Simple Liquid-Chromatographic Measurement of Vanillylmandelic Acid and Homovanillic Acid in Urine on Filter Paper for Mass Screening of Neuroblastoma in Infants

Junji Hanai,1 Tsunehiko Kawai,1 Yasumasa Seto,1 Nobuo Takasugi,1 Motoi Nishi,2 and Takeo Takeda3

We describe a simple and rapid method for measuring catecholamine metabolites in urine collected on filter paper. By this method, "high-performance" liquid chromatography, urinary vanillylmandelic acid and homovanillic acid can be measured within 15 min after being eluted from filter paper treated with tartrate buffer. One hundred urine samples can be pretreated in 2 h and 100-120 samples analyzed at 18-min intervals overnight in two chromatographic systems. Moreover, urinary creatinine also can be measured rapidly because of the use of microtitre plates. We applied this method in a mass screening program for early detection of neuroblastoma in infants. Between April 1986 and March 1987, two patients with neuroblastoma were detected. In all, neuroblastoma has been detected in 17 of 88 887 infants so examined since April 1981.

Additional Keyphrases: catecholamines · creatinine · electrochemical detection · incidence of neuroblastoma

The major acidic metabolites of catecholamines—vanillylmandelic acid (VMA) and homovanillic acid (HVA)—are excreted in large quantities in urine by patients with neuroblastoma (1-4). Therefore, urinary VMA and HVA are measured to detect and diagnose patients with neuroblastoma.

A mass-screening program for early detection of neuroblastoma in infants has been conducted in Japan in some districts (5), and the screening has been expanded into almost all local self-governing areas since 1986. In the screening method, urine collected on filter paper is used as a specimen, and a qualitative VMA test modified from the LaBrosse spot test (6), named the "VMA spot test" (7) or the "VMA dip test" (8), is generally applied. These tests, however, are not sensitive enough to detect all neuroblastoma patients, especially those who do not excrete VMA in the urine (9). Moreover, the intake of some foods or medicines can lead to false-negative or false-positive results (7, 10). To overcome these problems, we started a mass-screening program in which VMA and HVA in urine collected on filter paper are measured by "high-performance" liquid chromatography (HPLC). This program has been in operation in Sapporo City since April 1981 (11, 12).

Several methods for measuring VMA and HVA by HPLC have been described, but some require extracting the urine with an organic solvent (13, 14). Moreover, almost all of the methods for direct injection of diluted urine into the HPLC system require the use of fresh urine as specimens (15-17), making them less applicable to mass screening than are methods for urine collected on filter paper.

Here we describe a simple, rapid method for simultaneous measurement of VMA and HVA in urine collected on filter paper, in which we use an HPLC equipped with an electrochemical detector. We also describe some of the results by this method in a mass-screening program for early detection of neuroblastoma in infants in Sapporo City.

Materials and Methods

Reagents. VMA, HVA, and other catecholamine metabolites were purchased from Sigma Chemical Co., St. Louis, MO 63178; creatinine from Tokyo Kasei Kogyo Co., Tokyo, Japan; acetonitrile and ethyl acetate, "HPLC solvent grade," from Kanto Chemical Co., Tokyo, Japan; and picric acid solution (for use in creatinine determination) from Wako Pure Chemical Industries, Osaka, Japan. Other reagents were of analytical grade. All aqueous solutions were prepared with de-ionized water from a Milli R/Q system (Millipore Corp., Bedford, MA 01730).

Urine samples. Mothers of six- to 12-month-old infants were asked to collect urine samples by placing a piece of filter paper (no. 63, 30 × 56 × 1 mm; Toyo Roshi, Tokyo, Japan), which had been cut previously into comb-shaped strips (20 × 8 mm), on the infants' lower abdomen and covering it with a diaper. When the filter paper was wet, the mothers put it in the plastic bag provided and mailed it to our laboratory. The paper was then dried and stored in a refrigerator until assayed. We cut off one strip of the filter paper and used it as the urine sample.

