An Enzymatic Spectrophotometric Method for the Determination of Homogentisic Acid in Plasma and Urine

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Previous methods for the determination of homogentisic acid have been based upon its ability to reduce silver (1), phosphomolybdic acid (2), or iodine (3-5). The error introduced by other biological substances which have similar reducing properties is inconsequential in the determination of the relatively large amounts of homogentisic acid present in urine from a patient with alcaptonuria. Such errors, however, are substantial in the determination of the relatively low concentrations of homogentisic acid in the plasma of alcaptonuric patients. Some attempts were made to gain greater specificity by preliminary extraction of homogentisic acid into ether, and by the use of an iodometric method which took advantage of the increase in reduction of silver by homogentisic acid at pH 4.4 in the presence of colloidal gold to estimate levels of homogentisic acid in plasma. Nevertheless, these methods admittedly lacked specificity and precision when applied to alcaptonuric plasma.

We have developed a simple and rapid enzymatic spectrophotometric method for the determination of homogentisic acid which uses partially purified homogentisic acid oxidase and detects as little as 1 µg of homogentisic acid. This method has been used to determine the plasma levels of homogentisic acid in patients with alcaptonuria, the diurnal variation in this level, and the changes produced by feeding phenylalanine.

EXPERIMENTAL PROCEDURE

Principle—Homogentisic acid is oxidized by homogentisic acid oxidase to maleylacetoacetic acid. The latter product absorbs ultraviolet light with a molar extinction coefficient of 330 µM of 13,500 (7, 8). Homogentisic acid oxidase is specific for homogentisic acid as a substrate (gentisic acid, homogentisic acid lactone, and esters of the acid are not oxidized) (9, 10). The Michaelis-Menten constant, determined in our laboratory, is approximately 1.07 × 10⁻⁴ M. The high affinity permits the determination of small amounts of homogentisic acid and promotes a rapid and complete conversion of substrate to maleylacetoacetic acid.

Homogentisic Acid—The routine use of a standard homogentisic acid solution with each set of determinations is not necessary. However, a freshly prepared standard solution of the acid is used to check the activity of the homogentisic acid oxidase during purification of the enzyme and to assay the activity of stored enzyme preparations.

Purified homogentisic acid lactone, obtained from the California Corporation for Biochemical Research, was assayed spectrophotometrically with homogentisic acid oxidase, and spectrophotometrically after enzymatic hydrolysis of the lactone.¹ The analyses agreed closely, within 5% of the molar extinction coefficient of 13,500 at 330 µM (7, 8). Homogentisic acid was isolated from the urine of an alcaptonuric patient and purified (11). The crystalline homogentisic acid monohydrate had a melting point of 150-152°. The acid was also obtained commercially from the Cyclo Chemical Corporation. The purity of these preparations, based upon the molar extinction coefficient of maleylacetoacetic acid, varied from 85 to 93%.

Albumin Solution—An aqueous solution of crystalline bovine plasma albumin, purchased from the Armour Laboratories, containing 4.0 mg per ml is routinely added to the assay system to stabilize homogentisic acid. In the assay of plasma this reagent is not necessary.

Sodium Phosphate Buffer, pH 6.5—The optimal pH for homogentisic acid oxidase activity is slightly above 7.0 (9, 12), but a lower pH, 6.5, is preferable, since the acid is less susceptible to nonenzymatic oxidation to a melanin-like product.

Homogentisic Acid Oxidase—The method for the purification of homogentisic acid oxidase from rat liver is essentially the same as that described by Knox and Edwards (7) (see (8) also). In the alcohol fractionation step, the fraction that precipitates between 12 and 25% ethanol is saved and washed with 25% ethanol. In the next step, precipitation with ammonium sulfate, the fraction that precipitates between 40 and 70% saturation is saved. The precipitate is dissolved in a 1% aqueous solution of mercuric chloride and stored in evacuated tubes at -10°. The tubes of enzyme are reevacuated and frozen each time after use, and frozen preparations maintain nearly complete activity for several months. The specific activity of rat liver enzyme prep-

¹ Homogentisic acid lactone was converted to the free acid by incubation with human plasma which contains lactonase activity.
rations varies, but it is usually between 20 and 40 units per mg of protein. One unit is defined as an increase of 0.010 optical density units at 330 μm in one minute under the conditions of assay described below at 25°, with homogentisic acid as the substrate.

