Urinary Homogentisic Acid: Determination by Thin-Layer Chromatography

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A new method is described for qualitative and quantitative determination of urinary homogentisic acid. The method involves extraction of urine with ethyl acetate, thin-layer chromatography of the extract on Silica Gel G, elution of the homogentisic acid into water, and color development with Folin's phenol reagent. Absorbance is maximum at 750 nm and linear to a concentration of at least 5 mg of homogentisic acid per milliliter of urine. The method is highly specific for homogentisic acid; added gentisic acid, 3,4-dihydroxyphenylacetic acid, ascorbic acid, or L-3,4 dihydroxyphenylalanine do not interfere. The coefficient of variation "in-run" is 5.3%, "between-run" 6.8%. Using this method we have demonstrated a marked variation in day-to-day homogentisic acid excretion in a patient with alcaptonuria. This method, which offers some advantages over existing techniques, should be suitable for use in a clinical laboratory.

Additional Keyphrases: alcaptonuria • inherited metabolic disorders • day-to-day variation

Alcaptonuria, a rare hereditary metabolic disorder, is caused by a lack of the enzyme homogentisate oxidase (EC 1.13.1.5). Clinical features that suggest this diagnosis include arthritis, passage of urine that darkens on standing, and pigmentation of connective tissue and cartilage. The suspected diagnosis is confirmed by demonstrating the presence of homogentisic acid in the patient's urine.

Many tests for homogentisic acid have been described, but these procedures usually depend on the reducing properties of this compound and are thus not entirely specific (1–5). A published specific enzymatic procedure (6) is more suitable for research than for a clinical laboratory (6). The present report describes a method suitable for the qualitative and quantitative determination of homogentisic acid in the clinical laboratory.

Materials

We used a Model 2000 Photometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio 44074) equipped with a Beckman DU Model 2800 Monochromator and a tungsten lamp. Thin-layer chromatography plates were prepared with equipment obtained from Brinkmann Instruments, Inc., Cantiague Road, Westbury, N. Y. 11590. This equipment included 20 cm x 20 cm glass chromatography plates, the Desaga/Brinkmann TLC Applicator, Brinkman multiplate TLC tanks, aerosol reagent spray cans, and the Heraeus Drying Oven. After the preparation, Silica Gel G (0.25 mm thick) thin-layer chromatography plates were activated for 30 min at 110°–120°C in the oven.

Homogentisic acid, gentisic acid, ascorbic acid, 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylalanine (L-DOPA), and Silica Gel Type G were obtained from the Sigma Chemical Co., St. Louis, Mo. 63178. Phenol Reagent Solution (Folin–Ciocalteu), was obtained from Fisher Scientific Co., 711 Forbes Ave., Pittsburgh, Pa. 15219, and used as received, except that it was diluted with an equal volume of water. All other reagents used were of reagent grade. De-ionized water was used.

Methods

Standard solutions of homogentisic acid were prepared by dissolving the appropriate quantity of homogentisic acid in water. Two milliliters of standard solution or of urine were placed in 15-ml glass stoppered centrifuge tubes, acidified with hydrochloric acid (12 mol/liter) to a pH of 1 (pH testpaper), then saturated with sodium chloride. Four milliliters of ethyl acetate was added and the tubes were shaken vigorously for 2.5 min, either by hand or with a wrist-action shaker (Burrell Corp., Pittsburgh, Pa. 15219). The phases were allowed to separate and 20 μl of the ethyl acetate (upper) layer was spotted on a thin-layer chromatography plate. Six or seven samples were applied to each plate, including at least one standard solution of homogentisic acid. The plates were placed in a chromatography vessel that
contained about 100 ml of the upper layer of a mixture of benzene:acetic acid:water (2:3:1, by vol). Development was stopped when the solvent front had advanced 15 cm (time required, 30 to 50 min).

Although a single development of the plates usually gave adequate resolution, multiple development was used if the compounds under study had similar \( R_f \) values (e.g., homogentisic acid and DOPAC). In this technique, the chromatogram is developed, removed from the chamber, dried, and redeveloped in the same solvent.

After air drying 15 min, the plates were lightly sprayed, first with Folin's reagent and then with a solution of sodium carbonate (100 g/liter). The compounds of interest were visible as blue spots, and their \( R_f \) was recorded. The spots were scraped off with a spatula onto glassine paper and transferred to a 13 \( \times \) 100 mm test tube. An appropriate nonstained area of Silica Gel served as a blank.

