Urinary Organic Acids: Isolation and Quantification for Routine Metabolic Screening

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A method for isolating organic acids from acidified urine on an equimolar mixture of Porapak Q and Porapak T is described, and results are compared with extraction with ethyl acetate and ion exchange on DEAE-Sephadex. Average recoveries of 14C-labeled oxalic acid, lactic acid, succinic acid, α-ketoglutaric acid, citric acid, and cinnamic acid were equal to or better than those obtained with the spectrometric extraction method. The ion-exchange method gave higher recoveries for oxalic acid, lactic acid, and citric acid. The quantification of separated acids from reconstructed mass spectrometric ion traces is compared with quantification from the simultaneously recorded flame ionization detector response signals. A good correlation was obtained. With the present routine metabolic screening method we have detected several patients with inborn errors of metabolism.

Additional Keyphrases: column chromatography • automation • gas chromatography–mass spectrometry • inborn errors of metabolism • heritable disorders • anion-exchange

Analysis for organic acids in human urine by gas chromatography–mass spectrometry (GC-MS) is becoming quite common in clinical laboratories. By this technique it is possible to diagnose many inborn errors of lipid, amino acid, and carbohydrate metabolism and to monitor dietary and other forms of therapy (1–4).

Routine metabolic screening for inborn errors of metabolism requires access to a method that makes it practicable to process many samples and that at the same time fulfills reasonable criteria of specificity and reproducibility.

The compounds of interest cover a wide range of polarity, which makes the initial isolation step very critical. At present, extraction with ethyl acetate or diethyl ether, or both, is widely used. This type of liquid extraction yields poor analytical recoveries of the more-polar compounds (5–8) and, like all liquid-extraction methods, it is inconvenient for use with large numbers of samples. Initial isolation of an "organic acid fraction" by use of anion-exchange resins based on organic polymers or cellulose is an approach taken by several groups (2, 3). The more-polar compounds are well recovered, but the large amounts of sulfate and phosphate that are present in the isolated fraction cause problems in the chromatographic separation that follows (5, 6). It is also a disadvantage that a final aqueous solution is obtained, because removal of water is more cumbersome than evaporation of a solution containing mainly an organic solvent.

We report here our use of columns of ethylvinylbenzene–dimethylbenzene copolymers (Porapak®)1 for the initial isolation of an "organic acid fraction," followed by separation and identification by use of capillary gas chromatography–mass spectrometry. We have compared the isolation method on Porapak material with solvent extraction and with ion-exchange chromatography. The clinical usefulness of this new isolation method has been validated by analysis of more than 4000 samples sent to the laboratory for diagnostic and follow-up purposes.

Materials and Methods

Reagents and Instrumentation

All reagents were of analytical or "HPLC" grade. Urine samples were stored at −20 °C, with no additives.

For liquid-scintillation counting we used a Packard Tri-Carb Model 3320 instrument (Packard Instrument Co., Downers Grove, IL). As counting solution we used a 1:2 (by vol) mixture of Triton X-100 surfactant and a toluene solution containing 2,5-diphenyloxazole (PO) , 5 g/L, and 1,4-bis[5-phenyloxazol-2-yl]benzene (POPOP), 100 mg/L. To 10 mL of counting solution, 500 μL of the radioactive aqueous sample was added after adjustment to pH 9 with 0.1 mol/L sodium hydroxide. [2,3-14C]Sucinic acid (98%), and [1-14C]2-oxoglutaric acid (99%) were from NEN (New England Nuclear Chemicals, Dreieich, F.R.G.). [14C]Oxalic acid (97%), [side-chain-3-14C]Kinnamic acid (97%), [1,5-14C]Citric acid (98%), and [U-14C]Sodium-l-lactate (98%) were from Amer sham, Buckinghamshire, England. The radioactive materials were used as supplied, and the values for the radiochemical purities given in parentheses after the names of the compounds are those stated by the supplier for the batch delivered. For quench correction 14C-standard pills were used (LKB Wallac, S tockholm, Sweden). Porapak Q and Porapak T, mesh size 100–200, were from Waters Associates, Milford, MA. The material was wetted overnight with acetone and thoroughly washed with demineralized water before use.

