Determination of Vanillylmandelic Acid, Vanillactic Acid, and Homovanillic Acid in Dried Urine on Filter-Paper Discs by High-Performance Liquid Chromatography with Coulometric Electrochemical Detection for Neuroblastoma Screening

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We report a method for determination of vanillylmandelic acid, vanillactic acid, and homovanillic acid by high-performance liquid chromatography (HPLC), with coulometric electrochemical detection, for mass screening of neuroblastoma. Urine samples were collected on filter paper, dried, and then pretreated. The chromatographic procedure is reliable and fast, allowing for a large sample throughput for routine screening. Intricate extraction procedures and centrifugal separation are unnecessary. Screening for neuroblastoma by HPLC is rapidly gaining acceptance in Japan, and our method is being used at many screening centers. Of 26,571 infants screened in one year in Yokohama City, our method detected five with neuroblastoma.

Neuroblastoma, the most common and highly malignant tumor of childhood, has a very poor prognosis. Neuroblastoma is detected within one year of age and at stage I, II, or IV-s have the more favorable prognosis (1).

In Japan, mass screening for the early detection of neuroblastoma has been performed since 1973 by the spot test for vanillylmandelic acid (VMA) of Sawada et al. (2). Neuroblastoma screening of six-month-old infants has spread throughout the country.

In Yokohama City, this screening involved a VMA dip test (3) and thin-layer chromatography (TLC) (4) for several years. However, because of the assertion (5) that qualitative assays such as the dip test will fail to detect more than 60% of affected patients, and because of the complexity of the TLC technique for detecting VMA, vanillactic acid (VLA), and homovanillic acid (HVA), we investigated alternative methods for the fast, specific, and sensitive quantification of VMA, VLA, and HVA.

Several methods for measuring VMA and HVA by HPLC have been described, but some require extracting, with ethyl acetate, the urine collected on filter paper (5, 7), and others require the use of fresh urine samples for direct injection of diluted urine into the chromatograph (8, 9). However, our HPLC method requires neither, and so is more suitable for mass screening. Our method with coulometric electrochemical detection has been applied to mass screening of neuroblastoma in Yokohama City since February 1987. Here we describe its use for the assay of VMA, VLA, and HVA in dried urine samples on filter paper.

Materials and Methods

Apparatus

We used a Model LC-6A liquid chromatograph with a CTO-6A column oven, an SCL-6A system controller, and an SIL-6A auto injector (all from Shimadzu, Kyoto, Japan). The auto-injector was kept at 5°C in an RTE-5B refrigerating circulating bath (Neulab Instruments, Newington, NH). A 15 cm x 6 mm (i.d.) Shimadzu Shim-pack CLC-VMA column was packed with silica gel chemically bonded with hydrophilic functional group (5-µm particle size). The column was kept at 40°C.

For electrochemical detection of the eluting compounds, we used a Model 5100A Coulochem (ESA, Bedford, MA) system that consisted of a Model 5010 dual analytical cell positioned at the end of the chromatographic column (detector 1 set at +0.17 V, detector 2 set at +0.22 V) and a Model 5020 guard cell (set at +0.27 V) positioned between the pump and the autoinjector. The signals from detector 2 were recorded, via connection to a recorder-integrator (Shimadzu, Chromatopac C-R4A) operated in the peak-area mode.

Mobile Phase

The chromatographic mobile phase used routinely was 5 mmol/L tartaric acid solution containing 50 mL of acetonitrile per liter (pH 2.77). The flow rate was 1.0-1.4 mL/min.

Urine Specimens

In Yokohama City, four-month-old infants undergo physical examination at local health centers. At this time, parents are given thick filter paper (no. 63, 20 x 50 x 1 mm; Toyo Roshi, Tokyo, Japan) to soak up urine from a wet diaper when the infant is six to eight months old. The urine-soaked filter paper is then dried under a hair drier and mailed to the laboratory.

Preparation of Standard and Control Samples on Filter Paper

We prepared a stock solution by dissolving 40 mg each of VMA, VLA, and HVA in 100 mL of distilled water; 20 mL of this solution was kept frozen until required. Working standard solutions (10, 20, and 40 mg/L) were prepared by diluting the stock solution with distilled water. We soaked standard filter paper (larger stripe, 2 x 10 cm) with 1.9 mL of working solutions and dried them in a 60°C oven.

Similarly, we prepared filter paper standards for creatinine by soaking each strip with 1.9 mL of creatinine solution (200, 400, or 800 mg/L). The filter paper control was made by soaking it with 1.9 mL of control urine (pooled urine samples from normal infants).