Instruments. The chromatographic system consisted of a Model 638-50 HPLC chromatograph (Hitachi, Tokyo, Japan), a Model KSST-601 autosampler (Kyowa Seimitsu Co., Tokyo, Japan), a Model LC-4B amperometric detector with a glassy carbon TL-5A cell (Bioanalytical Systems Inc., West Lafayette, IN 47905), and a Model SIC 700B integrator (System Instruments Co., Tokyo, Japan). The analytical column was a 4 mm (i.d.) × 250 mm stainless-steel column packed with Hitachi gel No. 3013-O, a spherical porous polystyrene gel bonded with hydroxymethyl groups (5-μm average particle size). The column was kept at 40 °C with a Model 635-0335 temperature regulator (Hitachi). We also used flat-bottomed polystyrene 96-well microtitre plates (Sanko Junyaku Co., Tokyo, Japan) and measured absorbance in the wells with a Model NJ-2000 "microelisa" plate reader (Nippon InterMed, Tokyo, Japan).

Procedure. A strip (20 × 8 mm) of the urine-soaked filter paper was placed in a glass tube (10 mm i.d. × 80 mm) and stirred vigorously with 1.5 mL of tartrate buffer (50 mmol/L, pH 3.6) for 10 min with a vortex-type mixer, then centrifuged at 2000 × g for 10 min. We transferred 100-μL aliquots of the supernatant to a sample cup of the autosampler and to a well of the microtitre plate. The autosampler injected 20-μL aliquots into the HPLC system.

We calculated VMA and HVA concentrations by the external-standard method, comparing the peak heights of...
unknown samples with those for the standard. The mobile phase, a mixture of 500 mL of tartrate buffer (50 mmol/L, pH 3.6) and 70 mL of acetonitrile, was de-gassed before use. We detected all eluting compounds at a potential of +900 mV vs an Ag/AgCl reference electrode, at a flow rate of 1 mL/min. The column, packed with a porous polystyrene gel, was not washed with other solvents except with a mobile phase, so we replaced the top portion of the column with new packing material at approximately three-week intervals. By this method, more than 5000 samples could be analyzed with each column without solvent washing.

Creatinine was determined by the Jaffe reaction as follows: we placed 150 μL of an equimolar mixture of picric acid solution and 0.75 mol/L NaOH solution in the well of a microtiter plate that already contained 100 μL of sample supernate. After 25 min, we measured the well's absorbance at 510 nm with the plate reader and calculated the creatinine concentrations. Values for VMA and HVA were expressed as micrograms per milligram of creatinine.

Results

Analytical recoveries. A mixture of VMA and HVA at two different concentrations was added to pooled urine samples from two normal infants and from one patient with neuroblastoma. Aliquots of the solution were applied to a piece of filter paper, dried, and VMA and HVA were measured by the above procedure. Table 1 summarizes analytical recoveries.

Precision. Within- and between-run precision was evaluated at three different concentrations in infant-urine samples, including the urine of one patient with neuroblastoma, by analyzing the samples repeatedly during one full day and over a period of five days in duplicate. The results are summarized in Table 2.

Linearity and detection limits. The standard curves were linear up to at least 2 mg/L for VMA and 4 mg/L for HVA (r >0.99 each). Detection limits were 5 μg/L for VMA and 10 μg/L for HVA.

Method comparison. About 90 normal urine samples on

Table 1. Analytical Recoveries of VMA and HVA Added to Urine

<table>
<thead>
<tr>
<th>VMA, ng</th>
<th>Added</th>
<th>Recovery, %</th>
<th>HVA, ng</th>
<th>Original</th>
<th>Added</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>90.2</td>
<td>200</td>
<td>90.1</td>
<td>1000</td>
<td>97.0</td>
<td>400</td>
<td>97.0</td>
</tr>
<tr>
<td>500</td>
<td>200</td>
<td>92.9</td>
<td>1000</td>
<td>96.3</td>
<td>400</td>
<td>93.9</td>
</tr>
<tr>
<td>271.0</td>
<td>200</td>
<td>92.4</td>
<td>1000</td>
<td>93.2</td>
<td>400</td>
<td>90.0</td>
</tr>
<tr>
<td>500</td>
<td>200</td>
<td>92.6</td>
<td>1000</td>
<td>96.0</td>
<td>400</td>
<td>90.0</td>
</tr>
<tr>
<td>752.1</td>
<td>200</td>
<td>93.2</td>
<td>1000</td>
<td>96.0</td>
<td>400</td>
<td>90.0</td>
</tr>
<tr>
<td>500</td>
<td>200</td>
<td>98.1</td>
<td>1000</td>
<td>96.0</td>
<td>400</td>
<td>90.0</td>
</tr>
</tbody>
</table>