For capillary gas chromatography–mass spectrometry we used a MAT 44S quadrupole mass spectrometer–data system (Finnigan MAT, Bremen, F.R.G.). The mass spectrometer was operated in the electron impact mode at an ion source temperature of 220 °C and an electron voltage of 70 eV. The mass-spectrometric data were collected and evaluated with the SS300 Data System (software releases 4, 5, and 6).

The quadrupole analyzer was mass calibrated with bis-(perfluoroheptyl)-S-triazine up to m/z 1186 to yield relative peak intensities comparable to the mass spectrum obtained from a sector-field instrument. As a final test, we compared the mass spectrum of persilylated citric acid with that obtained from a sector-field instrument.

The mass spectrometer was interfaced through a slightly modified open split coupling (9) with solvent diverter to a Model 3700 gas chromatograph equipped with a Model 8000 autosampler (both from Varian, Palo Alto, CA) modified to work in conjunction with a previously described septumless injection port (10). The gas-chromatographic columns used were 25 m long, 0.5 mm i.d., open tubular Pyrex columns, statically wall-coated with SE-54 in our laboratory according to Grob (11) to yield a film thickness of 1.2 μm. At an average sample throughput of 10 samples a day the columns have a useful lifetime of about two months. The carrier gas was helium, used at the rate of 15 mL/min during injection.
(constant pressure regulation) and 3 mL/min during the temperature program (constant flow regulation). The temperature was programmed from 50 to 280 °C, at 7 °C/min. The initial temperature was held for 2 min, the final temperature for 8 min. At the column outlet the flow was diverted to the flame ionization detector (63%) and to the mass spectrometer (37%) through a glass-coated stainless-steel T-piece (Scientific Glass Engineering, Melbourne, Australia).

The flame-ionization detector-response signals were recorded and evaluated on a C-R2A integrator (Shimadzu Co., Kyoto, Japan).

The sample extracts derived from the solvent-extraction and Porapak methods were dried in a centrifugal evaporator (Savant Instrument, Hicksville, NY) modified to hold a maximum temperature of 82 °C.

Isolation of Organic Acids

Stock solutions for each of the radioactively labeled organic acids were made from 30 mL of pooled urine by adding 0.01 MBq of the labeled compound diluted with unlabeled material to give an additional 10 mg of the compound per liter in the stock solution.

From these stock solutions 2-mL portions were taken for the ethyl acetate extraction, ion-exchange separation, and the separation on Porapak.

Ion-exchange chromatography of urinary acids was performed according to Chalmers and Watts (12), except that we used methoxylamine hydrochloride instead of ethoxylamine hydrochloride.

We extracted 2 mL of urine with ethyl acetate after adding an equal amount of water saturated with sodium chloride and acidifying the sample with concentrated hydrochloric acid to pH <1. The sample was extracted three times with 3-mL portions of ethyl acetate, centrifuging to speed up phase separation. The combined extracts were taken to dryness in a centrifugal evaporator, followed by two repeated additions and evaporations of 1 mL of methylene chloride.

To prepare the adsorption columns for the isolation of urinary organic acids, we poured an equimolar slurry of Porapak Q and Porapak T in acetone into 15 × 0.8 cm columns plugged with glass wool, to yield columns 10 cm in height and containing about 1.5 g of material. About 700 mg of the Porapak mixture sufficed for adsorption of the organic acids from 2 mL of urine containing up to 500 mg of creatinine per liter. Before the acidified (pH <1) urine sample was applied, the column was washed with three column volumes of 0.1 mol/L hydrochloric acid. After 2 mL of acidified urine was applied to the column, it was washed twice with 1-mL portions of 0.1 mol/L hydrochloric acid. The organic acids were then eluted with about 4 mL of acetonitrile. The eluate was collected, accompanied by as little water as possible, and dried in the centrifugal evaporator. To facilitate the removal of collected water, 1 mL of methylene chloride was added twice during the evaporation procedure.