Procedure

In this method it is not necessary to extract catecholamine metabolites from the urine-soaked filter paper with ethyl acetate. We punched two 5-mm-diameter pieces of filter paper from each sample, standard solutions, and control urine sample and placed each pair of paper discs in a test tube containing 600 µL of 5 mmol/L sodium tartrate solution (pH 6.7). After mixing the contents for 5 s on a thermostatic mixer, then leaving the tube at room temperature for
1 h, we removed 75 μL of eluate for creatinine determination (see below). We kept 200 μL of the remaining eluate at 5 °C in a 200-μL autosampler vial in the refrigerated circulating bath. Of this, 10 μL was injected into the chromatographic column.

We calculated VMA, VLA, and HVA concentrations by the external-standard method, comparing the peak-areas of unknown samples with those for the standard.

**Determination of Creatinine**

We used a 96-well microplate, mixing 75-μL samples (eluted from filter paper, see above) with 75 μL of 1.2 mol/L trichloroacetic acid solution. To this we added 150 μL of an equivalent mixture of picric acid (35 mmol/L) and NaOH (1.6 mol/L) and shook the microplate on a mixer at room temperature for 30 min. We then measured the absorbance of each well at 510 nm with a Model MCC Titertek Multiskan (Flow Laboratory, Helsinki, Finland).

**Results**

**Chromatography**

Figure 1 shows chromatograms of a filter paper standard, the filter paper sample from a normal infant, and the filter paper sample from a patient with neuroblastoma. Retention times were 4.8 min (VMA), 6.9 min (VLA), and 7.9 min (HVA). All three compounds were well separated from other main metabolites of catecholamines. This result and the simplicity prompted routine use of our mobile phase.

**Linearity and Detection Limit**

To examine the linear dynamic range and minimum detection limit, we diluted and assayed a standard solution. For an injection of 10 μL of the elute from the standard solution-soaked filter paper under the conditions described, the smallest detectable concentration was 0.1 mg/L for each of the analytes VMA, VLA, and HVA. The calibration curves were linear to at least 100 mg/L for each.

**Choice of Eluate**

At an early stage of this study, we used tartaric acid (5 mmol/L, pH 2.77) to elute VMA, VLA, and HVA from the filter paper discs. However, in one sample, the results disagreed considerably with those determined on the next day. So we studied the effect of pH on the elution of VMA and HVA from the filter paper discs with 5 mmol/L tartaric acid (Figure 2) and now use pH 6 to 7. Because the pH of 5 mmol/L sodium tartrate is about 6.7, we routinely use it as the eluent. In the conditions described, 60% of the VMA and HVA in the urine-soaked discs is eluted, consistently.

**Correlation between the Present Method (HPLC) and TLC**

We assayed 85 urine samples from infants by the present method (HPLC, y) and TLC (x). The results are plotted in Figure 3. The data for VMA fit the regression equation $y = 0.74x + 0.31$ ($r = 0.93$). The data for HVA fit the regression equation $y = 0.78x + 1.24$ ($r = 0.94$).

**Discussion**

The present method is sensitive and precise for measuring VMA, VLA, and HVA. It does not involve a large investment in equipment or time, and we could analyze 80–100 samples per day with one HPLC system. Detector cells required cleaning after analysis of 8000–10 000 samples, and the chromatographic columns had to be replaced after analysis of 2500–5500 samples.

We believe that samples obtained from diapers and col-

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**Fig. 1. Chromatograms of a filter paper standard (A), and filter paper samples from a normal infant (B), and from a patient with neuroblastoma (C)**

Concentrations of VMA, VLA, and HVA (mg/L): (A) 20.0, 20.0, 20.0; (B) 2.8, 0.0, 4.6; and (C) 15.0, 0.0, 17.8. Concentrations of creatinine (mg/L): (B) 160 and (C) 140
Table 1. Infants with Neuroblastoma Detected by Mass Screening