Enzyme preparations should be essentially free from maleylacetoacetic acid isomerase activity. This can be readily determined by observing the stability of the product, maleylacetoacetic acid, at 330 μm after its formation from homogentisic acid. Homogentisic acid oxidase preparations may be slightly contaminated with p-hydroxyphenylpyruvic acid oxidase activity, and this contaminant would allow p-hydroxyphenylpyruvic acid to be oxidized to maleylacetoacetic acid if the α-keto acid were present in the biological material to be analyzed. Interference by p-hydroxyphenylpyruvic acid can be prevented by the addition of 0.001 M diethylthiocarbamate which completely inhibits p-hydroxyphenylpyruvic acid oxidase (13) without blocking homogentisic acid oxidase activity.

Method

Plasma Homogentisic Acid—Spectrophotometric measurements are made with a Beckman model DU spectrophotometer with ultraviolet attachment in 4 ml silica cells with a 10 mm light path. The control and experimental cuvettes contain 2.0 ml of 0.2 M sodium-phosphate buffer, pH 6.5, 0.47 ml of water, and 0.50 ml of plasma. An initial reading is taken at 330 μm and 0.03 ml of water and 0.03 ml of homogentisic acid oxidase (approximately 10 units) are added to the control and experimental cuvettes, respectively. Readings are taken at half-minute or minute intervals until the maximal absorption is reached.

The maximal optical density reading must be corrected for the slight absorption caused by homogentisic acid oxidase, and the value of this correction is determined by measuring the absorption of the enzyme, with the substitution of water for plasma in the above method. In most enzyme preparations the correction is less than 0.050. However, as enzyme preparations age, turbidity increases and centrifugation may be necessary to clarify the enzyme solution.

The corrected optical density reading divided by 0.0268 is equal to the number of μg of homogentisic acid in the plasma aliquot analyzed. The value, 0.0268, is the optical density change per μg of homogentisic acid oxidized to maleylacetoacetic acid under the above experimental conditions.

Urine Homogentisic Acid—Determination of homogentisic acid in urine is carried out essentially the same way as in plasma. However, urine from alcaptonuric subjects contains several mg of homogentisic acid per ml and must therefore be diluted until the aliquot to be analyzed contains between 5 and 25 μg. Addition of 0.05 ml of the 0.4% bovine plasma albumin solution to both the control and experimental cuvettes is made to stabilize homogentisic acid.

RESULTS AND DISCUSSION

Recovery of Homogentisic Acid Added to Urine and Plasma—Various amounts of homogentisic acid, between 2.5 and 20 μg,
The patients were given 0.1 g. L-phenylalanine per kg of body weight at 9:00 a.m. Urine samples were analyzed by the Briggs method (2).

### Table III

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time 6-26</th>
<th>Concentration of homogentisic acid</th>
<th>Extra homogentisic acid excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spectrophotometric method</td>
<td>Briggs method</td>
<td>g of homogentisic acid excreted/24 hrs</td>
</tr>
<tr>
<td>G. W.</td>
<td>9:00 a.m.</td>
<td>8.7</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>11:00 p.m.</td>
<td>20.5</td>
<td>25.4</td>
</tr>
<tr>
<td></td>
<td>1:00 p.m.</td>
<td>56.6</td>
<td>65.5</td>
</tr>
<tr>
<td>J. W. (son)</td>
<td>4:00 p.m.</td>
<td>51.6</td>
<td>68.8</td>
</tr>
<tr>
<td>D. W. (son)</td>
<td>9:00 a.m.</td>
<td>&lt;1.0</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>11:00 a.m.</td>
<td>&lt;1.0</td>
<td>13.3</td>
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<tr>
<td></td>
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<td>13.3</td>
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<tr>
<td></td>
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<td>&lt;1.0</td>
<td>13.3</td>
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### Table IV

<table>
<thead>
<tr>
<th>Patient</th>
<th>6-25 to 25</th>
<th>6-26 to 27*</th>
<th>6-27 to 28</th>
<th>Extra homogentisic acid excreted</th>
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</thead>
<tbody>
<tr>
<td>G. W.</td>
<td>3.55</td>
<td>7.91</td>
<td>12.91</td>
<td>3.75</td>
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<tr>
<td>J. W. (son)</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>D. W. (son)</td>
<td>0.19</td>
<td>0.06</td>
<td>0.14</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* Day of urine collection after oral administration of phenylalanine.