Before either solvent or column extraction, 200 μL of an internal standard solution of 3-chlorobenzoic acid and 5-pentynoic acid in water was added to each urine sample to give a concentration of 50 mg/L for each. When urine samples for routine metabolic screening are processed the amount of urine applied to the Porapak column depends on the creatinine value. Thus 10 mL of urine is applied when the creatinine value is <150 mg/L, 4 mL is applied when there is between 150 and 300 mg of creatinine per liter, and 2 mL is applied when the creatinine exceeds 300 mg/L.

Liquid-Scintillation Counting

All the dried samples from the solvent-extraction method, the ion-exchange method, and the Porapak separation method were dissolved in 2 mL of demineralized water, and the solutions were adjusted to pH 9 with sodium hydroxide. A 500-μL aliquot was then added to 10 mL of the liquid-scintillation cocktail. Radioactivity in all samples was counted twice the same day as prepared and again one day later, to check for chemiluminescence. The same procedure was followed after addition of the radioactive standard for quench correction.

Derivatization for Gas Chromatography–Mass Spectrometry

For gas chromatography–mass spectrometry the samples were derivatized with 200 μL of methoxylamine hydrochloride dissolved in redistilled pyridine (30 g/L, including 100 mg of tetrachloroanilic acid per liter as an external standard) for 45 min at 40 °C and with 200 μL of N,Obis(trimethylsilyl)-trifluoroacetamide (BSTFA) for 30 min at 80 °C to yield methoxime–trimethylsilyl derivatives.

Standard Curves for Method Comparisons

We prepared standard curves for 14 organic acids normally present in urine, using the Porapak and the solvent-extraction methods. Mixtures of all 14 acids were prepared in water containing, per liter, 200 mg of urea, 200 mg of hippuric acid, and 10, 20, 50, 100, or 200 mg of each of the organic acids. Twice, we took 2 mL of each standard mixture through the procedure described for use with urine samples. The signal from the flame ionization detector and the whole mass spectra in the mass range 50–650 amu (1.4-4 cycle time) were recorded simultaneously, with a flame ionization detector:mass spectrometer split ratio of 2:1. All components in the standard mixture were resolved on the capillary column, and calculations were based on the peak areas from the signal from the flame ionization detector and from the reconstructed specific-ion traces for each component (13–15).

Results

Comparison of Chromatograms after Initial Isolation by Porapak Adsorption, Extraction with Ethyl Acetate, and Ion-Exchange and Subsequent Derivitization

Figure 1 shows chromatograms of the derivatives of organic acids isolated from pooled urine by the three methods. The good agreement of the simultaneously recorded signals from the flame ionization detector and the mass spectrometer shows the good performance of the effluent splitter, and also that the mass-spectrometric cycle time is adequate to resolve the eluting gas-chromatographic peaks. The flame-ionization detector chromatogram from the ion-exchange isolation method was recorded with the attenuation increased by a factor of two as compared with the other chromatograms, because of the larger amounts of citric and hippuric acid recovered. A prominent feature of Figure 1 is that qualitatively and quantitatively more of the polar compounds (notably peaks 18, 22, 33, and 36 in Figure 1) but less urea and phosphoric acid (peaks 12 and 13 in Figure 1) are recovered with adsorption on Porapak than with solvent extraction with ethyl acetate. Another significant feature is the dramatic decrease in sulfuric acid and phosphoric acid recovered (peaks 8 and 13 in Figure 1) with the Porapak-adsorption method as compared with ion-exchange chromatography, although at the expense of some of the very polar polyhydroxycarboxylic acids that, together with urea and...
phosphoric acid, are found in the wash fraction preceding the elution with acetonitrile.

