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Note

Simultaneous determination of vanillylmandelic acid, homovanillic acid and creatinine in urine by reversed-phase ion-pair chromatography

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Neuroblastoma is one of the few malignant tumours that excrete unambiguous markers for diagnosis. About 95% of the patients studied were reported to excrete abnormal levels of either or both vanillylmandelic acid (VMA) and homovanillic acid (HVA) in their urine [1]. It is, therefore, very important to determine the urinary levels of both VMA and HVA in the screening or diagnosis of neuroblastoma. In Japan, spot-testing for VMA [2] or thin-layer chromatography for HVA and VMA [3] was initially used for mass screening purposes, but high-performance liquid chromatography (HPLC) has recently been introduced because of its greater accuracy [4]. Also, a new enzyme immunoassay method has been developed [5].

A practical method for the simultaneous determination of VMA and HVA in urine by HPLC was first proposed by Yoshida *et al.* [6]. Since then a number of HPLC methods have been reported including one using UV detection [7], another using fluorimetric detection [8] and several using electrochemical detection [9–14]. Some of these techniques require extensive pretreatment of the urine and are too complicated for routine analyses, but several of them are simple and rapid, allowing direct injection of diluted urine, and are in use for mass screening in Japan.

The measurement procedure for neuroblastoma screening is not complete if only the urinary VMA and HVA levels are measured. Creatinine (Cr) is used as an internal reference and the microgram values of VMA and HVA per milligram of Cr are employed as the indices for mass screening. For this purpose, Cr is also

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measured, usually by the Jaffe alkaline picrate colouring method. Neuroblastoma mass screening, therefore, now involves two measurement steps, which makes the automation of the whole procedure difficult.

We report here a simple, rapid HPLC method for the simultaneous determination of VMA, HVA and Cr in urine, using one analytical column, one clean-up precolumn and a column-switching technique with UV and fluorimetric detection.

EXPERIMENTAL

Reagents

HVA, VMA and Cr were obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and reagent-grade concentrated phosphoric acid (85%), sodium hydroxide, sodium azide and tetrabutylammonium hydroxide (10% aqueous solution) from Wako (Osaka, Japan).

We prepared the mobile phase and the washing solution by dissolving 11.5 g of phosphoric acid, 2.59 g of tetrabutylammonium hydroxide and 0.065 g of sodium azide in 900 ml of distilled water and then adjusting the pH of the solution to 5.6 with 1 *M* sodium hydroxide, followed by addition of distilled water to 1 l.

We prepared a stock standard solution by dissolving 5 mg each of VMA and HVA and 250 mg of Cr in 1 l of distilled water. We prepared a working standard solution every day by diluting the stock solution twenty-fold with distilled water. The stock solution was stable for several months when stored in a refrigerator.

Apparatus and chromatographic conditions

Two DS-3 liquid chromatographic pumps, an AO-30 column oven, a KT-21 degassing device (Showa Denko, Tokyo, Japan), an 870-UV ultraviolet detector,

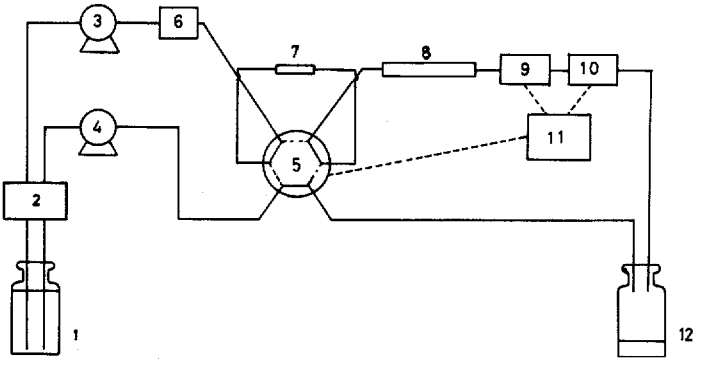

Fig. 1. Schematic diagram of the HPLC system for simultaneous determination of urinary VMA, HVA and Cr. 1 = Mobile phase and washing solution bottle; 2 = degassing device; 3,4 = pumps; 5 = electromagnetic six-way valve; 6 = autosampler; 7 = clean-up precolumn; 8 = analytical column; 9 = fluorescence detector; 10 = UV detector; 11 = integrator; 12 = waste bottle.

