Comprehensive Screening of Urine Samples for Inborn Errors of Metabolism by Electrospray Tandem Mass Spectrometry

James J. Pitt,* Mary Eggington, and Stephen G. Kahler

**Background:** Detection of abnormal metabolites in urine is important for the diagnosis of many inborn errors of metabolism (IEM). Rapid, comprehensive screening methods are needed.

**Methods:** We used electrospray ionization tandem mass spectrometry in positive- and negative-ion modes to detect selected metabolites in urine. For positive-ion analysis, samples were dried and butylated, whereas for negative-ion analysis, samples were merely diluted with the mobile phase. Analysis was by direct injection with multiple reaction monitoring for 32 metabolites in positive mode (amino acids and acylcarnitines) and 30 metabolites in negative mode (organic acids). Run time was 2.1 min in each mode.

**Results:** Interbatch CVs ranged from 4.8% to 32%, enabling quantification of many metabolites. The procedure was applied to controls (278 and 120 in positive- and negative-ion mode, respectively) and 108 IEM individuals representing 37 different IEM. In 105 IEM individuals, representing 36 different IEM, concentrations of one or more diagnostic metabolites were above the 99th percentiles of the control values.

**Conclusions:** The procedure is faster and less labor-intensive than conventional methods of testing for IEM by amino and organic acid profiling and has similar diagnostic sensitivity. The ability to include a greater range of metabolites offers the potential of a more comprehensive screening procedure.

The provision of reliable, functional biochemical tests for inborn errors of metabolism (IEM)\(^1\) will remain an important aspect of biochemical genetics laboratories for the foreseeable future, despite impressive advances in the ability to test for these conditions at the gene level. Biochemical tests that measure the function of an enzyme or protein or that detect the biochemical consequences of a deleterious gene defect can generally still be performed far more easily and inexpensively than the corresponding DNA test. This is exemplified by screening tests for IEM involving amino acids, organic acids, and acylcarnitines. A single screening test for each of these classes of metabolites can detect dozens of different IEM. Reliable screening tests for amino and organic acids in urine and blood samples have been available for many years. These tests usually rely on chromatographic separation to generate metabolite profiles. Amino acid profiles can be generated by ion-exchange chromatography \(^1\), thin-layer chromatography, paper electrophoresis \(^2\), or even gas chromatography \(^3\). Organic acid testing is generally performed with gas chromatography–mass spectrometry (GC-MS) after solvent extraction and derivatization \(^4\). A carylcarnitine analysis has been performed with HPLC \(^5\), GC-MS \(^6\), or GC of organic acids after hydrolysis of carnitine esters \(^7,8\). These processes are time-consuming and labor-intensive, and as typical runtimes are \(\geq 30\) min, throughput is also limited.

Many organic acid disorders can be successfully diagnosed by use of acylcarnitine profiles generated by electrospray ionization tandem mass spectrometry (ESI-TMS) without the need for chromatographic separation. In addition, amino acid profiles can also be generated from the same sample extract merely by altering the scan

---

\(^1\) Nonstandard abbreviations: IEM, inborn error(s) of metabolism; GC-MS, gas chromatography–mass spectrometry; ESI, electrospray ionization; TMS, tandem mass spectrometry; MRM, multiple reaction monitoring; OMIM, Online Mendelian Inheritance in Man accession number; THCA, tetrahydroxysterolaic acid; ASA, argininosuccinate; and MCADD, medium-chain acyl-CoA dehydrogenase deficiency.

Genetic Health Services Victoria, Murdoch Childrens Research Institute, Royal Children’s Hospital, Parkville 3052, Australia.

*Author for correspondence. Fax 61-3-8341-6388; e-mail jpit@cryptic.rch.unimelb.edu.au.

