Gas-Chromatographic-Mass-Spectrometric Determination of Urinary Acid Profiles of Normal Young Adults on a Controlled Diet

Thomas A. Witten, Steven P. Levine, Julia O. King, and Sanford P. Markey

Urinary acid metabolic profiles of 21 healthy young adults who were maintained on a palatable standard diet for three days have been studied by combined gas chromatography-mass spectrometry. Means and standard deviations of the excretion rates of individual acids were obtained with the aid of internal standards.

Additional Keyphrases: inborn errors of metabolism • diagnostic aid • normal values

Studies aimed at the determination of normal values of various endogenous urinary metabolites have been of two types—analytical techniques designed to quantitate some individual component or methods developed to study members of a related class of compounds. While quantitation of individual components is useful in the diagnosis of many disease states, it is advantageous to study a wide range of compounds when knowledge of unknown metabolic changes is being sought. We are interested in effects of pharmacologic agents on various metabolic processes as well as the detection and identification of inborn errors of metabolism.

Urinary organic acids reflect some of the major metabolic body functions. An enzyme defect in amino acid metabolism (I), a derangement of carbohydrate metabolism, or a catecholamine-producing neuroblastoma (2) may be readily diagnosed by identifying and measuring the abnormal amounts of specific metabolites excreted in urine.

Although high-resolution liquid chromatography has been widely used for metabolic profile studies (3), baseline excretion rates for most compounds have not been published. Gas-liquid chromatography (GC) may be utilized to screen derivatized urinary acids, but this technique alone does not absolutely identify each component. However, coupling gas chromatographs to mass spectrometers and computers permits both quantitation and unambiguous identification of many components. Such chromatographic analyses of a class of metabolites extracted from a biological fluid are called “metabolic profiles” (4).

Although important advances in understanding have been derived from chromatographic detection and identification of large and unusual peaks, we find no reports of systematic quantitation of organic acids in the urine of normal adults. Such information would be useful in the assessment of metabolic alterations associated with disease and in studies of the metabolic effects of drugs.

To provide this information, we have used GC and examined acid extracts of urines collected after overnight fast from 21 young adults by GC and GC-MS, and describe here the proportional contribution of the acids so far identified.

Materials and Methods

Subjects. Subjects were normal volunteers, 14 men and 7 women, who ranged in age from 21 to 29 (mean, 25 ± 3) years. They had no personal or family history of disease other than uncomplicated infectious diseases of childhood, and no significant abnormalities on physical examination. Liver-function tests were normal. Four of the seven women were taking contraceptive hormones, but no other subjects were receiving medication.

Diet and activity. The intake of different foods is known to affect the excretion of individual urinary organic acids (5, 6). The subjects were administered a standardized diet to minimize such fluctuations. A four-day intake (2600 cal/day) was planned to include foods that are familiar and acceptable, thus making it easier for the diet to be strictly followed (7). So that the diets might be as constant as possible, enough meat, cake, and frozen turnovers were procured and frozen to provide adequate amounts for the entire study. Canned fruits, vegetables, and juices, prepared without preservatives, were also obtained in advance. Fresh fruits, vegetables, milk, ice cream, and bread, purchased daily, were from the same supplier. Cheddar cheese was used for all evening snacks. Meals were prepared by the same staff for all subjects, by consistent methods of cooking. Subjects were instructed to take no additional food or drink, other than water, during the four-day test period.
Individuals taking part in the study pursued their normal daily occupations. They came to the metabolic kitchen at 0800, 1200, and 1700 hours each day to eat and to receive their evening snacks. On the third night, they slept in the metabolic ward, and that midnight they voided a urine that was discarded. At 0800 on the fourth morning, urine was collected before breakfast in chronic acid-washed (methanol-rinsed) glass bottles and kept refrigerated until analyzed. A procedural blank consisting of distilled water was processed in a manner identical to the urine samples.

