A Gas Chromatographic Procedure for Detection of Pathological Organic Aciduria

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A number of nonamino organic acids associated with inborn errors of metabolism can be detected by gas chromatography. The organic acids are extracted into ethyl acetate and diethyl ether, the extracts combined, and the solvents evaporated under nitrogen. The residue is taken up in carbon disulfide and an aliquot chromatographed on 5% neopentyl glycol adipate to separate short-chain fatty acids (C2 to 6). The remaining carbon disulfide is evaporated, the residue taken up in methanol, and the acids are methylated with diazomethane. The solvent is evaporated, the residue taken up in tetrahydrofuran, and an aliquot chromatographed on 15% diethylene glycol succinate to separate keto, hydroxy, and dicarboxylic acids. Chromatographic patterns for normal and abnormal urines have been established. Four cases of congenital methylmalonic aciduria have been diagnosed by this procedure.

Detection of short-chain nonamino acids in urine is an important laboratory resource for the diagnosis of inborn errors of metabolism, including congenital methylmalonic aciduria (1), isovaleric acidemia (2), propionic acidemia (3), green acyl dehydrogenase deficiency (4), and maple syrup urine disease (5). Several chromatographic techniques have been used to detect these acids (1–4, 6, 7) but to date no single procedure could be used for all. The following simple, rapid gas chromatographic procedure has been developed to obviate this problem, thus enabling screening for these and similar conditions.

Materials and Methods

Reagents

(a) Sodium chloride, A.G.
(b) Hydrochloric acid, 2 mol/liter.
(c) Ethyl acetate, “G.C.-Spectrophotometric” grade.
(d) Diethyl ether, anhydrous, A.G.
(e) Sodium sulfate, anhydrous, A.G.
(f) Methanol, “Nanograde” (Mallinckrodt Chem. Co., St. Louis, Mo.).
(g) N - Methyl - N’ - nitro - N - nitrosoguanidine (Aldrich Chem. Co., Inc., 2371 N. 30th St., Milwaukee, Wis.).
(h) Potassium hydroxide, 60 g/100 ml of water.
(1) Ethereal diazomethane, freshly prepared daily by bubbling nitrogen through a mixture containing 5 ml of reagent d, 5 ml of reagent h, and 200 mg of reagent g. The diazomethane evolved is collected into 50 ml of ethyl ether in a bottle surrounded by solid carbon dioxide. The procedure should be carried out in a well ventilated fume hood and the diazomethane should not be stored in bottles with ground-glass stoppers. We have not found it necessary to purify the reagent by distillation—a procedure made dangerous by its explosive nature.
(j) Tetrahydrofuran, redistilled over ferrous sulfate. Of a total of 500 ml, the first 50 ml and the final 100 ml of distillate should be discarded. Commercially available solvent contains 25 mg of butylated hydroxytoluene per 100 g, as a stabilizer; this gives rise to peaks that interfere with gas chromatography of organic acids.
(k) Carbon disulfide, “spectrophotometric” grade.
(l) Neopentyl glycol adipate, 5% with 2% phosphoric acid on 100–120 mesh Chromosorb W, acid-washed and dimethyl dichlorosilane-treated (Analabs, Inc., P.O. Box 501, North Haven, Conn).
(m) Diethylene glycol succinate, 15%, on 45–60
mesh Chromosorb W, acid-washed and dimethyl dichlorosilane-treated (same source as l).

Apparatus

(a) Test tubes, glass-stoppered, 16 × 150 mm.
(b) Test tubes, centrifuge, glass-stoppered, 17 × 136 mm.
(c) Stainless steel or glass columns, U-shaped, 8 ft. × 1/4 in. o.d.
(d) Dual-column gas chromatograph, Barber-Colman Model 5000, with dual-flame ionization detectors.

