Improved Colorimetry of Urinary 3-Methoxy-4-hydroxyphenylacetic Acid (Homovanillic Acid)

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Homovanillic acid is an important metabolite of dopamine, and a high proportion of patients with neuroblastoma excrete increased amounts of it in their urine. When this diagnosis is suspected, both homovanillic acid and vanilmandelic acid should be measured, because such a combined measurement reportedly leads to detection of 95% of cases. Although rapid and reliable chemical methods are available for vanilmandelic acid, the same is not true for homovanillic acid. We report here a colorimetric method for homovanillic acid that reasonably fills this void and that can be used in most clinical laboratories. In addition, we present normal values determined for children of various age groups.

Additional Keyphrases: pediatric chemistry · diagnosis of neuroblastoma · 3-methoxy-4-hydroxymandelic acid · 2-nitroso-1-naphthol-4-sulfonic acid as chromogen · normal values

In many instances, confirmation of a new or recurring neuroblastoma requires analysis for both vanilmandelic acid (VMA; 3-methoxy-4-hydroxymandelic acid) and homovanillic acid (HVA; 3-methoxy-4-hydroxyphenylacetic acid) in the urine. The former is reportedly present in above-normal amounts in 77% of cases, the latter in about 74%. With information from both tests, 95% of all cases can be detected (1, 2).

VMA can now be conveniently and reliably measured quantitatively in many clinical laboratories by methods that are relatively simple and require no special equipment (3, 4). The same is not true for HVA. The available methods lack sensitivity and are semiquantitative (5–7), involve techniques that are prolonged and relatively tedious (8, 9), or require equipment that is expensive and mandate special skills for interpretation (10, 11).

We describe here a relatively simple and rapid quantitative colorimetric method for urinary HVA. Used routinely in our laboratory for the past 20 months, it has proved to be both convenient and reliable.

Materials and Methods

Chemicals

Homovanillic acid, vanilmandelic acid, and tris(hydroxymethyl)aminomethane were from Sigma Chemical Co., St. Louis, Mo. 63178; chloroform from Mallinckrodt Inc., Los Angeles, Calif. 90058; and 2-nitroso-1-naphthol-4-sulfonic acid from K & K Laboratories, Inc., Plainview, N. Y. 11803, all in the purest grade available.

Procedure

Sample collection. Because of the difficulty in obtaining accurate 24-h urine samples from infants and children, untimed specimens were used. After an aliquot was taken for creatinine, the pH was promptly adjusted to less than 2.0 with HCl (6 mol/liter). Creatinine was determined the same day. The acidified samples were either refrigerated or frozen, depending on how promptly they were to be analyzed.

Extraction. We routinely run the test in duplicate. Into appropriately labeled tubes (polypropylene centrifuge tubes, 50 ml, cat. no. 25330; Corning Glass Works, Corning, N. Y. 14830) for blank, internal standard, and test, pipet the volume of urine containing 1 mg of creatinine, but not more than 4.5 ml. If this volume is exceeded, run the analysis on half the volume, and double the final result.

To each tube add 0.5 ml of 6 mol/liter HCl. To the internal standard tubes add 100 µl of a solution containing 200 mg of HVA per liter of 10 mmol/liter HCl (the equivalent of 20 µg of HVA).

Dilute the contents of all tubes to 5.0 ml with distilled water, add about 1.0 g of NaCl, and vortex-mix until it is dissolved. Add 30 ml of chloroform to each tube and shake vigorously for 5 min to extract the HVA (we used a mechanical shaker, the Eberbach Model 6000, variable speed). Centrifuge briefly to separate the layers, and aspirate the aqueous (top) layer and discard it. Transfer 25 ml of the chloroform solution to a second centrifuge tube with a 25-ml serological pipet. Add to each tube 2.0 ml of 41 mmol/liter tris(hydroxymethyl)-

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aminomethane (pH 8.5–9.0) and shake the tubes vigorously for 10 min. Centrifuge, remove the tris(hydroxymethyl)aminomethane (top) layer with a disposable Pasteur pipet, and pipet 1.6 ml into 1.0-cm cuvetts.

Chromogen formation. Add 0.4 ml of the color reagent (12 mmol/liter 2-nitroso-1-naphthol-4-sulfonic acid in 6.847 mol/liter ether alcohol) to each cuvet and mix. Then add 0.4 ml of 2 mol/liter HCl to the blank and 0.4 ml of nitrous acid solution (0.2 ml of 0.362 mol/liter NaNO2 in 5.0 ml of 2 mol/liter HCl, prepared just before use) to the internal standard and test.

