Urinary Organic Acids in Man. I. Normal Patterns

Alexander M. Lawson,1 Ronald A. Chalmers,2 and Richard W. E. Watts2

We studied qualitative pattern of urinary acidic metabolites excreted by normal persons. The results provide a basis on which to compare results for patients with potentially abnormal organic acidurias. A series of urinary polyhydroxy (aldonic and deoxyaldonic) acids has been identified. Most of these compounds have not been previously reported in human urine, except in connection with the present work, and are additional to the previously recognized urinary organic acids, which were also observed. Possible metabolic origins of some of the acids are briefly discussed.

Additional Keyphrases: DEAE-Sephadex anion-exchange chromatography • endogenous and exogenous acids • gas–liquid chromatography of trimethylsilyl esters and ethers • acidurias • inherited disorders of metabolism • polyhydroxy acids in urine • solvent extraction

There are about 50 diseases in which an inherited single enzyme defect causes a high concentration of acidic metabolites in the blood or urine (1–3). Dagleish et al. (4) proposed the use of gas chromatography for the study of the trimethylsilyl and methyl esters of organic acids extracted from physiological fluids, and reported data on a wide range of synthetic reference compounds. This work was extended to qualitative studies on urine from normal humans (5) with use of both solvent extraction and an anion-exchange method based on the use of diethylaminoethyl–Sephadex (6). Solvent extraction has also been used by many other workers [e.g., Jellum et al. (7)], but gives poor analytical recoveries of the more hydrophilic acids (2, 5). The methods used in the present work are also based on the use of DEAE-Sephadex for extraction, with stabilization of oxo-acids as ethoximes before trimethylsilylation, gas–liquid chromatography, and gas chromatography–mass spectrometry. This procedure has been quantitatively validated for a variety of organic acids (8, 9) and covers a wide range of potential urinary anionic constituents that have pKₐ values below about 5.5.

This paper reports the identification of the acidic metabolites observed in human urine by this procedure. Some of this work has been reported in preliminary form elsewhere (10).

Methods

Analytical Methods

An aliquot of the first morning urine specimen was promptly frozen at −70 °C and stored at −20 °C and −40 °C until analyzed. The longest storage time was eight weeks. The spectrum of organic acids encountered in the first specimen of urine voided on rising is very similar to that of the 24 h urine collection from the same subject (9), and is unchanged on storage.

The acidic metabolites from urine (an aliquot equivalent to 3.0 mg of creatinine) were quantitatively extracted onto a column of DEAE-Sephadex, from which they were eluted with a pyridinium acetate buffer. Ethoxime derivatives of the oxo-acids were prepared in the eluate, which was then freeze-dried under controlled conditions, and the trimethylsilyl esters and ethers of carboxyl and hydroxyl functions, respectively, were prepared. The components of the mixture of trimethylsilyl–ethoxime derivatives were separated by quantitative gas–liquid chromatography. These methods have been described previously (2, 8, 9).

The data were processed by an off-line computer program that was designed to exclude minor baseline fluctuations with relative peak areas of less than 1000 μV's (11). The minimum detectable amount (MDA) of the isolated peaks by this procedure is 1 ng per microgram of creatinine (equivalent to about 2 mg/24 h urine). For discrete peaks in more crowded regions of the chromatograms the MDA ranges from 2 to 5 ng per microgram of creatinine (3 to 8 mg/24-h urine). Some components in the complex chromatograms obtained are completely overlapped by larger peaks, or co-elute exactly with other components. These peaks cannot be detected or quantitated by gas chromatography alone, and their presence, when reported, has been observed by selected ion-monitoring or cyclic scanning with the mass spectrometer as described elsewhere (10).
Components were identified by their low-resolution mass spectra in conjunction with their gas-chromatographic retention data by use of a library file compiled from reference spectra determined during this work and data published in the literature (12-16).

Subjects

The detailed identification studies were made on urine samples from 10 healthy members of the laboratory staff, and ambulant subjects, between 1.5 and 83 years old, who were resident in the community and were randomly selected by complete households.