Procedure

Extraction of urine. Specimens of urine are stored at −20°C. One milliliter of urine is acidified to pH 1 with one or two drops of 2N hydrochloric acid, saturated with sodium chloride, and extracted twice with 3 ml of ethyl acetate and once with 3 ml of diethyl ether in a glass-stoppered test tube. The extracts are pooled, dried by shaking with anhydrous sodium sulfate, and the solvents evaporated at room temperature under a stream of nitrogen. The residue is dissolved in 100 μl of carbon disulfide, and 2 μl is used for chromatography on Neopentyl glycol adipate (see below). The remaining carbon disulfide is evaporated under nitrogen, the residue taken up in 100 μl of methanol, and ethereal diazomethane added until a persistent yellow color remains. The solvents are then evaporated carefully at room temperature, to avoid losses of volatile methyl esters, and the residue is redissolved in 100 μl of tetrahydrofuran for chromatography on the column of diethylene glycol succinate.

Gas chromatography. Two microliters of the carbon disulfide-soluble residue (the free acids) is injected onto the column of 5% neopentyl glycol adipate. The injector temperature is 180°C, detector temperature is 200°C, and the column temperature is programmed to rise 5°C/min in the range 100°C to 150°C.

Two microliters of tetrahydrofuran solution (the methyl esters) is injected onto the column of 15% diethylene glycol succinate. Both injector and detector temperatures are 200°C, and column temperature is maintained at 75°C for 5 min, after which it is programmed to rise 5°C/min to 200°C.

The carrier gas for both columns is nitrogen, the flow rate 45 ml/min.

Results and Discussion

Resolution of a standard mixture of short-chain fatty acids up to C-6, both straight and branched-chain, is shown in Figure 1. Normal urine shows only acetic acid with an occasional minor peak of propionic acid, and any marked deviation from this pattern is considered pathological. In particular, the acidurias characteristic of isovaleric acidemia, green acyl dehydrogenase deficiency (butyric and hexanoic aciduria), and propionic acidemia are easily differentiated from the normal pattern and from one another.

Table 1 shows retention data obtained for a number of methylated hydroxy, keto, and dicarboxylic acids on 15% diethylene glycol succinate under isothermal conditions. Normal urine occasionally shows significant peaks of methyl-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvic acid</td>
<td>0.20</td>
</tr>
<tr>
<td>α-Ketoisovaleric acid</td>
<td>0.21</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.24</td>
</tr>
<tr>
<td>α-Hydroxyisovaleric acid</td>
<td>0.32</td>
</tr>
<tr>
<td>β-Hydroxyisovaleric acid</td>
<td>0.34</td>
</tr>
<tr>
<td>α-Ketoisocaproic acid</td>
<td>0.36</td>
</tr>
<tr>
<td>Acetoacetic acid</td>
<td>0.45</td>
</tr>
<tr>
<td>α-Ketocaproic acid</td>
<td>0.55</td>
</tr>
<tr>
<td>Methylnalconic acid</td>
<td>0.55</td>
</tr>
<tr>
<td>β-Hydroxybutyric acid</td>
<td>0.56</td>
</tr>
<tr>
<td>α-Hydroxisocaproic acid</td>
<td>0.57</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>1.00</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>1.17</td>
</tr>
</tbody>
</table>

* All compounds as the methyl esters. Column: 15% DEGS on 45–60 Chromosorb W. Temperature: 75°C isothermal.
pyruvate and methyllactate as well as several minor unidentified components (Figure 2). The chromatogram obtained from the urine of an infant seven days old, with severe metabolic acidemia due to congenital methylmalonic aciduria, is shown in Figure 3. The major peak corresponds to the methyl ester of methylmalonic acid; in addition, large amounts of β-hydroxyisovaleric acid are present. The latter has been found in the urine of another patient with this disorder and its significance is currently under investigation. Urine from patients with maple syrup urine disease (branched-chain ketonuria) and diabetic ketoacidosis have also been chromatographed and are easily distinguished from normal urine.

If an abnormal chromatogram is obtained, other techniques (e.g., infrared spectrometry, mass spectrometry, or chromatography on other column supports) should be used for positive identification. For example, it is difficult with this system to differentiate between the methyl esters of β-hydroxybutyric acid (in ketoacidosis) and methylmalonic acid (in congenital methylmalonic aciduria and vitamin B_{12} deficiency). In this instance, either chromatography of trimethylsilyl derivatives on 3% XE-60 or mass spectrometry are useful in establishing positive identification.

We emphasize that this procedure provides a rapid (2 h) means of screening for a group of clinically indistinguishable and often treatable disorders. In this context, an inability to identify an abnormal compound positively represents only a minor inconvenience.

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References