Absorbance measurement and calculations. After addition of the color reagents, the contents of all tubes are mixed and the tubes are allowed to stand at room temperature for exactly 20 min, after which the absorbance (A) of the internal standard and test is determined at 500 mn, with the blank as reference. It is not necessary to remove the excess color reagent, because an appropriate blank is used. The concentration of the analyte is calculated as follows:

\[
\frac{A_T - A_S}{A_I - A_S} \times 20 = \mu g \text{HVA/mg creatinine}
\]

where \( A_T \) = absorbance of the test and \( A_S \) = absorbance of the internal standard (standard + test).

Tests that give results greater than about 100 \( \mu g \) of HVA per milligram of creatinine should be repeated with use of a smaller volume of urine, and the result multiplied appropriately.

Results and Discussion

Chemical Basis of the Reaction

The only present method for HVA measurement that appears to approach the criteria of ease and rapidity of analysis, adequate quantitation, and reasonable accuracy, and does not require costly equipment or special techniques, is based on the reaction between HVA and 1-nitroso-2-naphthol (12). This method apparently has been widely and successfully used for several years and presumably has given satisfactory information (13), although the complete procedure and its evaluation have never been published.\(^1\) This reagent, however, forms an unstable colored complex with HVA. Furthermore, the stability of the complex decreases with increasing HVA concentration. This requires special procedural observations and appropriate dilutions if one is to get reliable results, because the colored complex forms and then fades rapidly, especially when HVA concentration are greatly above normal.\(^2\)

The chemical basis of the reaction with 1-nitroso-2-naphthol is unknown, but presumably the nitroso group is the initiating force bringing about reaction. It is thought to be through an electrophilic attack by the activated nitroso group (=N+O\(^-\)) upon the aromatic

\(^1\) Bio-Science Laboratories, Van Nuys, Calif. 91405; personal communication.

\(^2\) Knight, J. A., and Haymond, R. E., unpublished results.

HVA nucleus and the para side-chain (–CH\(_2\)CO\(_2\)H), thereby forming the unstable colored complex, which is possibly of cyclic configuration.\(^2\) We reasoned that stabilization of the polarized nitroso group would enhance its reactivity and lead to a more stable complex. Hence a strong electron-withdrawing group (-SO\(_2\)H, -NO\(_2\), -CO\(_2\)H) placed appropriately on the 1-nitroso-2-naphthol molecule should improve the reaction. Therefore, 1-nitroso-2-naphthol-4-sulfonic acid was considered to be an excellent candidate. This compound is not commercially available, but 2-nitroso-1-naphthol-4-sulfonic acid is, and readily reacts to produce a relatively stable red complex that absorbs light maximally at 500 nm (Figure 1). The method is very sensitive, producing an absorbance of about 0.008–0.009 A/\( \mu g \) of HVA in the original sample. In addition, the standard curve is linear to at least 100 \( \mu g \) of HVA per milligram of creatinine.

Reaction Specificity

Essentially three different groups of chemical compounds of biological interest react with 1-nitroso-2-naphthol in the presence of nitric acid and (or) nitrous acid to produce generally unstable pink to purple complexes, which soon fade, leaving pale-yellow to colorless solutions. These include certain substituted hydroxyindoles (14, 15), selected para-substituted phenols (16, 17), and various guaiacol derivatives (12). The para-substituted phenols (tyrosine, tyramine, etc.) require nitric acid for reaction and therefore do not interfere here because only nitrous acid is present. The hydroxyindoles such as 5-hydroxyindole acetic acid likewise do not interfere because they react to produce
deep-purple or violet compounds (green with the color agent we used). More importantly, their concentrations are always essentially negligible in the patients under consideration.

The guaiacol derivatives in urine primarily include VMA and HVA. 1-Nitroso-2-naphthol does not react with VMA, but the color reagent we used does so. Its overall interference is insignificant, however, because (a) VMA is relatively insoluble in chloroform, less than 15% being extracted, and (b) it reacts with 2-nitroso-1-naphthol-4-sulfonic acid to give an absorbance that is about 33% of that for HVA in equimolar amount. This means that a urine containing four- to fivefold the normal concentration of VMA adds no more than 2 to 3 µg/mg of creatinine to the apparent total HVA.

The metanephrines also react, but even when their concentrations are greatly above normal they add little to the total concentration since their mean excretion by patients with phaeochromocytoma, is reportedly about 2.5 mg/24 h (18). 3-Methoxy-4-hydroxyphenylethyl-ene glycol also reacts, but is not appreciably extractable into chloroform, because humans excrete only 2 to 11% of this compound in unconjugated form (19).

Other nonreacting substances that may be present in urine include vanillic acid, vanillin, 3-methoxytyrosine, epinephrine, norepinephrine, glycerol guaiacolate, acetylsalicylic acid, phenacetin, and a variety of other substituted phenol and catechol derivatives.