an 820-FP fluorescence detector, an electromagnetic six-way valve (Japan Spectroscopic, Tokyo, Japan), an AS-23 autosampler and a Labchart 80 integrator (System Instruments, Tokyo, Japan) were connected as shown in Fig. 1. Two columns were used: an analytical column and a clean-up precolumn for preventing the constituents eluting later than HVA from entering the analytical column. A similar liquid chromatographic set-up is reported in ref. 15. Both the analytical column (150 mm × 4.6 mm I.D.) and the precolumn (20 mm × 4.6 mm I.D.) were packed with 5-μm DE polyacrylate gel (Showa Denko), which has a pore size of 50 Å and is usually used as packing for reversed-phase liquid chromatography. The two columns were maintained at 43°C in the column oven. When the switching valve was in the solid line mode, 50 μl of the urine sample were injected and first retained on the precolumn. Of the urine constituents for analysis, Cr was retained for the shortest length of time, then VMA and last HVA. As soon as the HVA left the precolumn, the valve was switched to the dotted line mode and the precolumn was connected with the washing solution. The urine constituents that had left the precolumn were separated in the analytical column and detected by the UV detector (235 nm) and the fluorescence detector (excitation, 280 nm; emission, 320 nm) connected in series. The switching was controlled at 2.5 min after injection by the integrator via a time-control relay. Then, 14 min later, when the analysis was over, the switching valve was returned to the solid line mode and the next sample was injected. The integrator had two channels and received the signals from the two detectors, drawing the chromatogram for Cr from the UV detector and that for VMA and HVA from the fluorescence detector. It determined the concentrations of Cr, VMA and HVA and calculated the microgram values of VMA and HVA per milligram of Cr. The flow-rates of the mobile phase and the washing solution were both 1.0 ml/min.

For a purity check of the urinary Cr peaks, a Shimadzu SPD-6A photodiode array detector was used.

Urine samples

At periodical physical examinations of infants in the Tokyo metropolitan area, parents were given a filter paper (No. 2, 20 × 50 × 1 mm, Toyo Roshi, Tokyo, Japan) for collection of the urine of their infants. The filter paper was wetted with the urine, dried and mailed direct or via a local health centre, to one of the clinical laboratories in charge of mass screening. Dried urine samples thus mailed to the Tokyo Association of Health Services were used in this study. Six 5 mm diameter pieces were punched out from each filter paper. After addition of 400 μl of distilled water, the pieces were stirred in a test-tube for 30 min on a vibrator, and 50 μl of the supernatant were injected into the liquid chromatograph. Furthermore, the supernatant was analysed for correlation studies by the method used routinely in the Tokyo Association of Health Services for mass screening of neuroblastoma. In this method, VMA and HVA are determined by HPLC with coulometric electrochemical detection (ED) and Cr by the Jaffe method [14].

RESULTS AND DISCUSSION

Chromatography

Fig. 2 shows chromatograms of the working standard solution, a normal urine sample and urine sample from a patient with neuroblastoma. The upper chromatograms are from the fluorescence detector, indicating VMA and HVA peaks, and the lower are from the UV detector, indicating a Cr peak. The retention times of Cr, VMA and HVA were 2.6, 5.9 and 10.9 min, respectively. The separation of the peaks was satisfactory. There was no interference from the constituents of previous samples when multiple samples were successively injected, because constituents eluting later than HVA were prevented from entering the analytical column by use of the precolumn and the column-switching technique. The timing of switching (2.5 min) was determined by first injecting a working standard solution with the analytical column disconnected and recording a chromatogram from the fluorescence detector.

Check of the purity of the creatinine peaks

The purity of the creatinine peaks in the UV chromatograms of urines from infants was checked by overlapping the spectrograms of the creatinine peaks of urine samples with that of an authentic creatinine peak from 210 to 280 nm. The purity P as defined by the following equation was on the average more then 0.99.

$$P = \sum_{\lambda} S_1(\lambda) \cdot S_2(\lambda) / \sqrt{\sum_{\lambda} (S_1(\lambda))^2} \sqrt{\sum_{\lambda} (S_2(\lambda))^2}$$