Table 2. Precision Data for VMA and HVA

<table>
<thead>
<tr>
<th>Urine no.</th>
<th>VMA, ng</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
<th>VMA, ng</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run (n = 5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>90.2</td>
<td>3.1</td>
<td>3.4</td>
<td>4.4</td>
<td>4.4</td>
<td>3.1</td>
<td>3.4</td>
<td>4.4</td>
</tr>
<tr>
<td>2</td>
<td>271.0</td>
<td>6.6</td>
<td>2.4</td>
<td>1.9</td>
<td>1.9</td>
<td>6.6</td>
<td>2.4</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>752.1</td>
<td>32.3</td>
<td>3.3</td>
<td>4.4</td>
<td>4.4</td>
<td>32.3</td>
<td>3.3</td>
<td>4.4</td>
</tr>
<tr>
<td>Between-run (n = 5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>84.7</td>
<td>2.4</td>
<td>2.8</td>
<td>3.3</td>
<td>3.3</td>
<td>2.4</td>
<td>2.8</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>290.1</td>
<td>6.7</td>
<td>2.3</td>
<td>3.5</td>
<td>3.5</td>
<td>6.7</td>
<td>2.3</td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
<td>865.7</td>
<td>16.7</td>
<td>1.9</td>
<td>2.9</td>
<td>2.9</td>
<td>16.7</td>
<td>1.9</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Fig. 1. Chromatographic separation of aqueous standard mixture MHPG, 3-methoxy-4-hydroxyphenylglycol; VLA, vanilllic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxy-3-indoleacetic acid; VA, vanillic acid
six months to one year of age, were determined by the present method; however, values under a detection limit for each analyte were eliminated. The results (Table 3) demonstrated that the values were in agreement with those given in previous reports (3, 16, 19). Taking the rate of retest into account, we have established cutoff values of 18 μg per milligram of creatinine for VMA and 28 μg per milligram of creatinine for HVA and have used them since April 1987. These values approximately correspond to the mean + 3SD.

Results of the screening. The present method has been applied to measurements of VMA and HVA in mass screening since April 1986. Of 15 661 infants screened, two patients with neuroblastoma were detected. During the period from April 1981 to March 1987, 17 of 88 887 infants have been detected and diagnosed as having neuroblastoma. Table 4 shows the values for urinary VMA and HVA of the patients with neuroblastoma detected by the mass screening.

Discussion

Several studies have suggested that almost all patients with neuroblastoma can be detected and diagnosed with high reliability by measuring urinary VMA, HVA, and other catecholamine metabolites, because 90% or more of such patients show an increased urinary excretion of these metabolites (1-4, 9, 21).

In Kyoto City, Japan, mass screening of neuroblastoma in infants has been conducted by use of the "VMA spot test" since 1973 (7). In our mass-screening program, begun in 1981, quantitative measurements of urinary VMA and HVA by HPLC have been made (II). In the beginning we assayed urinary VMA and HVA after their extraction from filter paper with organic solvent. However, this pretreatment was very complicated, making it impossible to process a large number of samples quickly.

By the method described above, we can pretreat 100 urine samples within 2 h, that is, in a quarter to a fifth of the time required by the organic solvent extraction method. Moreover, 100 to 120 samples can be analyzed at 18-min intervals overnight by two HPLC systems, thus enabling us to assay about 25 000 samples a year. The new method has made it possible to screen all the infants born in Sapporo City in a year—about 20 000 a year.

From April 1981 to March 1987, 88 887 infants from six months to one year of age have been screened. This number corresponds to about 75% of all the live-births in Sapporo City during this period. Of these, 17 infants were detected and diagnosed as having asymptomatic neuroblastoma. This incidence of neuroblastoma is about one per 5200 infants, considerably higher than reported in a previous paper (5).

The method applied in mass screening for neuroblastoma should be sensitive enough to detect every affected patient and capable of distinguishing false-negative or false-positive cases. The present method ensures accurate measurement of urinary VMA and HVA and thus enables precise detection of this condition. Our results have confirmed the usefulness of this method for early detection of neuroblastoma in infants and its easy application in mass screening of infants.
This study was supported by a grant-in-aid for Research of Mass Screening Systems from the Ministry of Health and Welfare of the Japanese Government.

References