Advantages

The current method has several distinct advantages over that in which 1-nitroso-2-naphthol is used (13):

1. The complex is more stable, resulting in a linear relationship between concentration and instrument response to at least 100 µg of HVA per milligram of creatinine. Beyond this, the curve begins to flatten. One will not, by this method, misinterpret a very high value as being normal.

2. It is more rapid and convenient, because it requires fewer reagents and extractions.

3. Chloroform extracts HVA from urine much more efficiently than does toluene (8). At 25 µg of HVA per milligram of creatinine, we note an average 88% extraction through the entire procedure when using chloroform. At 100 µg/mg, the absolute recovery is about 83%. In contrast, the recovery when toluene is used is reportedly in the 30–50% range (8).

4. The current technique requires no more than 27 ml of urine, usually less, depending on the creatinine concentration. This is often a more readily obtained volume than the 60 ml required in the other method—for example in the case of infants. In addition, this smaller volume of urine is easier to work with and extraction is more efficient.

5. The tris(hydroxymethyl)aminomethane/chloroform interface, in the second extraction, is much sharper than is true for the corresponding tris(hydroxymethyl)aminomethane/toluene mixture, where small buffer droplets are suspended in the toluene layer, sometimes making it difficult to get a clean separation.

Precision, Reliability, and Reference Values

Within-run and day-to-day reproducibility studies yielded means of 19.9 ± 1.0(SD) and 19.5 ± 1.7, respectively. The corresponding coefficients of variation were 5.0 and 8.7%.

We have used this method routinely during the past 20 months and find it to be both convenient and reliable. During this period, we have detected six new cases of neuroblastoma and analyzed frozen urine from five previous ones. Of these 11 cases, both the HVA and VMA were elevated in eight. In two, both were normal or borderline elevated; in each of these, the tumor was highly undifferentiated, a situation known to result in normal values occasionally (20). In one case, the VMA was increased and the HVA was normal. In none of these few cases have we seen an increased HVA with a normal VMA, although this situation is not uncommon (1, 2, 21). We did have three cases in which the VMA was moderately increased (two- to fourfold normal) while the HVA was markedly increased (up to 20-fold normal). In one other instance, both were greatly increased, but even here the HVA value was considerably higher than the VMA value (1000 vs. 213 µg/mg creatinine). Occasional cases without tumor have been seen in which the values were borderline or mildly above normal. This is not surprising, because the reaction is not completely specific. However, in every case of neuroblastoma that we have seen in which the HVA value was increased, it was at least twice the upper limit of normal. Where borderline-elevated values have been noted, and the patient had no tumor, assay results for repeat specimens were usually normal.

We have evaluated some urine samples from healthy children of different age groups (Table 1). Most of these

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Table 1. Normal Values for Urinary HVA in Children

<table>
<thead>
<tr>
<th>Age</th>
<th>1–12 months</th>
<th>1–2 years</th>
<th>2–5 years</th>
<th>5–10 years</th>
<th>10–15 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. subjects</td>
<td>42</td>
<td>42</td>
<td>55</td>
<td>48</td>
<td>42</td>
</tr>
<tr>
<td>Range</td>
<td>2.9–46.8</td>
<td>7.0–45.6</td>
<td>1.7–34.3</td>
<td>0.6–21.8</td>
<td>1.6–26.8</td>
</tr>
<tr>
<td>Mean</td>
<td>23.7</td>
<td>23.1</td>
<td>15.9</td>
<td>10.0</td>
<td>8.3</td>
</tr>
<tr>
<td>SD</td>
<td>10.2</td>
<td>9.2</td>
<td>6.4</td>
<td>4.7</td>
<td>4.9</td>
</tr>
<tr>
<td>Normal range</td>
<td>3.3–44.1</td>
<td>4.7–41.5</td>
<td>3.1–28.7</td>
<td>0.6–19.4</td>
<td>0–18.1</td>
</tr>
</tbody>
</table>

(±2 SD)

* Values expressed as micrograms of HVA per milligram of creatinine.
children were admitted for elective surgery and samples were obtained before the operative procedure. We chose to measure HVA in untimed specimens, because reliable 24-h urine samples are almost impossible to obtain in infants and children. The normal values indicated here compare favorably with those previously reported by a different method (22), although ours are a little higher. It is seen that the concentration of HVA, expressed as micrograms per milligram of creatinine, decreases with the subjects' age, being particularly high during the first two years. Preliminary data suggest that normal values may be up to 50–55 μg/mg of creatinine during the first two to three postnatal months (most of the children studied in this group were six to 12 months old).

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References