Liquid-Chromatographic Assay for Urinary Homovanillic Acid, with Fluorescent Detection

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We describe a procedure for measuring urinary homovanillic acid by "high-performance" liquid chromatography. The pH of urine samples is adjusted, and they are chromatographed directly on an octyl silica column. The effluent is reacted with a ferricyanide reagent, to convert homovanillic acid to a product that is fluorescent at 420 nm on excitation at 320 nm. The standard curve is linear up to a homovanillic acid concentration of 20 mg/L; the detection limit is 0.3 mg/L. The method is evaluated for precision, recovery, potential interference, and reference values, and compared (r = 0.99) with a published spectrophotometric method. The proposed method is suitable for the routine analysis for homovanillic acid in urine.

Homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid, HVA) is the major urinary metabolite of the catecholamine, dopamine. Measurements of HVA along with 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA) have been advocated in the detection and monitoring of patients with neuroblastoma (1, 2).

As an alternative to the laborious ultraviolet (3) or colorimetric (4) procedures for HVA, chromatographic techniques have been used. For gas chromatography, sample derivatization is required (2). The specificity and selectivity of internal standards in this procedure have also been questioned (5). In the development of liquid-chromatographic procedures, detector sensitivity and specificity have been the major obstacles. Electrochemical detection (6) has been a promising approach to this problem. We report here our work with a liquid-chromatographic procedure with a fluorescent detection system that we find to be sensitive, specific, and applicable to the clinical laboratory.

Materials and Methods

Apparatus

**Liquid chromatograph:** Model 850 (E.I. DuPont and Co., Clinical Systems Div., Wilmington, DE (19898)) with a 4.6 × 25 cm CLC-1 column (DuPont), packed with octyl silica and maintained at room temperature. A guard column (CLDG-1) containing Permaphase ODS (DuPont) is used to extend the life of the analytical column. A mobile-phase flow rate of 1 mL/min is used.

**Column effluent monitor:** HVA is detected by reacting the column effluent with potassium ferricyanide to produce a fluorescent product. With a Model 3 proportioning pump (Technicon Instruments Corp., Tarrytown, NY 10591), an alkaline potassium ferricyanide reagent is pumped into the column at 0.8 mL/min. A three-port tee fitting (polypropylene) is used for this post-column reagent addition. The reaction mixture then flows through 92 cm of polyethylene tubing with an internal diameter of 1.12 mm. The tubing is wrapped around a rod (6 mm diameter) to form a mixing coil. Fluorescent products in the reaction stream are monitored with a Model 512 spectrofluorometer (Perkin-Elmer Corp., Norwalk, CT 06856) with a 25-μL flow cell (Hellma Cells, Inc., Jamaica, NY 11424) having a 1-cm lightpath. The excitation wavelength is set at 320 nm and emission at 420 nm.

Reagents

A stock standard (1 g/L) of 4-hydroxy-3-methoxyphenylacetic acid (Sigma Chemical Co., St. Louis, MO 63178) is prepared in 10 mmol/L HCl. Working standards (1.25–20 mg/L) are prepared by appropriate dilutions of the stock standard in mobile phase. Mobile phase is prepared by adding 70 mL of acetonitrile (HPLC grade; Fisher Scientific Co., Pittsburgh, PA 15219) and 2 mL of formic acid to 930 mL of water. The volume of acetonitrile was gradually decreased to 50 mL during four months to maintain a constant retention time for HVA.

A stock 2.5 g/L solution of potassium ferricyanide is prepared in potassium borate buffer (1 mol/L, pH 10.5). The working potassium ferricyanide reagent is prepared by making a 100-fold dilution of the stock solution with borate buffer.

Procedures

**Chromatography:** A 2-mL aliquot of standard or acidified urine sample is diluted with 2 mL of 0.6 mol/L formic acid. The pH of the urine sample is adjusted to 2.9 with 2 mol/L NaOH. The injection loop (volume about 50 μL) of the liquid chromatograph is filled by injecting 150 μL of sample. After chromatographic separation and detection, the peak height of the fluorescent HVA reaction product is measured and the concentration of HVA in the urine is calculated from comparison to the peak heights for the HVA standards.

**Other:** The measurement of urine creatinine was based on the method of Chasson et al. (7). Urinary VMA was assayed by the method of Pisano (8). Urines used in the correlation and reference value studies were collected in bottles containing 12 mL of HCl (6 mol/L). Samples not assayed within 24 h were stored frozen. All compounds used in the interference study were purchased from Sigma Chemical Co.