Analytical Recovery of $^{14}$C-Labeled and Unlabeled Acids from Porapak Columns

Table 1 shows the radioactivity accounted for on use of the three isolation methods with pooled normal urine supplemented with six $^{14}$C-labeled organic acids. Recovery of succinic acid, 2-oxoglutaric acid, and cinnamic acid from Porapak compares well with that by the ion-exchange method, whereas the more polar compounds—oxalic acid, lactic acid, and citric acid—are less well recovered from the Porapak material. Recovery of oxalic and cinnamic acid from Porapak is equal to the recovery obtained with the solvent-extraction method. The other radiolabeled compounds we studied are better recovered with adsorption on Porapak than with the ethyl acetate extraction method, notably 66% of citric acid recovered vs <10%, and 97% of 2-

| Table 1. Recovery of Selected Organic Acids as a Percent of Initial Radioactivity (Mean ± SD) |
|---------------------------------|---------------------------------|---------------------------------|
| Porapak | Solvent extraction | Ion-exchange |
| n = 10 | n = 10 | n = 3 |
| Lactic acid | 34.1 ± 5.4 | 22.6 ± 0.9 | 81.1 ± 2.4 |
| Oxalic acid | 30.6 ± 5.8 | 31.3 ± 1.4 | 94.2 ± 2.0 |
| Succinic acid | 93.6 ± 2.2 | 74.0 ± 3.2 | 94.4 ± 1.8 |
| 2-Oxoglutaric acid | 97.0 ± 1.2 | 58.4 ± 2.5 | 83.9 ± 2.2 |
| Citric acid | 66.3 ± 4.5 | 8.7 ± 0.5 | 83.5 ± 1.1 |
| Cinnamic acid | 94.5 ± 3.1 | 95.4 ± 3.6 | 99.2 ± 3.8 |

Fig. 1. Flame ionization detector traces (left) and simultaneously recorded total ion current traces (right) of capillary gas-chromatographic separations of methoxime-trimethylsilyl derivatives of organic acids extracted from human urine by three different methods: adsorption on Porapak (A), ethyl acetate extraction (B), and ion exchange on DEAE-Sephadex (C).

Identities of selected peaks: 1, lactic acid; 2, 2-hydroxysuccinic acid; 3, oxalic acid; 4, oxalic acid; 5, 3-hydroxypropionic acid + reagent artifact; 6, p-cresol; 7, 3-hydroxypropionic acid; 8, sulfonic acid; 9, 3-hydroxy-2-methylbutyric acid; 10, 3-hydroxysalicylic acid; 11, 2-ethylhexanoic acid; 12, urea; 13, ethylmalonic acid + pyrophoric acid; 14, unidentified compound; 15, succinic acid; 16, methylleucine acid; 17, uracil; 18, erythro-4-deoxyerythro-5-methyl-2-carboxylic acid; 19, glutamic acid; 20, 2-deoxyerythro-5-methyl-2-carboxylic acid; 21, adipic acid; 22, 2-thiolactic acid; 23, unidentified compound; 24, furan-2,5-dicarboxylic acid; 25, erythroxylic acid; 26, 2-oxoglutaric acid; 27, 3,4-methylenedioxypentanoic acid; 28, 3-hydroxy-3-methylglycine acid; 29, 4-hydroxyphenylacetic acid + 2,5-furandicarboxylic acid; 30, glycylfurane-2-carboxylic acid; 31, trans-aconitic acid; 32, citric acid lactone; 33, citric acid; 34, hippuric acid + 3-hydroxyphénylacetic acid; 35, 3-deoxyarabinohexonic acid; 36, tyrosine; 37, unidentified compound; 38, glucuronic acid; 39, 3-hydroxyhippuric acid; 40, 4-hydroxyhippuric acid; A, 4-pentynoic acid (internal standard); B, 3-chlorobenzoic acid (internal standard); C, tetracosanoic acid (external standard); Glu, glucuronides and glutamic acid-conjugated compounds.
oxoglutaric acid recovered vs 58%. If the columns were washed with twice the amount of 0.1 mol/L hydrochloric acid we have recommended here, nearly all lactic acid and all oxalic acid were found in the wash fraction.

No radioactivity was recovered from the Porapak columns after either an additional elution with acetonitrile or after the acetone wash. After all the recovery experiments had been run, no radioactivity could be detected in the column material when it was examined as a slurry in the scintillation cocktail.