Reagents. N,O-Bis(trimethylsilyl)trifluoracetamide (BSTFA) silylation reagent, “OV17” liquid phase, and “Chromosorb W, HP” (80/100 mesh) stationary phase were obtained from Pierce Chemical Co., Rockford, Ill. N,O-Bis(trimethylsilyl-d18silyl)-acetamide was purchased from Merck and Co. “Nanograde” Mallinckrodt solvents were used. Methylmalonic acid (MMA) was purchased from J. T. Baker Chemical Co.

Extraction [after the method of Hammond and Goodman (8)]. To 10.0 ml of urine in a glass-stoppered centrifuge tube was added 3.5 g of NaCl, and the pH of the solution was adjusted to about 1 with concentrated HCI. As an internal standard, 1.0 ml of a 0.5 mg/ml solution of MMA in ethyl acetate was added. To the whole was added 15.0 ml of ethyl acetate and the tube was shaken vigorously for 1 min. After centrifugation, 14 ml of the organic phase was transferred to a 50-ml glass-stoppered centrifuge tube. The extraction was repeated with ethyl acetate, then twice with diethyl ether and the pooled extracts were dried with 3 g of anhydrous Na2SO4. The organic extract was evaporated to about 2 ml under a gentle stream of nitrogen, then transferred to a reaction vial and evaporated completely. Trimethylsilylation was achieved by adding 100 μl of BSTFA and heating at 60°C for 1 h. A 2 mg/ml external standard of MMA was prepared by dissolving MMA in BSTFA and heating at 60°C.

Gas-chromatography. Quantitative analytical runs were performed with a Hewlett-Packard 7620A GC fitted with a flame ionization detector (FID). GC conditions were: nitrogen flow, 15 ml/min; injector and detector temperature, 230°C; column oven program, 50°C to 190°C at 2°C/min; ½-inch by 8-foot stainless steel column containing 1% OV17 on 80–100 mesh Chromosorb W.

Quantitation. Peak areas were obtained by planimetry. The GC-FID response scale for all urinary acids was calibrated by bracketing each test run with the MMA-TMS external standard. The MMA internal standard in each test urine was corrected to 100% recovery and all other component areas normalized by the same fraction. The normalized component areas were then corrected for creatinine content and total urine volume. [Creatinine was determined (9) in duplicate with a Technicon Basic AutoAnalyzer.] Final results were expressed in terms of micrograms of component per milligram of creatinine, as well as milligrams of component per kilogram body weight per 24 h.

Retention indices. Retention indices, expressed in terms of methylene unit (MU) values (10) were calculated by linear interpolation and extrapolation from two known peak-retention times [MMA and p-hydroxyphenylacetic acid, which have MU values in our system of 13.19 and 17.75, respectively (11)]. MU values are relatively independent of changes in temperature, flow rates, and column liquid loading for a given liquid phase and are thus useful in inter- and intra-laboratory comparisons.

Mass spectrometry. In the GC-MS-computer system we used a Beckman GC 45 gas chromatograph, a ceramic frit GC-MS interface, an Associated Electrical Industries MS 12 mass spectrometer, and a Digital Equipment Corporation PDP8/I computer (12, 13). A complete mass spectral scan was obtained every 5 s. Mass spectra were recorded at 70 eV ionizing voltage, 100 μA trap current, and at an ion source temperature of 180°–220°C.

GC-MS studies were performed on a urinary extract treated with BSTFA and repeated with an identical sample derivatized with deuterob-BSA. The addition of deuterium labels to the trimethylsilyl derivatives of the urinary acids can change the mass spectra of the particular ion fragments that contain this derivative group, but without changing the relative abundances of these ions to a great extent. This is an invaluable aid in the interpretation of the mass spectra of unknown urinary acids. The GC retention indices and mass spectra of compounds under investigation were compared to those of pure reference compounds (14).