Received March 21, 2002; accepted July 19, 2002.
functions of the mass spectrometer. With these techniques, profiles can be generated in ~2 min and many aspects of the overall process can be automated (9, 10). Increasing numbers of centers around the world are adopting this technology for dried-blood-spot newborn screening (11–14) as it can detect ~20 IEM, many of them serious and life-threatening if untreated. However, many IEM do not produce an abnormal acylcarnitine profile or increase in plasma amino acids; hence, they are not detected by this approach. Many other diagnostically important metabolites cannot be easily incorporated into existing newborn-screening programs that use dried-blood-spot samples.

Clearly, the need to perform comprehensive testing for IEM in urine samples from symptomatic patients will remain for the foreseeable future. The present communication describes the development of TMS methods suitable for rapid and comprehensive testing of urine samples and their application to the diagnosis of a wide variety of IEM.

**Materials and Methods**

Unlabeled calibration materials were obtained from Sigma-Aldrich or synthesized in house. Stable isotope amino acids and other stable isotope compounds were from BDH or Sigma-Aldrich. Archival urine samples from techniques, profiles can be generated in /H11011 sized from [2H3]carnitine and the corresponding acyl acids and other stable isotope compounds were from Sigma-Aldrich or synthesized in house. Stable isotope amino anoyl chloride (16 17), [13C2]glycine was synthesized from [13C2]glycine and hexanoyl chloride (16, 17). Other reagents were obtained from BDH or Sigma-Aldrich. Archival urine samples from IEM patients were stored at −5 to −20 °C for periods of up to 11 years. Diagnoses were confirmed by the findings of characteristic clinical symptoms, metabolite abnormalities detected on at least two occasions by conventional amino acid analysis or GC-MS organic acid analysis, and/or demonstration of an enzyme deficiency or DNA mutation. Control urine samples were obtained from those submitted to our laboratory and that showed no abnormalities on routine amino acid analysis by paper electrophoresis and organic acid screening by GC-MS. Urine creatinine was determined using a Jaffe reaction on a Cobas Mira analyzer (F. Hoffmann-La Roche).

Calibrator solutions were prepared in an aqueous matrix to simulate the composition of urine samples. This matrix contained 30 mmol/L urea, 11.4 mmol/L sodium chloride, 7.8 mmol/L potassium chloride, 1.5 mmol/L potassium dihydrogen orthophosphate, and 7.6 mg/L bovine serum albumin. For monitoring recoveries and for quality-control purposes, a pooled urine sample was prepared and diluted to a creatinine concentration of 1.0 mmol/L. Two enriched urine samples were prepared by diluting the same pooled urine sample to a creatinine concentration of 1.0 mmol/L and adding abnormal amounts of metabolites. The added concentrations of these metabolites were 0.125 and 0.5 times the concentrations of the highest calibrator (see Tables 1 and 2).

 Twenty-microliter aliquots of calibrators and aliquots of urine containing 20 nmol of creatinine were placed in the wells (300-μL volume) of a 96-well polypropylene microtiter plate (Porvair Sciences Ltd.).

**SAMPLE PREPARATION FOR POSITIVE-ION ANALYSIS**

We added 10 μL of internal standard solution containing labeled amino acids and acylcarnitines (see Table 3 for compositions) to each well. The samples were evaporated to dryness under a stream of air at 65 °C. We then added 50 μL of n-butanol–acetyl chloride (9:1 by volume), sealed the plate with paraffin film, and placed it on a rotary shaker for 20 min. The plate was then incubated at 65 °C for 20 min. After cooling, the supernatant was decanted into clean wells and evaporated under an air stream at 65 °C. The samples were reconstituted in 200 μL of acetonitrile–water (1:1 by volume) by mixing on a rotary shaker for 20 min.

**SAMPLE PREPARATION FOR NEGATIVE-ION ANALYSIS**

For negative analysis, we added 200 μL of a solution containing 5 μmol/L hexanoyl-[13C2]glycine in acetonitrile–water (1:1 by volume) to each well.