We made a further comparison between recovery by the solvent-extraction method and Porapak-adsorption method by simultaneous quantification of 14 unlabeled organic acids with the flame ionization detector and the mass spectrometer (13-15), using reconstructed specific ion traces after establishing that standard curves were linear by either method (see below). For this quantitative comparison, pooled urine could not be taken as for the recovery experiments with radiolabeled acids and the routinely prepared standard curves, because interfering compounds would have made quantification with the flame ionization detector impossible. Therefore we used an "artificial urine" containing urea and hippuric acid to compare the two methods. Table 2 gives the results obtained from the analysis of an aqueous standard solution containing 50 mg of each compound per liter. The measured areas from the flame ionization detector and from the reconstructed specific ion traces are given in relation to the area under the internal standard peak (recovered to the same extent by both extraction methods). No correction for detector response differences was made. Recoveries were calculated relative to the area obtained from directly derivatized standards dissolved in pyridine (50 mg of each compound per liter) with no urea or hippuric acid added (cf. Table 2). Overall recoveries obtained with the Porapak method as assessed by this approach were equal to or better for all compounds, except oxalic acid, than those with the solvent-extraction method. The Porapak method gives much better overall recoveries for (e.g.) citric acid (53% vs 8%) and 2-oxoglutaric acid (69% vs 4%).

The Porapak columns can be reused at least 50 times with no deterioration in performance. To regenerate the columns, they are washed with four column volumes of acetone, followed by at least 10 column volumes of demineralized water. We could detect no compound in blank runs from the washed columns.

Quantification of Compounds in the Isolated Organic-Acid Fraction

Compounds in the isolated organic-acid fraction can in principle be quantified by measuring the response from the flame ionization detector, from the total ion current, or from the area under a specific ion in a reconstructed ion chromatogram (13-15) as well as from single or multiple ion monitoring (16-18). For 14 compounds we prepared standard curves based on five different concentrations. The aqueous standard solutions were taken through the isolation procedure, in duplicate. Both flame ionization detection and measurement from reconstructed mass-spectrometric ion traces gave linear standard curves in the range of 0-200 mg/L (Figure 2, panels F and M). None of the calculated regression coefficients were less than 0.98. The intercept with the y-axis gives an estimate of the detection limit of the two isolation procedures.

The sensitivity of the mass-spectrometric detection depends on the intensity of the ion selected for quantification. If, as here, a flame ionization detector is used in parallel, it also depends on the split ratio used.

We also calculated linear-regression lines to correlate the method based on reconstructed ion chromatograms from whole mass spectra with the flame ionization detector response-based quantification reference method (Figure 2, panel M/F) and to correlate the Porapak method with the solvent-extraction method (Figure 2, panel P/E). The two quantification and isolation methods correlated well, as shown by the calculated regression coefficients, which all exceed 0.98. Calculated coefficients of variation from different runs were in the range of 1-7% for all compounds except lactic acid and oxalic acid, for which CVs >10% were occasionally found.

Table 2. Peak Areas (Mean of Five Determinations, SD <10%) of Selected Organic Acids as a Percent of Internal Standard Area (3-Chlorobenzoic acid) Measured with a Flame Ionization Detector (FID) and by Mass Spectrometry (MS)

<table>
<thead>
<tr>
<th>Compounds (50 mg/L)</th>
<th>Standard</th>
<th>Solvent extraction</th>
<th>Porapak adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FID</td>
<td>MS</td>
<td>FID</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>219</td>
<td>150</td>
<td>6</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>190</td>
<td>122</td>
<td>12</td>
</tr>
<tr>
<td>3-Hydroxybutyric acid</td>
<td>233</td>
<td>97</td>
<td>9</td>
</tr>
<tr>
<td>2-Hydroxyisovaleric acid</td>
<td>219</td>
<td>177</td>
<td>20</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>179</td>
<td>184</td>
<td>217</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>172</td>
<td>173</td>
<td>17</td>
</tr>
<tr>
<td>Adipic acid</td>
<td>275</td>
<td>178</td>
<td>18</td>
</tr>
<tr>
<td>3-Methyladipic acid</td>
<td>125</td>
<td>86</td>
<td>93</td>
</tr>
<tr>
<td>2-Oxoglutaric acid</td>
<td>198</td>
<td>180</td>
<td>45</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetic acid</td>
<td>296</td>
<td>224</td>
<td>25</td>
</tr>
<tr>
<td>trans-Aconitic acid</td>
<td>229</td>
<td>171</td>
<td>65</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>326</td>
<td>180</td>
<td>36</td>
</tr>
<tr>
<td>Citric acid</td>
<td>273</td>
<td>182</td>
<td>118</td>
</tr>
<tr>
<td>Vanillylmandelic acid</td>
<td>297</td>
<td>193</td>
<td>256</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>205</td>
<td>198</td>
<td>110</td>
</tr>
<tr>
<td>3-Chlorobenzoic acid</td>
<td>213</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Mass-to-charge ratios of the ions reconstructed for quantification.