The chromatographic procedure is compared with two spectrophotometric methods, the first of which is based upon the reaction of HVA with 1-nitroso-2-naphthol. The full procedure and its evaluation are not published, and the analyses were performed by Bio-Science Laboratories, Van Nuys, CA 91405. Because of discrepancies with samples from a patient with neuroblastoma, details of the procedure were obtained from them, and we evaluated the linearity of the procedure. The second spectrophotometric method used in the correlation study is based upon the procedure of Knight and Haymond (4).

For fluorescent scanning of peaks found in the chromatograms, the chromatograph and proportioning pumps are stopped when the desired fluorescent peak is in the flow cell. Excitation spectra were obtained with the emission wave-
length set at 420 nm, emission spectra with the excitation wavelength set at 320 nm.

**Results**

Figure 1, a chromatogram of a urine assayed by our procedure, shows a major fluorescent peak at 15 min. By comparison to chromatograms of HVA standards and by assaying urine supplemented with HVA, this peak is identifiable as the fluorescent product of HVA. Additional fluorescent peaks are observed on chromatograms of urine samples but are resolved from HVA. Several of these peaks have been identified (see below). To verify that HVA is indeed converted to a fluorescent product by reaction with ferricyanide under our conditions, and to be sure there were no compounds present with native fluorescence, we replaced the ferricyanide reagent with borate buffer (1 mol/L pH 10.5). Under these conditions a chromatograph of the urine sample showed no fluorescent peak at 15 min.

For HVA standard, fluorescent peak height (y) and HVA concentration (x) were linearly related over a range of 1.25 to 20 mg/L, the regression equation being $y$ (in mm) = $12.1x - 4.8$ ($r = 0.999$).

The lowest concentration of HVA distinguishable from baseline noise with 95% confidence is 0.3 mg/L.

Intra-assay precision was determined by analysis of 10 samples of two patients' urines. The mean for the first urine was 5.8 mg/L (SD, 0.11; CV, 1.7%), for the second urine, 17.5 mg/L (SD, 0.28; CV, 1.6%). Inter-assay precision was determined in 11 separate runs by measuring aliquots from a lyophilized pool and from two frozen urine pools. The mean for the lyophilized pool was 2.5 mg/L (SD, 0.13; CV, 5.0%), for the first frozen pool, 7.3 mg/L (SD, 0.30; CV, 4.1%), and for the second frozen pool, 12.4 mg/L (SD, 0.35; CV, 2.8%). The frozen pools used in the precision study were initially adjusted to pH 2.9 and were found to be stable for at least four months at $-10 \degree C$. Analytical recovery of HVA (Table 1) ranged from 96 to 101%.

Possible interfering substances were studied. Chromatograms of urine from patients who were taking salicylate show a major fluorescent peak with a retention time of about 18 min, which is chromatographically resolved from the HVA peak. This fluorescent peak represents o-hydroxyhippuric acid (salicylic acid) and this salicylate metabolite fluoresces without reaction with potassium ferricyanide. A fluorescent peak is also seen in chromatograms from patients on methyl dopa medication. This peak has a retention time similar to salicylic acid, but in this case the fluorescence is ferricyanide dependent. We believe the compound to be a methyl dopa metabolite. The following compounds were shown not to interfere with the determination of HVA: VMA, metanephrine, normetanephrine, epinephrine, norepinephrine, 5-hydroxyindoleacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, gentisic acid, and salicylic acid.

Figure 2b shows a chromatogram of a urine from a 17-year-old patient with a confirmed diagnosis of neuroblastoma. A further 20-fold dilution of the patient's urine was chromatographed (Figure 2c) and showed a peak with a retention time identical to the chromatographed HVA standard (Figure 2b). HVA values for this patient ranged from 39 to 175 mg/24 h for the four urine specimens so assayed. VMA was also increased in these same urine samples, with values ranging from 25 to 101 mg/24 h. Individuals on leva-dopa medication have also shown increased urinary HVA. For one such patient the 24-h output was 74 mg, or 52 mg/g of creatinine.

We also studied the urinary excretion of HVA by 31 adults who had no history of neuroblastoma or pheochromocytoma and who were not on leva-dopa medication. The 24-h excretion ranged from 1.1 to 7.0 mg (mean, 3.6 mg/24 h). For 26 of these urines the mean was 2.7 mg of HVA per gram of creatinine (range 1.0 to 13.3 mg/g).