**TMS**

Loop injections (20 μL) were made via a Gilson 215 autosampler (Gilson Inc.) into a mobile phase of acetonitrile–water (1:1 by volume) infused at a flow rate of 30 μL/min, maintained by a HP 1100 binary pumping system (Agilent Technologies), into the Z-spray electrospray ion source of a Quattro LC tandem mass spectrometer (Micromass). The source was operated with a capillary voltage of 3.2 kV (positive-ion mode) or 2.2 kV (negative-ion mode) and a temperature of 70 °C. Argon was used as the collision gas at a pressure of 0.25 Pa. Fragmentation transitions, cone voltages, and collision energies were optimized during manual loop injections of pure calibrators. Positive- and negative-ion data were acquired in separate batched runs. Multiple reaction monitoring (MRM) for various metabolites was performed under the conditions shown in Tables 1 and 2, and data were acquired for 1.3 min after injection. The total cycle time between injections was 2.1 min.

Data were processed using Neolynx software (Micromass) with MRM signals averaged between 0.6 and 1.3 min and baseline subtraction of signals from 0.15 to 0.45 min. Raw data were exported to an Excel spreadsheet file (Microsoft) for calculations. Ratios of the MRM signals of the metabolites relative to the internal standards were used to construct calibration curves and calculate concentrations in the urine samples. The internal standards used for each metabolite are listed in Tables 1 and 2.

Four calibrants with concentrations 0 (i.e., blank), 0.25, 0.5, and 1 times the concentrations of the highest calibrators listed in Tables 1 and 2 were run with every batch. Calibrators were not available for a few metabolites, and these are indicated in Tables 1 and 2. For these, it was...
Table 1. Tandem mass spectrometric assays for positively charged metabolites in urine.

<table>
<thead>
<tr>
<th>No.</th>
<th>Metabolite</th>
<th>Transition, m/z</th>
<th>Cone voltage, V</th>
<th>Collision energy, eV</th>
<th>Internal standard</th>
<th>Linearity, R</th>
<th>Highest calibrator, µmol/L</th>
<th>CV, %</th>
<th>Recovery, %</th>
<th>CV, %</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycine</td>
<td>132–76</td>
<td>30</td>
<td>10</td>
<td>2</td>
<td>0.995–1.000</td>
<td>1000</td>
<td>11</td>
<td>71–131</td>
<td>5.4</td>
<td>92–132</td>
</tr>
<tr>
<td>3</td>
<td>Alanine</td>
<td>146–44</td>
<td>25</td>
<td>12</td>
<td>4</td>
<td>0.997–1.000</td>
<td>250</td>
<td>12</td>
<td>85–124</td>
<td>9.3</td>
<td>96–123</td>
</tr>
<tr>
<td>5</td>
<td>Proline</td>
<td>172–70</td>
<td>25</td>
<td>12</td>
<td>12</td>
<td>0.985–1.000</td>
<td>250</td>
<td>12</td>
<td>85–124</td>
<td>9.3</td>
<td>96–123</td>
</tr>
<tr>
<td>6</td>
<td>Valine</td>
<td>174–72</td>
<td>25</td>
<td>12</td>
<td>8</td>
<td>0.978–1.000</td>
<td>250</td>
<td>16</td>
<td>72–120</td>
<td>11</td>
<td>101–122</td>
</tr>
<tr>
<td>7</td>
<td>Guanidinoacetate</td>
<td>174–101</td>
<td>25</td>
<td>15</td>
<td>19</td>
<td>0.911–1.000</td>
<td>125</td>
<td>13</td>
<td>96–138</td>
<td>10</td>
<td>45–75</td>
</tr>
<tr>
<td>9</td>
<td>Leucine/Isoleucine/Hydroxyproline</td>
<td>188–86</td>
<td>25</td>
<td>12</td>
<td>12</td>
<td>0.976–1.000</td>
<td>250</td>
<td>12</td>
<td>66–97</td>
<td>9.8</td>
<td>98–120</td>
</tr>
</tbody>
</table>

assumed that the response was the same as the internal standard, and the concentration values were thus calculated in arbitrary units. Also included with each batch were the unenriched and enriched urine samples. Imprecision and analytical recovery from the enriched urine samples were determined from consecutive batches run over a period of 5 weeks. During this period the instrument was also used for various other analyses, such as...
acylcarnitines and amino acids in dried-blood-spot samples, peptides, and phospholipids. Apart from routine ion source cleaning performed at approximately weekly intervals, no other precautions were taken to minimize ion source contamination.

