitivity decreases with fouling the surface of glassy carbon electrode.

In this work, the procedure of clean-up of the acids was elaborated by using chromatography on a column of QAE-Sephadex A-25 and a simple and reliable HPLC was established for the fine determination of HVA, VMA and 5-HIAA in urine. The three acids are separated by isocratic elution, and are stably monitored by not only EC but also UV detector.

EXPERIMENTAL

Materials and reagents

HVA, VMA and 5-HIAA were obtained from Tokyo Kasei (Tokyo, Japan). QAE-Sephadex A-25 strong basic anion exchange gel was from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of reagent grade and were obtained from Wako Pure Chemicals (Osaka, Japan).

A working standard solution of HVA, VMA and 5-HIAA in 10^{-3} M HCl contained 25, 25 and 12.5 mg/l, respectively. The solution was stable for a month at -80°C . The new solution was used daily.

Apparatus

We used a liquid chromatograph model LC-3A (Shimadzu, Kyoto, Japan) equipped with Rheodyne 7125 injection valve with 20- μl sample loop, 300 mm $\times$ 4.0 mm I.D. column packed with Nucleosil 5 C_{18} (average particle diameter of 5 μm ; Marcherey-Nagel, Düren, G.F.R.), variable-wavelength UV detector SPD-2A (Shimadzu) and EC detector EC-8 (Toyo Soda, Tokyo, Japan). The column temperature was controlled at 45°C with a water jacket. For injections into the liquid chromatograph we used a Hamilton No. 701 10.0 μl syringe (Hamilton, Reno, U.S.A.).

Preparation of the column of QAE-Sephadex A-25

QAE-Sephadex A-25 conditioned as acetate form was rinsed and decanted ten times with water. The gel slurried in water was poured into a glass column (internal diameter of 9.5 mm) up to 28 mm height. The column was washed with water prior to use.

Procedures

One ml of urine mixed with 4 ml of 0.03 M hydrochloric acid (HCl) was allowed to pass through the column of QAE-Sephadex A-25 (acetate form; 28 mm $\times$ 9.5 mm I.D.). The column was washed with 8 ml of 0.01 M HCl. The adsorbed HVA, VMA and 5-HIAA were eluted from the column with 10 ml of 0.2 M sodium chloride (NaCl) in 0.01 M HCl. To the effluent were added 3 g of NaCl and 1 ml of 1 M citric acid-sodium hydroxide buffer (pH 3.0). After the resulting mixture had been extracted with 10 ml of ethyl acetate by shaking for 1.5 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 5- μl aliquots of the solution were injected into the chromatograph.

Chromatographic conditions

An isocratic elution mode of the reversed phase HPLC was employed: column, Nucleosil 5 C₁₈; eluent, 0.05 M citric acid/acetonitrile (7:1, v/v). The flow rate of the eluent was 0.5 ml/min, and the column temperature was maintained at 45°C. The UV detector was used at 280 nm, and the sensitivity was set at 0.02 absorbance units (a.u.) at full scale. The oxidation potential of the EC detector was maintained at 0.75 V vs. Ag/AgCl reference electrode, and the sensitivity was set at 320 nA at full scale.

RESULTS AND DISCUSSION

Clean-up of urine

The three acids directly extracted from urine into ethyl acetate were not separated from the other constituents in the urine by isocratic elution HPLC on a reversed phase column. We adopted a purification method using anion exchange Sephadex gel. Figure 1 shows the elution profiles of 10 µg authentic substances of HVA, VMA and 5-HIAA, respectively, applied to the column of QAE-Sephadex A-25 as 5 ml of solution in 0.024 M HCl. At the acidity of 0.02-0.03 M HCl, HVA, VMA and

Fig. 1 Elution profile of HVA, VMA and 5-HIAA from the QAE-Sephadex A-25 column (acetate form; 28 mm x 9.5 mm I.D.)

5-HIAA were adsorbed on QAE-Sephadex A-25. These substances retained on the gel were not desorbed by washing the column with 0.01 M HCl and were eluted with 0.2 M NaCl in 0.01 M HCl. The recoveries of the three acids from the QAE-Sephadex A-25 column were almost quantitative on the basis of the amounts applied.

A column of strong basic anion exchange polystyrene resin such as Dowex 1-X2 was examined for another purification method. From the column, however, although HVA and VMA were eluted¹⁰⁾, 5-HIAA would not be desorbed even with 3 M NaCl.