than 5%, and the precision of the method is adequate for alcaptonuric urine and for most samples of alcaptonuric plasma (0.5 ml or 1.0 ml analyzed, contains at least 2 μg). To increase the precision in analyses of plasma with low concentrations of homogentisic acid, the amount of plasma analyzed per cuvette can be increased to 1.5 ml if a photomultiplier attachment is used. Higher sensitivity may also be obtained by using the semi-micro modification in which the total liquid volume per cuvette is 1.0 ml.

Plasma which has been frozen at -10° for one week loses about 25% of its homogentisic acid content, and the loss is only slightly less if ascorbic acid (0.001 M) has been added as an antioxidant. Frozen acidified urine samples, however, maintain homogentisic acid without any detectable loss for at least one week.

**Homogentisic Acid Levels in Normal and Alcaptonuric Plasma**—Numerous assays performed on plasma from many nonalcaptonuric individuals have failed to demonstrate homogentisic acid. If this acid is present in normal plasma, its concentration must be less than 1 μg per ml, the lower limit of detection by this method. Homogentisic acid added to normal plasma can be completely recovered even after several hours, and the failure to find homogentisic acid in normal plasma is not caused by its enzymatic removal by homogentisic acid oxidase.

The concentration of homogentisic acid in the plasma of six alcaptonuric individuals varied considerably, and values ranged from 5.0 to 11.0 μg per ml in the fasting state (Table I). The values gradually increased during the day, and an example of the diurnal variation in the plasma concentrations in a 38-year-old male alcaptonuric patient on a regular hospital diet is illustrated in Table II.

Calculation of the renal clearance of homogentisic acid in this patient (Table II) indicates that this acid is secreted by the kidney. This finding is in agreement with earlier conclusions of Neuberger et al. (11) based upon renal clearance studies in a 7-year-old alcaptonuric girl.

**Effect of Feeding Phenylalanine to Alcaptonuric and Non-alcaptonuric Subjects**—It seemed probable that the diurnal variation observed was caused by the catabolism of phenylalanine and tyrosine in the diet, since these amino acids are the only known precursors of homogentisic acid. Experiments were, therefore, carried out to determine the effect of feeding phenylalanine to an alcaptonuric patient and his two nonalcaptonuric sons. L-Phenylalanine, 0.1 g per kg of body weight, was administered orally after being mixed with ice cream, and plasma samples were taken at various intervals for homogentisic acid analyses. The results of these studies are summarized in Table III. Plasma levels of homogentisic acid determined by the specific enzymatic method were about 10 μg per ml lower than when determined by the nonspecific chemical method of Briggs (2), based upon the reduction of molybdate. In addition, the results indicate that there was no detectable homogentisic acid in the plasma of the two sons of the alcaptonuric patient after ingestion of phenylalanine. Since there is general agreement that alcaptonuria is inherited as a recessive trait (10, 14), children of an alcaptonuric subject should theoretically be carriers of the disease. Homogentisic acid oxidase is missing in alcaptonuric liver (15), and it might be expected that carriers of the abnormal gene would have a reduced amount of enzyme activity as do carriers of phenylketonuria (16–18); this deficiency might be revealed by a suitable loading test.

Urine analyses also showed that the alcaptonuric subject excreted nearly all of the phenylalanine ingested as extra homogentisic acid, but no homogentisic acid was excreted by the presumably heterozygous nonalcaptonuric sons. Possibly a tolerance test with homogentisic acid itself would be a more sensitive means of showing a defect in the rate of metabolism in carriers of alcaptonuria. A tolerance test based upon this principle is now being evaluated.

### Summary

An enzymatic spectrophotometric method has been described for the specific and quantitative determination of homogentisic acid in plasma and urine. The method is based upon the enzymatic conversion of homogentisic acid to maleylacetoacetic acid with purified homogentisic acid oxidase, and the absorption of the resulting product is measured at 330 μm. The method has been applied to the analysis of plasma and urine of normal and alcaptonuric individuals and the effect of feeding phenylalanine on these levels has been determined.

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