At this point the test tubes containing the silica gel could either be stored (in the refrigerator or freezer) for one to three days, or one could proceed with the analysis, depending on the schedule of the laboratory. When ready to proceed, 2 ml of water is added to each tube, and the tubes periodically mixed during the following 3-h elution period. After the tubes are centrifuged at 2000 rpm for 10 min, 1 ml of supernatant fluid is removed and placed in a clean 13 \( \times \) 100 mm tube. Folin's reagent, 250 \( \mu \)l, and 1 ml of sodium carbonate solution (200 g/liter) are added and the rack of tubes is placed in a preheated 95\(^\circ\)C water bath for 1 min. After the tubes are cooled to room temperature, they are again centrifuged at 2000 rpm for 10 min and the absorbances of the supernatant fluids at 750 nm are determined. The spectrophotometer is set at zero absorbance with the reagent blank prepared from an unstained portion of the Silica Gel G plate. The absorbance of this reagent blank is very close to that of de-ionized water.

**Results**

**Chromatographic Separation**

The \( R_f \) of homogentisic acid averaged 0.17, ranging day to day from 0.13 to 0.20. Thus, it is essential that a homogentisic acid standard be included on each plate, because daily variation in \( R_f \)'s on thin-layer plates is greater than is the case with paper chromatograms. The present chromatographic system adequately separates homogentisic acid from the following aromatic acids (listed in order of increasing \( R_f \) values): 3-hydroxykynurenic, 3,4-dihydroxymandelic, 3-methoxy-4-hydroxymandelic, homogentisic, DOPAC, 3-hydroxyanthranilic, salicylic, gentisic, p-hydroxyphenylpyruvic, 5-hydroxyindoleacetic, caffeic, p-hydroxyphenylacetic, p-hydroxybenzoic, indolelactic, indoleacetic, indoleacrylic, homovanillic, vanillic, and ferulic acid.

**Absorption Spectra**

Figure 1 shows the absorption spectra of 2 mg and 4 mg of homogentisic acid standard solutions taken through the extraction and chromatographic procedure. Absorbance is maximal at 750 nm.

**Recovery**

To determine the efficiency of the extraction procedure, we dissolved homogentisic acid (2 or 3 mg/ml) in either water or the urine of normal subjects, which was then extracted with ethyl acetate. After chromatography, elution, and color development, the absorbance was measured at 750 nm, and the results compared with those obtained when homogentisic acid was directly dissolved in ethyl acetate and the remainder of the analytical procedure carried out. Recovery of homogentisic acid from water and urine after extraction was similar, and ranged from 81 to 97% (91 \pm 5.3% SD).

To determine the approximate loss during chromatography, we dissolved homogentisic acid in ethyl acetate, chromatographed, eluted into water, and after color development the absorbance was measured and compared to the absorbance obtained when the ethyl acetate solution of homogentisic acid was placed directly into a tube, the ethyl acetate evaporated, water added, and color developed. The absorbance of the material obtained after chromatography was 95% that of the material directly transferred. This may not represent a precise recovery, for it was necessary to spray the plates with Folin's reagent to identify the homogentisic acid spot before it was removed.

Although the recovery of homogentisic acid during this procedure was acceptable (80–90%), we corrected for this loss by taking standard solutions of homogentisic acid through the entire extraction and chromatographic procedure along with the urine samples being analyzed. The remainder of the calculations are made with use of such standards.

**Calibration Curve**

Figure 2 shows the calibration curve obtained when standard solutions of homogentisic acid were taken through the extraction and chromatographic procedure.
procedure. Absorption is linear to at least a 5 mg/ml concentration. Because the calibration curve is linear, it is necessary to include only one or two standard solutions in each analytical run.

Specificity

Urine from normal subjects contained no measurable homogentisic acid. Table 1 shows that the following compounds do not interfere with recovery of homogentisic acid: gentisic acid, 3,4-dihydroxyphenylacetic acid (DOPAC), ascorbic acid, or L-DOPA.

Precision

To determine the precision of the method, we prepared multiple aliquots of urine from a 24-h urine from a 46-year-old-man with alcaptonuria. The “within-run” mean 24-h urinary homogentisic acid excretion was 3428 mg (SD, 181; CV, 5.3% for 20 replicate samples). “Between-run” precision, determined on different days, was 6.8%.

Homogentisic Acid Excretion in Alcaptonuria

Table 2 shows the 24-h urinary homogentisic acid excretion by a 46-year-old man with alcaptonuria. There is a 2.5-fold variation in amount excreted per 24-h (4.932 g to 1.984 g). This spontaneous variation in homogentisic acid excretion in alcaptonuria has previously been noted, and it must be kept in mind when one is evaluating therapeutic approaches to alcaptonuria (7).