**Results**

**Positive-ion data**
The analytical performance of several positive metabolites determined in eight consecutive batches of samples is given in Table 1. Calibrators were prepared in a matrix that approximated the composition of urine to compensate for ionization suppression effects that occur in the presence of salts. Most correlation coefficients were in the range 0.95–1.00, indicating good linearity of calibration. It thus appears that the suppression effects occur equally for the metabolites and internal standards and that quantification can be achieved provided that ion ratios relative to the internal standards are used for the calculations. Between-batch variability was determined from the CVs of the two enriched urine samples and ranged from 5.4% to 15% at the higher concentration.

**Table 2. Tandem mass spectrometric assays for negatively charged metabolites in urine.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Metabolite</th>
<th>Transition, m/z</th>
<th>Cone voltage, V</th>
<th>Collision energy, eV</th>
<th>Internal standard</th>
<th>Linearity, R</th>
<th>Highest calibrator, μmol/L</th>
<th>CV, %</th>
<th>Recovery, %</th>
<th>Low control&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CV, %</th>
<th>Recovery, %</th>
<th>High control&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>Glycolate</td>
<td>75–47</td>
<td>25</td>
<td>12</td>
<td>74</td>
<td>0.985–1.000</td>
<td>125</td>
<td>16</td>
<td>17–440</td>
<td>18</td>
<td>19–178</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>Lactate</td>
<td>89–43</td>
<td>20</td>
<td>10</td>
<td>74</td>
<td>0.939–1.000</td>
<td>250</td>
<td>23</td>
<td>69–118</td>
<td>32</td>
<td>100–264</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>3-Hydroxypropionate</td>
<td>89–59</td>
<td>20</td>
<td>8</td>
<td>74</td>
<td>0.889–1.000</td>
<td>20</td>
<td>8.4</td>
<td>73–165</td>
<td>13</td>
<td>76–222</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>4-Hydroxybutyrate</td>
<td>103–67</td>
<td>20</td>
<td>13</td>
<td>74</td>
<td>0.999–0.999</td>
<td>125</td>
<td>8.3</td>
<td>84–115</td>
<td>14</td>
<td>75–139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>3-Hydroxyisobutyrate</td>
<td>103–73</td>
<td>20</td>
<td>10</td>
<td>74</td>
<td>0.959–0.999</td>
<td>125</td>
<td>12</td>
<td>64–140</td>
<td>9.7</td>
<td>142–216</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>Glycerate</td>
<td>105–75</td>
<td>20</td>
<td>10</td>
<td>74</td>
<td>0.993–0.999</td>
<td>125</td>
<td>7.6</td>
<td>68–83</td>
<td>21</td>
<td>72–149</td>
<td></td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>C4:1 Dicarboxylate</td>
<td>115–71</td>
<td>20</td>
<td>10</td>
<td>74</td>
<td>0.989–1.000</td>
<td>250</td>
<td>11</td>
<td>97–123</td>
<td>30</td>
<td>53–117</td>
<td></td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>3-Hydroxyisovalerate</td>
<td>117–59</td>
<td>25</td>
<td>10</td>
<td>74</td>
<td>0.989–1.000</td>
<td>125</td>
<td>7.8</td>
<td>80–114</td>
<td>5.4</td>
<td>113–146</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>2-Hydroxyisovalerate</td>
<td>117–71</td>
<td>30</td>
<td>12</td>
<td>74</td>
<td>0.995–1.000</td>
<td>50</td>
<td>6.9</td>
<td>78–109</td>
<td>23</td>
<td>77–147</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>C4 Dicarboxylate</td>
<td>117–73</td>
<td>20</td>
<td>10</td>
<td>74</td>
<td>0.986–0.999</td>
<td>250</td>
<td>15</td>
<td>74–125</td>
<td>9.6</td>
<td>97–135</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>5-Oxoproline</td>
<td>128–84</td>
<td>30</td>
<td>12</td>
<td>74</td>
<td>0.994–1.000</td>
<td>500</td>
<td>14</td>
<td>93–166</td>
<td>12</td>
<td>63–99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>C5 Dicarboxylate</td>
<td>131–87</td>
<td>25</td>
<td>10</td>
<td>74</td>
<td>0.994–1.000</td>
<td>125</td>
<td>16</td>
<td>48–131</td>
<td>13</td>
<td>63–102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>Phosphoethanolamine</td>
<td>140–79</td>
<td>30</td>
<td>12</td>
<td>74</td>
<td>0.846–0.998</td>
<td>250</td>
<td>45</td>
<td>12–101</td>
<td>24</td>
<td>68–131</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>C6:1 Dicarboxylate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>143–99</td>
<td>15</td>
<td>7</td>
<td>74</td>
<td>0.987–0.999</td>
<td>50</td>
<td>13</td>
<td>151–234</td>
<td>7.1</td>
<td>101–137</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>C6 Dicarboxylate</td>
<td>145–85</td>
<td>25</td>
<td>12</td>
<td>74</td>
<td>0.988–1.000</td>
<td>125</td>
<td>12</td>
<td>57–80</td>
<td>18</td>
<td>35–107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>2-Ketoglutарат</td>
<td>145–101</td>
<td>20</td>
<td>8</td>
<td>74</td>
<td>0.970–1.000</td>
<td>250</td>
<td>28</td>
<td>20–287</td>
<td>18</td>
<td>53–93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>HydroxyCS-dicarboxylate</td>
<td>147–85</td>
<td>25</td>
<td>10</td>
<td>74</td>
<td>0.991–1.000</td>
<td>125</td>
<td>4.6</td>
<td>163–366</td>
<td>11</td>
<td>52–117</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Obtained with pooled urine control enriched with concentrations 0.125 times the concentration of the highest calibrator (n = 8).