*Recoveries as measured against the relative areas from the derivatized standards dissolved in pyridine.

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Validation of Clinical Usefulness

Figure 2 shows examples of chromatograms obtained with the Porapak method for urines of patients with different inborn errors of metabolism. The compounds most characteristic of each disease are clearly visible, and minor compounds of interest can also be discerned. Thus, in a case of maple-syrup urine disease, the largest peak is produced by 2-hydroxyisovaleric acid (peak 50, Figure 3, panel MSUD, about 80 mg/L), but even 2-oxoisovaleric acid, 2-oxo-3-methylvaleric acid, 2-oxoacapric acid, and 2-hydroxyisovaleric acid in the range of 10 to 40 mg/L (peaks 45, 50, 52, and 53, respectively, Figure 3, panel MSUD), characteristic of maple-syrup urine disease, can be seen. In the case of methylmalonic aciduria and in the case of methylcrotonylglycinuria due to biotinidase (EC 3.5.1.12) deficiency (19) the characteristic presence of methylcitric acid (peak 67, Figure 3, panels MM and MCG, >60 and 200 mg/L, respectively) can be noted in addition to the main diagnostic metabolites, methylmalonic acid (peak 51, Figure 3, panel MM, >400 mg/L) and 3-hydroxyisovaleric acid and 3-methylcrotonylglycin (peaks 10 and 60, respectively, Figure 3, panel MCG, >200 and 40 mg/L, respectively). Detection of the huge amount of lactic acid (peak 1, Figure 3, panel PC, >300 mg/L) in a case of pyruvate carboxylase (EC 6.4.1.1) deficiency (20) poses no problem, and even in the heavily contaminated sample of a case of ornithine carbamoyltransferase (EC 2.1.3.3) deficiency, orotic acid (peak 64, Figure 3, panel OCT, 95 mg/L) is easily detected by our screening method.

The urine from an untreated patient with hereditary tyrosinemia shows the presence of succinylacetone and succinylacetocacetate (peak multiples 59 and 72, Figure 3, panel TYR, both above 50 mg/L), which are required as diagnostic criteria for this disease (21) but are easy to overlook (22).

Discussion

Non-ionic polymers have been used to isolate (e.g.) steroids and water pollutants before their analysis with gas chromatography–mass spectrometry (23). Dressler (23) stated that for acidic compounds neither of the sorbents is suitable, but we tested several commercially available adsorbents such as Tenax, Amberlite XAD resins, Sephadex LH-20, Lipidex 1000 and 5000 (24), and octadecyl-modified silica (Sep-pak*) but found them unsatisfactory because considerably more water or fewer components were recovered than with the solvent-extraction method. The best results were obtained with Porapak Q, but very polar compounds such as oxalic acid were not recovered as well as with the ethyl acetate method. We therefore tested the more-polar Porapak T material, but here less-polar compounds such as decanedioic acid were not recovered as well as with the solvent-extraction method. We found a one-to-one mixture of Porapak Q and Porapak T to be a good compromise in polarity between the nonpolar Porapak Q and the more-polar Porapak T.