To investigate the influence of pH on ethyl acetate extraction of metabolites, the extraction yields at different pH values were evaluated. The results are shown in Table 1. As the yield of VMA is lower at pH 2-5, this acid shall be extracted at pH 1. At pH 1, however, 5-HIAA was labile. Thus, the extraction of HVA, VMA and 5-HIAA into ethyl acetate was found to be optimal at pH 3.

HPLC

The chromatograms shown in Fig. 2 demonstrate our results with two types of

chromatographic detector. In Fig. 2-a, the HPLC column effluent was monitored by using a EC detector. Figure 2-b shows a chromatogram of the same urine extract for which UV detector is used at 280 nm.

Under the conditions described, VMA, 5-HIAA and HVA were eluted at 8.4, 17.6 and 21.0 min, respectively and were well separated from the other constituents in urine.

Table 1 Recoveries of HVA, VMA and 5-HIAA extracted with ethyl acetate from aqueous solution at pH values between 1 and 5

pH of aqueous phase	Recovery*(%)		
	HVA	VMA	5-HIAA
1	100	66	84
2	98	62	89
3	98	61	90
4	96	33	95
5	96	14	90

*To 5 ml of buffer at each pH value were added 1 g of NaCl and 5 ml of ethyl acetate containing 0.2-0.6 mg of authentic substances. After the mixture had been shaken for 1.5 min, 4 ml of the organic phase were taken and evaporated to dryness. The residue was dissolved in 4 ml of methanol and the solution was analyzed by the present HPLC.

Working curve

The working curves of authentic substances added to urine were linear over the each range of 1-25 mg/l for

HVA and VMA and 12.5 mg/l for 5-HIAA, in both UV and EC detection.

The recoveries of HVA, VMA and 5-HIAA were 99 %, 61 % and 88 % over the same range, respectively.

The detectable limit, based on a signal-to-noise ratio 2, was found to be 0.1 mg/l for HVA and 5-HIAA and 0.2 mg/l for VMA, in both UV and EC detection.

Reproducibility and comparison between the detection methods

The reproducibilities of the methods are summarized in Table 2. Figure 3 depicts the correlation between the detection methods. The UV detection was compared with EC detection by measuring sample simultaneously with both detectors. These results support the present method to be reliable for practical purposes. In the following works, chromatographic peaks were quantified by measuring absorbance

Fig. 2 Chromatograms of urine extract from a normal person obtained by (a) EC and (b) UV detection methods

at 280 nm because the maintenance of UV detector was somewhat easier than that of EC detector.

Table 2 Reproducibility of assays (n=6)

		Coefficient of variation(%)			
		Within-day		Between-day	
		UV	EC	UV	EC
HVA	(1.7 mg/l)	5.0	5.3	5.8	6.9
VMA	(1.5 mg/l)	6.9	6.9	7.7	8.5
5-HIAA	(1.2 mg/l)	5.7	6.8	9.3	8.5

Fig. 3 Correlation of EC and UV detection methods for determination of HVA, VMA and 5-HIAA in urine. EC and UV measurements were made simultaneously for each sample (n=26)

Validity of the method

The urinary excretions of HVA, VMA and 5-HIAA in eight normal persons and two patients with neuroblastoma were determined by the present HPLC coupled with UV detection. The results are summarized in Table 3. The values in normal persons are consistent with those reported in the literatures¹¹⁾⁻¹³⁾. HVA as well as VMA was largely excreted in patients with neuroblastoma⁶⁾¹⁰⁾.

Table 3 Urinary excretion ($\mu\text{g}/\text{mg}$ of creatinine*) of HVA, VMA and 5-HIAA in human

Subjects	HVA	VMA	5-HIAA
Normal persons (n=8)	$2.6 \pm 1.3^{**}$	$1.4 \pm 0.6^{**}$	$2.0 \pm 1.2^{**}$
Neuroblastoma	54	12	12
Neuroblastoma	330	49	N.D.

*Creatinine content in urine specimens was determined by HPLC described elsewhere¹⁴⁾. **mean $\pm$ S.D.

In conclusion, liquid chromatographic analysis combined with a simple clean-up

procedure using a Sephadex anion exchange gel provides a rapid, simple and reliable method to determine urinary levels of HVA, VMA and 5-HIAA. The present HPLC coupled with UV detection is especially adequate and useful in screening of catecholamines or serotonin secreting tumors because of the simplicity of the apparatus and the procedure.

Furthermore, the urinary excretions of the three metabolites in not only human but also animal such as mouse, rat or dog could be determined by the present HPLC coupled with EC detection. The details are described elsewhere.

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Keyword phrases

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