<sup>b</sup>Obtained with pooled urine control enriched with concentrations 0.5 times the concentration of the highest calibrator (n = 8).

<sup>c</sup>Typically refers to 3-methylglutarate.

<sup>d</sup>Calibrator was not available. This metabolite was quantified by assuming the same response as the internal standard.
Several additional metabolites were included that are not usually part of the panel tested for as part of dried-blood-spot testing by TMS: guanidinoacetate, δ-amino levulinate, cystine, and homocystine. These metabolites are all markers for IEM but in some cases may be increased only in urine and not in blood (e.g., cystinuria).

**NEGATIVE-ION DATA**

Many metabolites associated with IEM can be successfully analyzed by ESI in negative-ion mode without the need for derivatization. Fig. 1 shows the negative-ion electrospray spectrum of urine from a patient with molybdenum cofactor deficiency [Online Mendelian Inheritance in Man accession number (OMIM) 252150] compared with a control urine. The samples were prepared by simple dilution of the urine with the mobile phase and analyzed by single-stage MS. Several significant and diagnostically relevant observations can be made: relative to the sulfate signal, there were increased signals attributable to sulfite, thiosulfate, and S-sulfocysteine, indicating sulfite oxidase deficiency as a consequence of the decreased availability of the molybdenum cofactor. Increased xanthine and hypoxanthine signals indicate deficiency of xanthine oxidase, which requires the same cofactor. This simple and rapid analysis thus serves to distinguish this condition from isolated sulfite oxidase deficiency (OMIM 272300) in which the abnormalities in purine metabolism do not occur (18). With established diagnostic procedures, this diagnosis would usually require at least two separate analyses: amino acid analysis to identify S-sulfocysteine and HPLC analysis of purines to identify increased xanthine excretion. Citrate excretion was also increased, presumably the result of secondary inhibition of the Krebs cycle or oxidative phosphorylation, as there was a significant lactic acidosis in this patient.