Results

Metabolite Identification

Figure 1 shows a typical chromatogram of the trimethylsilyl (TMS) and TMS-ethoxime derivatives of the acidic metabolites extracted from the urine of a normal subject. The chromatogram represents an aliquot of urine equivalent to 12 µg of creatinine; the gas chromatograph (Hewlett-Packard 5750) was operated at an amplification of 10^3 X 20 (electrometer range X integrator attenuation). The qualitative pattern of metabolites shown in Figure 1 has proved to be consistent for the great majority of specimens from normal individuals examined during the course of this work, although certain peaks were only detectable in a fraction of the population studied. The prevalence of the major metabolites is shown in Table 1.

In addition to the compounds enumerated in Figure 1, small amounts of lactic, pyruvic, methylmalonic, oxalic, 3-hydroxybutyric, and furan-2,5-dicarboxylic acids were also found in some cases. Large amounts of tartaric acid were occasionally observed; this compound appears to be of dietary origin. 5-Hydroxymethyl-2-furoic acid, furan-2,5-dicarboxylic acid, and 2-deoxy-pentonic acid were observed in unusually high amounts in the urine of several subjects within 1 to 2 h of ingestion of sweets or chocolates or of heat-sterilized fruit juice preparations.

![Figure 1](image-url)

**Table 1. Prevalence of Excretion of Detectable Amounts of the Major (and Some Minor), Urinary Acidic Metabolites, Illustrating the Consistency of the Excretory Pattern Shown in Figure 1**

<table>
<thead>
<tr>
<th>No. of peak in Fig. 1</th>
<th>Metabolite</th>
<th>No. of subjects excreting detectable amounts</th>
<th>Prevalence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Oxalic acid</td>
<td>239</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td>Sulfate</td>
<td>420</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Benzoic acid</td>
<td>38</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>Phosphate</td>
<td>420</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>Succinic acid</td>
<td>58</td>
<td>14</td>
</tr>
<tr>
<td>9</td>
<td>4-Deoxytetronic acid</td>
<td>416</td>
<td>99</td>
</tr>
<tr>
<td>10</td>
<td>3-Deoxytetronic acid</td>
<td>395</td>
<td>94</td>
</tr>
<tr>
<td>11</td>
<td>2-Deoxytetronic acid</td>
<td>414</td>
<td>99</td>
</tr>
<tr>
<td>13</td>
<td>5-Hydroxymethyl-2-furoic acid</td>
<td>48</td>
<td>11</td>
</tr>
<tr>
<td>14 + 15</td>
<td>Tetronic acids a</td>
<td>420</td>
<td>100</td>
</tr>
<tr>
<td>16</td>
<td>2-Oxoglutaric acid</td>
<td>147</td>
<td>35</td>
</tr>
<tr>
<td>18</td>
<td>4-Hydroxyphenylacetic acid</td>
<td>382</td>
<td>91</td>
</tr>
<tr>
<td>20</td>
<td>2-Deoxypentonic acid</td>
<td>413</td>
<td>98</td>
</tr>
<tr>
<td>22</td>
<td>Aconitic acid c</td>
<td>406</td>
<td>97</td>
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<td>24</td>
<td>Hippuric acid</td>
<td>289</td>
<td>69</td>
</tr>
<tr>
<td>27</td>
<td>Citric acid</td>
<td>413</td>
<td>98</td>
</tr>
<tr>
<td>30</td>
<td>Gluco-1,5-lactone</td>
<td>411</td>
<td>98</td>
</tr>
<tr>
<td>33</td>
<td>Glucuronic acid</td>
<td>270</td>
<td>84</td>
</tr>
<tr>
<td>34 + 35</td>
<td>Gluconic plus glucaric acids d</td>
<td>401</td>
<td>96</td>
</tr>
</tbody>
</table>

* Total no. of subjects was 420 (age range, 1.5 to 83 years).

b Always both erythronic and threonic acids, with the former acid comprising 55-75% of the combined peak.

c Assumed to be cis-aconitic acid, this being the naturally occurring form of this acid.

d Generally with glucaric acid as the major constituent.
Benzoin and 4-hydroxyphenylacetic acids were the most frequently detected aromatic acids, and very low amounts of homovanillic (3-methoxy-4-hydroxyphenylacetic) and 5-hydroxyindole-acetic acids were also observed. The amounts of these latter compounds were generally less than about 7 ng per microgram of creatinine (10 mg/24 h).