Results

Figure 1 shows a typical GC metabolic profile of silylated urinary acids. The lower tracing shows the

![Fig. 1. Urinary acid profile for a normal woman (upper tracing) and a procedural blank (lower). Methylmalonic acid internal standard (H) has been added to both samples. (The relative areas of procedural blank peaks vary.)](image-url)
Table 1. Mean Urinary Acid Excretion Rates of 21 Normal Subjects

<table>
<thead>
<tr>
<th>MU</th>
<th>Identity</th>
<th>μg/mg creatinine, ±SD</th>
<th>mg/kg per 24 h, ±SD</th>
<th>mg/kg per 24 h (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A,B,C</td>
<td>BSTFA impurities</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12.1</td>
<td>α-hydroxybutyric</td>
<td>&lt;5</td>
<td>&lt;0.1</td>
<td>0.30 (30)</td>
</tr>
<tr>
<td>12.3</td>
<td>β-hydroxybutyric</td>
<td>29 ± 20</td>
<td>0.62 ± 0.40</td>
<td>—</td>
</tr>
<tr>
<td>12.7</td>
<td>β-hydroxyisovaleric</td>
<td>9.8 ± 7.6</td>
<td>0.22 ± 0.18</td>
<td>—</td>
</tr>
<tr>
<td>13.0</td>
<td>procedural impurity</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>13.19</td>
<td>internal standard-methylmalonic</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>13.6</td>
<td>procedural impurity and acetoacetic</td>
<td>—</td>
<td>—</td>
<td>0.04 (30)</td>
</tr>
<tr>
<td>13.9-14.2</td>
<td>urea and phosphoric</td>
<td>17 ± 17</td>
<td>0.36 ± 0.39</td>
<td>—</td>
</tr>
<tr>
<td>14.4</td>
<td>succinic</td>
<td>18 ± 12</td>
<td>0.41 ± 0.24</td>
<td>0.04-0.16 (28)</td>
</tr>
<tr>
<td>14.7</td>
<td>unidentified</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>15.1</td>
<td>procedural impurity</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>15.5</td>
<td>unidentified</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>15.8</td>
<td>procedural impurity</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16.2</td>
<td>unidentified</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16.6</td>
<td>adipic</td>
<td>2.8 ± 1.7</td>
<td>0.062 ± 0.033</td>
<td>&lt;0.18 (29)</td>
</tr>
<tr>
<td>16.85</td>
<td>β-methyladipic</td>
<td>6.2 ± 3.8</td>
<td>0.14 ± 0.087</td>
<td>—</td>
</tr>
<tr>
<td>17.2</td>
<td>5-hydroxymethyl-2-furoic</td>
<td>9.5 ± 11.5</td>
<td>0.22 ± 0.27</td>
<td>0.18 (37)</td>
</tr>
<tr>
<td>17.4</td>
<td>tartaric</td>
<td>5.7 ± 2.4</td>
<td>0.13 ± 0.052</td>
<td>—</td>
</tr>
<tr>
<td>17.75</td>
<td>p-hydroxyphenylacetic</td>
<td>9.6 ± 3.9</td>
<td>0.21 ± 0.087</td>
<td>0.09-0.58 (32,38)</td>
</tr>
<tr>
<td>18.2</td>
<td>2,5-furandicarboxylic</td>
<td>4.3 ± 4.9</td>
<td>0.11 ± 0.12</td>
<td>0.06 (32)</td>
</tr>
<tr>
<td>18.6</td>
<td>envelope containing</td>
<td>7.4 ± 5.1</td>
<td>0.17 ± 0.11</td>
<td>—</td>
</tr>
<tr>
<td>18.9</td>
<td>aconitic (Y), citric</td>
<td>11 ± 7.0</td>
<td>0.21 ± 0.27</td>
<td>—</td>
</tr>
<tr>
<td>19.2</td>
<td>isocitric (Z), &amp; tetronic (X-Z)</td>
<td>15 ± 14</td>
<td>0.39 ± 0.35</td>
<td>1.9-18 (28)</td>
</tr>
<tr>
<td>19.4</td>
<td>dihydroxyphenylpropionic</td>
<td>34 ± 45</td>
<td>2.3 ± 7.3</td>
<td>—</td>
</tr>
<tr>
<td>19.6</td>
<td>BB</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>19.8, 20.6</td>
<td>CC, DD hippuric</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a Mass spectrum obscured by urea.  b Liquid chromatography.  c Colorimetry.  d Extraction and recrystallization.