To test the stability of homogentisic acid during prolonged storage, urine samples were acidified to pH 3 and stored frozen for nine months. The amount found in the sample of March 4, 1972, was only 9% less after nine months of storage (4.932 g to 4.489 g); that found in the sample of March 16, 1972, was only 4% less after nine months (3.846 g to 3.703 g). This stability is surprising in view of the ease with which homogentisic acid can be oxidized. In the only previous study of the stability of this compound during storage, there was no measurable loss of homogentisic acid from frozen acidified urine samples for at least one week (6).

Discussion

Chemical techniques for determination of urinary homogentisic acid usually depend on the potent reducing properties of this compound. Reduction of silver nitrate by homogentisic acid in an alkaline medium was one of the earliest methods used, but it was not specific for homogentisic acid as silver nitrate is also reduced by urinary uric acid (1).

The second analytical method used depended on the reduction of phosphomolybdic acid in an acidic solution (2). Phosphomolybdic acid is also reduced by p-hydroxyphenylpyruvic acid, L-DOPA, and ascorbic acid (3).

Homogentisic acid can reduce iodide, and this furnished a third approach to its analysis. However, gentisic acid, hydroquinone, catechol, and L-3,4-DOPA can interfere with this analytical method, for they also reduce iodide (4).

In a widely used technique, homogentisic acid is extracted from urine with ethyl ether, followed by reduction of silver by homogentisic acid at pH 4.4 in the presence of colloidal gold (4). This laborious method is only moderately accurate, and despite the use of a 2-h time curve, there is potential interference by gentisic acid (4).

Homogentisic acid can be quantitatively measured by paper chromatography, but this method is tedi-

<p>| Table 1. Measurement of Added Homogentisic Acid (2 mg/ml) in Urine in the Presence of Other Compounds |</p>
<table>
<thead>
<tr>
<th>Other compound</th>
<th>Homogentisic acid found (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentisic acid</td>
<td>2.02</td>
</tr>
<tr>
<td>Gentisic acid</td>
<td>1.71</td>
</tr>
<tr>
<td>DOPAC</td>
<td>2.03</td>
</tr>
<tr>
<td>DOPAC</td>
<td>1.97</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>2.16</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>2.09</td>
</tr>
</tbody>
</table>

* Concentration: 2 mg/ml of urine, 20 μl of extract chromatographed except in the case of DOPAC. Because the Rf for homogentisic acid (Rf 0.17) and DOPAC (Rf 0.26) are similar, only 5 μl of ethyl acetate extract was chromatographed so we could obtain smaller spots with complete resolution between the compounds.

| Table 2. 24-h Urinary Homogentisic Acid Excretion by a Patient with Alcaptonuria |
|------------------|-----------------------|
| Date of 24-h collection | Homogentisic acid (g/24 h) |
| 2/28/72           | 2.646                 |
| 3/4/72            | 4.932                 |
| 3/7/72            | 4.090                 |
| 3/16/72           | 3.846                 |
| 5/8/72            | 1.984                 |
| 10/22/72          | 4.347                 |
ous and its specificity not well defined (5). Seegmiller et al. (6) described an extremely specific and sensitive method based on the enzymatic conversion of homogentisic acid to maleylacetoacetic acid with purified homogentisate oxygenase. Homogentisate oxygenase is not commercially available, and its isolation and purification are complex procedures for a clinical laboratory. Because of deterioration during storage, the enzyme must be periodically repurified from rat liver. This enzymatic method of analysis is most suitable for a research laboratory specializing in metabolic disorders such as alcaptonuria.

We were recently consulted about two patients with suspected alcaptonuria. Both patients had arthritis, a common finding in alcaptonuria, and their urine samples became black after alkalization with sodium hydroxide. One patient was receiving aspirin therapy, and a salicylate metabolite, gentisic acid, was responsible for darkening the urine. The other patient was receiving L-DOPA for Parkinson's disease, and DOPAC, a metabolite of L-DOPA, produced the black urine. DOPAC excretion has already caused misleading reports about the presence of homogentisic acid in the urine of patients receiving L-DOPA (8). Thus, specificity is an important prerequisite for a satisfactory test for alcaptonuria. The present technique has the required specificity, for it distinguishes homogentisic acid, gentisic acid, and DOPAC.

The present method is a modification of a technique previously described for determining urinary homovanillic acid (9). It is not only specific and precise, but quite convenient. Folin's phenol reagent is commercially available and has a long shelf life. Although we prepare our own thin-layer chromatography plates, excellent premade plates can be purchased from commercial sources. Because of increasing drug abuse, the necessary thin-layer chromatography techniques are already in use in many clinical laboratories for the detection of urinary drug metabolites.

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References