Except for lactic acid, oxalic acid, and citric acid, recoveries from the Porapak columns exceeded 80% for the compounds studied by the radioactive tracer method (cf. Table 1). Table 2 summarizes an attempt to assess and compare overall recoveries by the solvent-extraction method and the Porapak adsorption method by simultaneous flame ionization detection and mass spectrometry. To get resolved peaks for quantification with the flame ionization detector, we had to use an artificial urine, although, owing to matrix effects, overall recoveries were lower for all compounds as compared with supplemented urine (Table 1). In Table 2, internal-standard-corrected values for area response were calculated and compared. The values obtained for “artificial urine” were compared with the values obtained for directly derivatized standards dissolved in pyridine.

Except for oxalic acid, all compounds studied were recovered equally well or better with adsorption on Porapak than by solvent extraction. For 2-oxoglutaric acid and citric acid, recoveries were more than fivefold greater with Porapak as compared with solvent extraction.

Lactic acid and oxalic acid are not well recovered by either technique, but one does not run the risk of overlooking any significant increase, as for instance of lactic acid in cases of lactic acidosis (e.g., Figure 3, panel PC). If one wishes to quantify these acids more accurately, one should use specific methods based on enzymic reactions.

Sulfuric acid and phosphoric acid are recovered with the ion-exchange isolation method, leading to problems in the following gas-chromatographic separation. The quality of the chromatograms obtained with Porapak adsorption or extraction with ethyl acetate is better than with the ion-exchange technique (cf. Figure 1). Reportedly, sulfuric acid

Fig. 2. Linear regression lines for 14 components of normal urine (1 to 14 as in Table 2) from the standard curves (left side; duplicate values at 10, 20, 50, 100, and 200 mg/L) and the corresponding correlations of the different detection and isolation methods (right side)

Abbreviations are as follows: M, mass-spectrometric quantitation; F, flame-ionization detector quantitation; P, Porapak adsorption method; E, ethyl acetate extraction method; M/F, correlation between mass-spectrometric method and simultaneous quantification with the flame-ionization detector; P/E, correlation of the Porapak-isolation method against the ethyl acetate extraction method.
and phosphoric acid can be removed by precipitation with barium hydroxide, but this would introduce a further step and also the potential risk of coprecipitation of organic acids (5, 25, 26). More different compounds (including several neutral compounds) are recovered with the Porapak procedure than with ethyl acetate extraction. For example, 2-oxoproline at concentrations of interest for the diagnosis of 2-oxoprolinuria is detected after Porapak adsorption.

The three isolation procedures give reproducible yields, which makes it possible to quantify excretions with the aid of an internal standard—at least if recovery exceeds 90%. Irrespective of which isolation procedure is used, a very complex mixture is obtained. Capillary gas chromatography with use of a flame ionization detector suffices to detect the large abnormal patterns that are seen in many classical cases of inborn errors, but this technique cannot be generally used for quantification or even identification, because of overlapping components (13, 15, 16, 27). With the mass-spectrometric technique as used in our laboratory, a mass spectrum is recorded every 1.4 s. Thus it is possible to inspect any peak of interest to establish its purity. For example, orotic acid is usually eluted close to or together with acetic acid, but a mass spectrum recorded from this peak reveals the presence of orotic acid, which is important.

Fig. 3. Total ion-current chromatograms of methoximated and silylated compounds isolated from the urine of different patients by the Porapak method and separated by capillary gas chromatography.