dependent of diet, exercise, or urine volume (15, 16). However, other studies have shown that the constancy of creatinine excretion is both a variable function of the above factors (17) and also highly dependent on the subject under investigation (18). The possible masking effect of creatinine output on important metabolic variations has been noted in several studies (19-22). Therefore, the factors of diet, activity, and age have been kept constant in this study, and the results are reported in terms of both micrograms of component per milligram of creatinine and milligram of component per kilogram of body weight per 24 hours. Note that the variance of the excretion rates for individual components is about the same whether expressed in terms of body weight or creatinine output (Table 1).

Systematic errors are known to be present in these data, owing to the use of a single internal standard for the calculation of extraction efficiency and PID response. A study by Hammond has shown that the recoveries of isovaleric, hexanoic, and methylmalonic acids from urine by a modification of this extraction method are 46%, 68%, and 91%, respectively (23). Although aromatic acids containing as many as three polar groups may be extracted quantitatively by this method, it is known that citric and isocitric acids (aliphatic acids with four polar groups) are only poorly extracted (24). The internal standard (MMA, peak H) recovery from the urines of all normal subjects in this study was 88.9% (SD, 13.6%). The components present in the procedural blank.

Quadruplicate extractions and GC analyses were performed on one urine sample, to determine the reproducibility of the method. As might be expected, there exists an increasing variance with diminishing peak area. The mean of the relative standard deviations for peaks representing more than 15 μg/mg of creatinine is 9.3%, and for smaller peaks it is 19%.

Table 1 shows the means and standard deviations of excretion rates of 15 acid components from the urines of 21 young normal adults (the single sample from each described above). The wide range of standard deviations (±100% of the means) is noteworthy in view of the care that was taken to ensure dietary constancy. No relationship between peak size and variance such as appears in the quadruplicate analyses seems to exist.

It should be emphasized that the data presented here are for urinary acid excretion in the resting state. (All samples were collected at 0800 hours.) This was necessary because this study was followed by an investigation, which will be reported elsewhere, of the effects of ethanol upon acid excretion rates.

Discussion

The practice is widespread of expressing the urinary excretion of metabolic products in terms of a unit weight of urinary creatinine. It has long been thought that daily urinary creatinine excretion is in-
recoveries of β-hydroxybutyric, β-methyladipic, and p-hydroxyphenylacetic acids by this method are 44%, 82%, and 85%, respectively. These systematic errors may account for some of the differences between excretion rates determined by this and by other techniques.

Gas-chromatographic resolution is another limiting factor. In the case of the Krebs Cycle acids and the tetronic acids (threonine and erythronic acids), resolution of each component is not possible. While the other peak envelopes are at least partially resolved (Figure 1, peaks S–W), there are errors inherent in using the perpendicular-drop method of area assessment (25).

The wide range of organic acid excretion rates among normal subjects may be attributed to individual metabolic differences. Even with a defined diet, it is apparent that individual metabolic variations have a marked effect on the excretion of organic acids. These findings confirm those of Young et al. (5, 6), who have studied the effects of a chemically defined diet on the excretion patterns of carbohydrates, amino acids, and ultraviolet-absorbing constituents of urine by thin-layer and high-resolution ion-exchange chromatography.

In summary, we have presented the mean and range of urinary excretion of some organic acids in normal young adults. This information should be useful in the interpretation of gas-chromatographic metabolic profile data in cases other than acidurias associated with major enzymic defects such as branched-chain ketoaciduria and isovaleric aciduria (26, 27).

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References