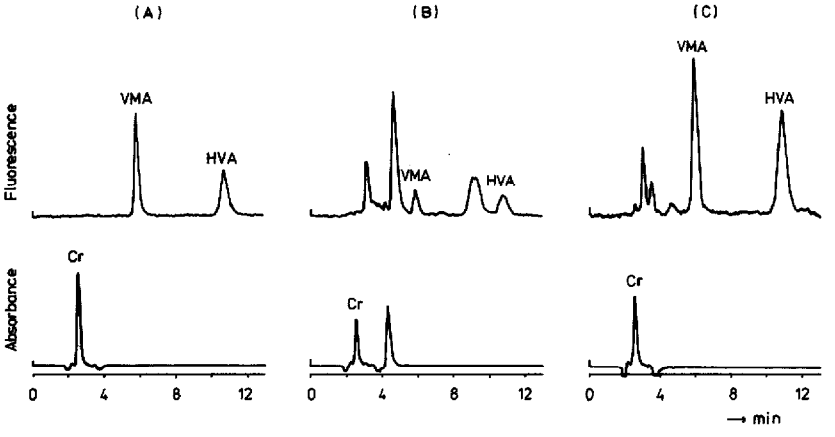

Fig. 2. Chromatograms of (A) a working standard solution containing 0.25 μg/ml VMA, 0.25 μg/ml HVA and 0.0125 mg/ml Cr, (B) a normal urine sample containing 0.057 μg/ml VMA, 0.100 μg/ml HVA and 0.0063 mg/ml Cr and (C) a urine sample from a neuroblastoma patient containing 0.386 μg/ml VMA, 0.567 μg/ml HVA and 0.0093 mg/ml Cr. The upper chromatograms were obtained with a fluorescence detector and the lower chromatograms with a UV detector.

where λ is the wavelength and S_1 and S_2 respectively are the absorption spectra of the urinary and authentic creatinine.

Calibration curves

The calibration curves for VMA, HVA and Cr obtained by injecting standard solutions containing 0.125, 0.25, 0.50 and 1.0 mg/l VMA and HVA and 6.25, 12.5, 25.0 and 50 mg/l Cr were all linear and passed through the origin. The concentrations of VMA, HVA and Cr in the urine samples obtained by extraction from the urine filter papers fall within the above concentration ranges and correspond to *ca.* 2.5% of those in the original urine samples. For calibration purposes, Kinoshita *et al.* [14] used standard filter papers that had been soaked with standard solutions and dried. We used standard solutions because they are more stable in storage and simpler to handle in operation than standard filter papers. It had previously been found by the comparison method that the correlation between the microgram values of VMA and HVA per milligram of Cr obtained with the two calibration methods is good, the correlation coefficient r for VMA being 0.99 and that for HVA 0.98 (unpublished data).

Precision

We assessed the precision of the present method by repeated analyses ($n=10$) of three diluted urine samples containing VMA in the range 0.079–0.31 mg/l, HVA in the range 0.069–0.414 mg/l and Cr in the range 3.76–14.98 mgl/l. The coefficients of variation (C.V.) were 2.3–3.9, 2.1–3.6 and 1.4–1.9% for the VMA, HVA and Cr concentrations, respectively. The C.V. for the retention times were 0.4–0.9%.

Correlation with the HPLC–ED method

We assayed 200 urine samples by the present method (y) and by the HPLC–ED method (x). The correlation between the two methods for the VMA concentrations fits the regression equation $y = 0.915x - 0.001$ ($r=0.990$) and that for the HVA concentrations fits the regression equation $y = 1.004x - 0.007$ ($r=0.972$). Good correlation was also observed for the creatinine concentrations (Fig. 3).

As for the microgram values of VMA and HVA per milligram of Cr, the regression equations were $y = 1.005x - 0.016$ ($r=0.968$) and $y = 1.057x + 0.151$ ($r=0.860$), respectively.

The mean values and the S.D. based on the assay by the present method were 8.8 ± 2.35 μg/mg of Cr for VMA and 18.2 ± 4.91 μg/mg of Cr for HVA, respectively. On the other hand, the mean values and the S.D. by the HPLC–ED method were 9.16 ± 2.40 μg/mg of Cr for VMA and 17.70 ± 3.38 μg/mg of Cr for HVA, respectively. Thus, the cut-off values for normal urine samples, which are arbitrarily taken as having mean values plus 2.5 times the S.D. are 14.7 and 30.6 μg/mg of Cr for VMA and HVA, repectively, by the present method and

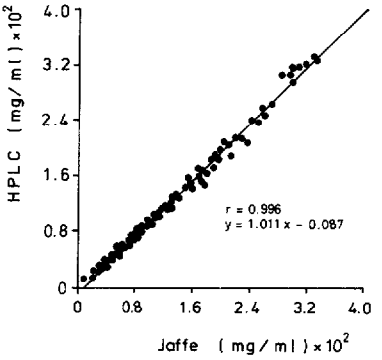

Fig. 3. Correlation for Cr determination between the present method (HPLC) and the Jaffe method.

15.2 and 26.15 μg/mg of Cr for VMA and HVA, respectively, by the HPLC–ED method.

In ref. 4, the mean values and the S.D. obtained in mass screening by HPLC at six major clinical laboratories in different parts of Japan are described. The mean values and the S.D. (in parentheses) for VMA and HVA are 12.1 (6.1) and 18.5 (6.8), 10.5 (3.6) and 18.4 (5.5), 8.63 (3.23) and 14.3 (4.53), 9.02 (2.8) and 14.7 (3.76), 9.63 (2.20) and 16.0 (3.76), and 7.17 (2.82) and 16.2 (4.61) μg/mg of Cr. The values reported by most laboratories are very close to those obtained in this study.

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