Because of an error in the acquisition program the elapsed time shown includes the oven cool-down time with the exception of the MM and OCT panels, where the clock was started at injection time. Differences in spectrum numbers arise because different columns have been used throughout the years. Abbreviations used are: MSUD, maple syrup urine disease (treated); MM, methyl malonic aciduria (treated); MCG, 3-methylcrotonylglycinuria; PC, pyruvate carboxylase deficiency; OCT, ornithine carbamyltransferase deficiency; TYR, hereditary tyrosinemia. In addition to the peak identities given in the legend to Fig. 1, the following peaks are identified: 41, ethylene glycol; 42, tiglic acid; 43, pyruvic acid; 44, phenol; 45, 2-oxo-isovaleric acid; 46, acetoacetic acid; 47, 2-hydroxybutyric acid; 48, 3-hydroxybutyric acid; 49, 2-hydroxyisovaleric acid; 50, 2-oxo-3-methylvaleric acid; 51, methylmalonic acid; 52, 2-oxo-isocaproic acid; 53, 2-hydroxyisocaproic acid; 54, thymol; 55, fumaric acid; 56, 2-hydroxy-2-methylleucinic acid; 57, 5-oxo-2-tetrahydrofuranacetic acid; 58, glutamic acid; 59, succinylacetone; 60, 3-methylcrotonylglycine; 61, 4-oxo-6-hydroxyheptanoic acid; 62, phenylalanine (mono- and dimethylylpyr derivative, respectively); 63, octanedic acid; 64, orotic acid; 65, vanillic acid; 66, homovanillic acid; 67, methyldric acid; 68, vanillylmandelic acid; 69, 4-hydroxyphenylactic acid; 70, 4-hydroxyphenylacetic acid; 71, glucose; 72, succinylacetatase; 73, palmitic acid; 74, 3-hydroxydecanedioic acid; 75, uric acid; 76, oleic acid; 77, N-phénylacetylglutamine; U, uncertain identification; D, sample contaminant; R, reagent artefact.

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in order not to overlook cases of ornithine carbamoyltransferase deficiency (Figure 3, panel OCT). Most compounds have at least one specific ion within a given retention-time window and a reconstructed ion chromatogram can then be used for quantification (13-15).

Because quantification of resolved peaks with the flame ionization detector is well established (28, 29), we simultaneously recorded the signals from the detector and from the mass spectrometer. The mass-spectrometric quantification gives results that correlate well (r > 0.99) with those obtained from the simultaneously recorded signal from the flame ionization detector (Figure 2, panel M/F). For complex mixtures with unresolved components the mass-spectrometric quantification is clearly preferable, and therefore it is routinely used in our laboratory. In conjunction with a library search program and standard curves from urine supplemented with the compounds of interest, quantitative data are obtained automatically (15, 30).

The described isolation procedure has now been in routine use for five years in our laboratory, with up to 20 samples a day, and was run in parallel to the solvent-extraction method for half a year in the beginning. The usefulness of the method has been shown by the fact that we detected different cases of metabolic disorders such as 3-methylcrotonylglycinuria (19), pyruvate carboxylase deficiency (20), tyrosinemia, methylmalonic aciduria, and three cases of orotic aciduria (ornithine carbamoyltransferase deficiency) that had gone undetected with the solvent-extraction method (unpublished). Because of the mild isolation conditions and good reproducibility of our method it has been possible to follow the quantitative variation in excretion of (e.g.) succinylacetacetae and succinylacetone in tyrosinemic patients during treatment (31). The mild separation attained through adsorption on Porapak has also been found useful in studies of mercapturic acids in our laboratory (32, 33) and by others (34).

Even with high-resolution capillary chromatography and the use of a good mass-spectrometric system, one will encounter peaks that cannot be properly identified or quantified. In these cases, one must resort to subfractionation of the initial isolate by chromatographic techniques and (or) to the use of different methods of derivatization (35-39). Quantification from reconstructed ion traces is practicable in screening programs and sufficiently sensitive for the amounts in question. For accurate quantification of trace amounts—for example, in amniotic fluid—the addition of labeled internal standards and analysis by selected ion monitoring is more appropriate.

In a review article, Gates and Sweeley (40) stated: "Barring a major breakthrough in GC-MS design, it seems likely that repetitive scanning GC-MS will be restricted to the exhaustive quantitative analysis of a relatively small number of samples, and will not be used widely for broad-scale screening programs" and "Few of the extraction procedures typically used for metabolic profiling are completely satisfactory; most will need considerable modification before they can be considered routine, easy-to-use procedures." We believe that, with the presently described approach and considering the availability of fully computerized mass spectrometers at acceptable prices, metabolic screening analysis of an "organic acid fraction" can now be more widely made routine in clinical laboratories.

This study was supported by grants from the Swedish Medical Research Council (03X-585) and from Torsten and Ragnar Söderbergs Stiftelse.

References
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