To study the hydrophobic aromatic acids in more detail and to explore the possibility that the large amounts of sulfate and phosphate that are extracted onto DEAE-Sephadex obscured the presence of any organic acid derivatives appearing in the same regions of the gas chromatograph, we re-extracted the eluate from the DEAE-Sephadex column—after ethoxime formation, freeze-drying, reconstitution to a volume of 2.0 ml with water, acidification with HCl, and saturation with NaCl—with diethyl ether (three times) and ethyl acetate (three times). The combined ether and ethyl acetate extracts were evaporated in a stream of dry nitrogen and trimethylsilylated with the same volume (1 ml) of reagent that was used to produce the sample giving the chromatogram shown in Figure 1. This procedure gives poor extraction of the more hydrophilic compounds (e.g., the polyhydroxy acids) but accentuates the more hydrophobic and aromatic acids. Figure 2 shows a typical gas–liquid chromatogram obtained in this way, at the same amplification as that in Figure 1, to illustrate the relative concentrations of these acids as compared to those shown in Figure 1. The metabolites may be further accentuated by using a fivefold more concentrated injection, and Figure 3 illustrates a typical chromatogram of these minor hydrophobic acidic metabolites.

We were unsuccessful in our attempts to remove sulfate and phosphate before DEAE-Sephadex extraction by precipitation as the barium salts at pH 2 and pH 6.5, or of phosphate as magnesium ammonium phosphate at pH 6.2. These procedures resulted in coprecipitation of most of the organic anionic components, particularly those with multiple carboxyl functions, and gave chromatograms resembling those produced by direct extraction of urine with ether and ethyl acetate [for example, see Chalmers and Lawson (2)]. Similarly, it was not possible to separate the inorganic anions by fractionation on short columns of DEAE-Sephadex because the multiple $pK_a$ values of phosphate resulted in the appearance of this anion in most of the fractions containing the organic anions.

**Discussion**

Any study of many ambulant subjects living normally in the community (17), of neonates and infants (9), and of mentally subnormal patients (3, 9) precludes the use of 24-h urine collections or of accurately timed samples from completely fasting individuals. We used the first specimen of urine voided on rising in the present work.
The detailed medical histories of the normal persons used in these studies were known, and they included few problems from acidic drug metabolites (17).

The aldonic and deoxyaldonic acids found in the present work have, except for the tetronic (5) and gluconic (18) acids, only recently been reported in urine in connection with the present work (2, 9, 10). Studies reported elsewhere (19) showed that the amounts of these compounds, other than 2-deoxytetronic acid, changed little in association with large fluctuations in the composition of the diet. This suggests that they may be mainly endogenous.

Glucuronic, gluconic, and glucaric acids, and pentoses and deoxypentoses are formed from glucose. The oxidation of pentoses and deoxypentoses could yield the pentonic and 2-deoxypentonic acids we observed. Erythronic acid is usually the main component of the double peak of the tetronic acids on the gas chromatogram and presumably arises by oxidation of D-erythrose, which is itself formed from D-glucose. Ascorbic acid is metabolized via threonic acid (20, 21) as well as via L-lyxonic and L-xylonic acids (pentonic acids). It could therefore contribute to the observed range of urinary organic acids.

The possible origins of the deoxytetronic acids are obscure and little is known about the occurrence of deoxysugars, except 2-deoxyribose, in human metabolism. The excretion of 2-deoxytetronic acid changed more than that of the other deoxytetronic acids in an experiment in which a high glucose diet was given (19), which may indicate that the immediate metabolic precursors of this compound are more closely related to glucose than are those of the other deoxytetronic acids.