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age at primary screening, months</th>
<th>Sex</th>
<th>Dip-test</th>
<th>VMA</th>
<th>VLA</th>
<th>HVA</th>
<th>Creatinine concn, mg/L</th>
<th>Stage of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>F</td>
<td>-</td>
<td>6.0(100.0)</td>
<td>0.0</td>
<td>15.2(253.3)</td>
<td>290</td>
<td>II</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>F</td>
<td>-</td>
<td>14.6(51.0)</td>
<td>0.0</td>
<td>8.0(27.6)</td>
<td>300</td>
<td>III</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>M</td>
<td>+</td>
<td>82.9(236.9)</td>
<td>0.0</td>
<td>44.2(126.3)</td>
<td>350</td>
<td>II</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>F</td>
<td>±</td>
<td>13.1(145.6)</td>
<td>0.0</td>
<td>17.8(197.8)</td>
<td>90</td>
<td>II</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>F</td>
<td>±</td>
<td>12.4(155.0)</td>
<td>0.0</td>
<td>9.2(115.0)</td>
<td>80</td>
<td>II</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>F</td>
<td>-</td>
<td>16.2(49.1)</td>
<td>0.0</td>
<td>30.6(92.7)</td>
<td>330</td>
<td>II</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>M</td>
<td>-</td>
<td>11.2(48.7)</td>
<td>0.0</td>
<td>12.0(52.2)</td>
<td>230</td>
<td>II</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>F</td>
<td>-</td>
<td>10.0(125.0)</td>
<td>0.0</td>
<td>12.2(240.0)</td>
<td>80</td>
<td>II</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>M</td>
<td>-</td>
<td>14.4(40.0)</td>
<td>0.0</td>
<td>15.8(43.8)</td>
<td>360</td>
<td>II</td>
</tr>
</tbody>
</table>

By HPLC

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age at primary screening, months</th>
<th>Sex</th>
<th>VMA</th>
<th>VLA</th>
<th>HVA</th>
<th>Creatinine concn, mg/L</th>
<th>Stage of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>8</td>
<td>M</td>
<td>2.2 (12.2)</td>
<td>0.0</td>
<td>6.9 (38.3)</td>
<td>180</td>
<td>I</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>M</td>
<td>16.5(117.9)</td>
<td>0.0</td>
<td>20.6(147.1)</td>
<td>140</td>
<td>IV</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>M</td>
<td>6.0 (22.2)</td>
<td>0.0</td>
<td>8.7 (24.8)</td>
<td>270</td>
<td>II</td>
</tr>
<tr>
<td>13</td>
<td>7</td>
<td>M</td>
<td>2.2 (18.3)</td>
<td>0.0</td>
<td>2.9 (24.2)</td>
<td>120</td>
<td>II</td>
</tr>
<tr>
<td>14</td>
<td>7</td>
<td>F</td>
<td>15.8 (83.2)</td>
<td>0.0</td>
<td>14.5 (73.8)</td>
<td>190</td>
<td>II</td>
</tr>
</tbody>
</table>

*Cutoff values by TLC: VMA, 20.0 mg/g creatinine, HVA, 45.0 mg/g creatinine.

*bCutoff values by HPLC: VMA, 18.0 mg/g creatinine, HVA, 32.0 mg/g creatinine.

lected onto filter papers are suitable for mass determination of VMA, VLA, and HVA. However, it is necessary to dry the urine-soaked filter paper promptly, to avoid decomposition of creatinine, HVA, and VMA by bacteria (10).

In Japan, after the experiences in Kyoto and Nagoya City (11, 12), mass screening for neuroblastoma by various procedures (6—9) spread to other cities and prefectures, and neuroblastoma was detected early in many infants. Recently, Kikuchi and Tsutsui (5) suggested that quantitative assays such as the spot test or the dip test may miss more than 60% of the neuroblastomas. Our experience supports this contention.

In Yokohama City, neuroblastoma screening was done by the dip test (all specimens) and TLC (only about 60% of the specimens) from October 1982 to January 1987. TLC was performed in principle on specimens with a positive or intermediate dip-test result or with creatinine concentrations >110 mg/L. During this period, 105 454 urine samples were screened and nine affected patients were discovered (Table 1). However, the dip test detected only three of these patients. Therefore, TLC was needed for the initial screening, in addition to the dip test. However, TLC requires complicated pretreatment of samples, i.e., extraction of VMA, VLA, and HVA from filter paper with ethyl acetate. Therefore, since February 1987, we have been performing mass screening for neuroblastoma by HPLC. If the VLA concentration exceeds 1.0 mg/L, we re-assay the sample by HPLC or TLC. (This is an arbitrary cutoff value, not a physiologically based one.) Of the 26 571 infants screened so far by HPLC, five cases of neuroblastoma have been subsequently identified, for an incidence of 1:5300 infants (Table 1).

The use of HPLC, especially in combination with coulometric electrochemical detection, has facilitated many new studies of catecholamine biochemistry (13, 14). We consider the present method to be the method of choice for simultaneous determination of VMA, VLA, and HVA in dried urine on filter paper discs in mass screening for neuroblastoma.

References