We initially compared results by the chromatographic assay

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**Table 1. Analytical Recovery of HVA Added to Urine**

<table>
<thead>
<tr>
<th>Added</th>
<th>Expected</th>
<th>Observed</th>
<th>Recovery</th>
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<tbody>
<tr>
<td>0</td>
<td>—</td>
<td>2.3 ± 0.1</td>
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</tr>
<tr>
<td>5</td>
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<td>7.1 ± 0.1</td>
<td>4.8</td>
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<tr>
<td>10</td>
<td>12.3</td>
<td>12.2 ± 0.1</td>
<td>9.9</td>
</tr>
<tr>
<td>15</td>
<td>17.3</td>
<td>17.5 ± 0.4</td>
<td>15.2</td>
</tr>
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* Average and range of duplicate determinations.
with those by a spectrophotometric method that involves extraction with organic solvent and reaction of HVA with 1-nitroso-2-naphthol. We found a discrepancy in our data for a urine from a patient with a surgically and histologically diagnosed neuroblastoma. Chromatographically, the value was 111 mg/L; it was 1.8 mg/L by the spectrophotometric procedure. Figure 2b shows a chromatogram of the urine as assayed by the HPLC procedure. Concerned by this discrepancy, we further verified the identity of the HVA peak by spectral scans. The excitation and emission spectra were identical to the scans of an HVA standard peak (Figure 3). The spectrophotometric assay was evaluated for linearity by assaying various dilutions of the urine from the patient with neuroblastoma. Dilutions of two- four- and 40-fold resulted in final calculated values of 32, 77, and 111 mg/L, respectively. Using pure HVA standards we find that the lack of linearity is the result of color fading or nonreactivity of 1-nitroso-2-naphthol as the concentration of HVA in the extracts increase.

As a more reliable method for comparison, we set up the procedure of Knight and Haymond (4), comparing results for 20 urine samples from normal individuals and from patients with neuroblastoma or who were being treated with leva-dopa. With the results of the chromatographic procedure on the y-axis, the regression equation is 

$$y = 0.81x - 1.8 \quad (r = 0.99).$$

**Discussion**

In 1965, Corrodi and Werdinius (9) reported that HVA is rapidly oxidized to a fluorescent compound (2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diacetic acid) by alkaline potassium ferricyanide. Fluorometric procedures were subsequently reported (10, 11) for measurement of HVA in urine, involving ion-exchange chromatography, oxidation by alkaline potassium ferricyanide, and fluorescent measurement. Hoeldtke (12) found that salicylate and an unidentified salicylate metabolite interfere with these fluorometric methods, even when aspirin is taken in fairly low dosage (600 to 900 mg). We have used this fluorometric reaction as a means of detecting HVA after reversed phase chromatography, with use of a simple post-column reaction system. Interference by aspirin medication is obviated by our chromatographic separation, and we believe that the unidentified metabolite of salicylate reported by Hoeldtke is salicylic acid.

We find that urine, after proper pH adjustment, can be assayed directly by our procedure. Acceptable precision is attained by our technique of filling the injection loop without the use of an internal standard. The analytical column is protected by a guard column. By repacking the guard column after each 50 to 70 injections we have experienced no major deterioration in column performance throughout the course of this study.

Comparison of our results with a procedure based on the reaction of HVA with 1-nitroso-2-naphthol resulted in a major discrepancy for a urine from a patient with neuroblastoma. The chromatographic procedure measured a value that was at least 10-fold our upper limit of normal while the spectrophotometric assay measured a normal value. Results of repeat analyses by both procedures were substantially the same as before. Non-linearity of the 1-nitroso-2-naphthol reaction has been previously reported (4), but the finding was based on unpublished data. We confirm this, and find that the final absorbance for an HVA concentration of 200 mg/L is the same as the absorbance for an HVA concentration of <10 mg/L. There was no non-linearity problem with the method of Knight and Haymond (4); in their method the 1-nitroso-2-naphthol-4 sulfonic acid forms a more stable colored product with HVA. Data by this method correlated with the chromatographic procedure, both for normal individuals and patients with neuroblastoma or on leva-dopa medication.

The major advantages of our assay include the limited sample preparation, the simplicity of our post-column reaction design, and the accuracy of the quantitation. The proposed method is an excellent alternative to spectrophotometric techniques for laboratories that have the appropriate instrumentation.

We are grateful to Dr. Hose Maccora (Mayo Clinic, Rochester, MN) for his assistance and suggestions during the initial phase of this study.

**References**