The excretion of 5-hydroxymethyl-2-furoic acid, furan-2,5-dicarboxylic acid, tartaric, and 2-deoxypentonic acids was associated with ingestion of chocolates or confectionary or heat-sterilized fruit juice preparations. 2-Oxo-3-deoxyglucose is excreted after the intravenous administration of heat-treated fructose solutions (22). We suggest that this compound is oxidized to a 2-deoxypentonic acid in vivo, and that all of this group of compounds are derived directly from the diet. Pettersen and Jellum (23) also concluded that urinary 5-hydroxymethyl-2-furoic acid is of dietary origin. This compound was reported in urine by Mrochek and Rainey (24) and Witten et al. (25), and occurred in 48 of the 420 urine samples we examined (Table 1). 5-Hydroxymethyl-2-furaldehyde and furan-2,5-dicarboxaldehyde are formed when reducing sugars, especially fructose, are heated with free amino acids (23). The aldehydes are presumably oxidized to the corresponding acids in vivo and these are excreted in the urine. Tartaric acid is also derived directly from the diet.

The analysis of the combined ether and ethyl acetate extracts of the freeze-dried eluate from the DEAE-Sephadex column permits the more detailed semi-quantitative study of the small amounts of aromatic and other hydrophobic acids present in urine from normal persons (Figures 2 and 3). Ethyl acetate is the most useful single extractant for this purpose, although methyl acetate extracts larger quantities of citric acid (Chalmers and Lawson, unpublished observations). The examination of the regions of the chromatograms occupied by sulfate and phosphate is also possible, although the trimethylsilyl derivatives of these inorganic anions normally obscure little of the chromatograms obtained with the nonpolar OV 101 (and polar OV 25) columns used in this work (Figure 1). There is considerable loss of the hydrophilic acids, and this procedure is only recommended as being supplementary to the routine method when small amounts of hydrophobic acids are of interest.

The removal of sulfate and phosphate as their barium salts (and of phosphate as magnesium ammonium phosphate), proved unsuccessful in the present work, because most of the organic acids are co-precipitated. This was to be expected, because most of the polyhydroxy acids and those with multiple carboxylic acid functions are insoluble in aqueous media as their barium salts, these salts often being used in the isolation of such acids during preparative work. This is exemplified by the very low recoveries of organic acids such as citric acid that have been reported (26) when a barium hydroxide precipitation stage was introduced into a procedure based on similar methods. Additionally, many organic acids are unstable when an alkaline medium (26) is used, and these procedures cannot be recommended.

The acidic metabolites excreted in low amounts that we observed on use of solvent extraction of the DEAE-Sephadex extract (Figures 2 and 3) include aliphatic dicarboxylic acids and 3-hydroxy-3-methylglutaric acid as well as aromatic acids derived from aromatic amino acid metabolism (for example, 4-hydroxyphenylacetic acid) and from catecholamine degradation (for example, homovanillic and vanilmandelic acids). Some of the other acids detected are of dietary origin (for example, 3-hydroxyphenylhydralacrylic acid (27)). The use of solvent extraction of the DEAE-Sephadex extract is thus a useful procedure for the semi-quantitative study of these hydrophobic acids.

Our studies show a diverse range of anionic compounds in urine, some of which have been observed for the first time during the course of this work. The aldonic and deoxyaldonic acids appear to be an important part of the organic anionic composition of normal urine [some 30 to 35% of the total organic acid excretion (17)], but their importance in disease remains to be assessed. Improved extraction and derivatization methods combined with gas chromatography–mass spectrometry enables the range of compounds to be reliably defined, and this provides a background against which an abnormal organic aciduria caused by an inborn error of metabolism or other factors can be viewed. The effects of dietary alterations, studies on individual variations in excretion, and the frequency distributions and quantitative excretion ranges of the urinary acidic metabolites in the populations studied are described elsewhere (17, 19).
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References