The presence of these expected signals in the negative-ion spectra as well as several others that would be expected in normal urine samples (e.g., hippurate) suggested that this mode of analysis may be of more general utility and be applicable to many IEM in which anionic species are excreted. This is particularly relevant to several organic acidurias that do not produce abnormal acylcarnitines. Clearly, TMS is preferable to single-stage MS in terms of specificity, especially in the case of isomeric organic acids that have the same molecular weight. This is illustrated for the case of 3-hydroxybutyrate, 4-hydroxybutyrate, and 3-hydroxyisobutyrate, shown in panels A, B, and C, respectively, of Fig. 2. Fragmentation of the parent ion of 103 m/z produces a major fragment at m/z 57 in the case of 4-hydroxybutyrate, but this fragment is not produced by 3-hydroxybutyrate or 3-hydroxyisobutyrate. By selecting the m/z 103 m/z transition for MRM, it was thus possible to measure 4-hydroxybutyrate independently in the presence 3-hydroxybutyrate and 3-hydroxyisobutyrate, and by careful selection of appropriate MRM transitions, it was possible to independently measure several isomeric organic acids in this fashion. In some cases, no suitable transition to resolve isomeric organic acids was found. This is illustrated for the case of succinate and methyl-

---

**Table 3. Internal standards for positive-ion analysis.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Internal standard</th>
<th>Concentration, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>[2H₂]Glycine</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>[2H₄]Alanine</td>
<td>250</td>
</tr>
<tr>
<td>8</td>
<td>[2H₆]Valine</td>
<td>200</td>
</tr>
<tr>
<td>12</td>
<td>[2H₃]Leucine</td>
<td>200</td>
</tr>
<tr>
<td>13</td>
<td>[2H₃]Dimitrine</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>[2H₃]Methionine</td>
<td>100</td>
</tr>
<tr>
<td>19</td>
<td>[2H₃]Phenylalanine</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>[2H₄]Tyrosine</td>
<td>200</td>
</tr>
<tr>
<td>38</td>
<td>[2H₃]Cystine</td>
<td>100</td>
</tr>
<tr>
<td>43</td>
<td>[2H₃]Homocystine</td>
<td>100</td>
</tr>
<tr>
<td>17</td>
<td>[2H₃]Carnitine</td>
<td>100</td>
</tr>
<tr>
<td>32</td>
<td>[2H₃]Isovaleryl carnitine</td>
<td>10</td>
</tr>
<tr>
<td>36</td>
<td>[2H₃]Octanoylcarnitine</td>
<td>10</td>
</tr>
</tbody>
</table>

---

Fig. 1. Negative-ion spectra of urine samples.
Negative-ion single MS spectra of urine samples from a control (A) and an individual with molybdenum cofactor deficiency (B). Increased metabolites are indicated by up arrows; decreased metabolites are indicated by down arrows.
There is only one fragment (m/z 73) produced from the parent ion (m/z 117) of methylmalonate, and this fragment is also produced by succinate. By monitoring the m/z 117→73 transition by MRM we were thus measuring both succinate and methylmalonate, and the signal should more appropriately be designated C4 dicarboxylate. In most cases of methylmalonic acidemia, including those analyzed as part of the present study (see below), the methylmalonate concentrations in urine greatly exceed that of succinate, so that succinate interference will not generally lead to a false-positive result. However, situations such as bacterial contamination may occasionally cause increased succinate concentrations in urine (19). Additional GC-MS analysis would be required in these situations. Alternatively, an additional MRM scan function for the m/z 117→99 transition, specific for succinate, could be included to check for succinate interference. A study of the TMS characteristics of anionic species indicated that a large number of metabolites of diagnostic relevance to IEM could be detected by simple dilution, direct injection into the electrospray ion source, and monitoring of the appropriate MRM transition in negative-ion mode. The TMS conditions for the detection of these metabolites are given in Table 2. Although the majority were organic acids, some other relevant anionic metabolites were also included, such as the amino acids S-sulfocysteine and phosphoethanolamine and the taurine conjugate of tetrahydroxycholestanoic acid (THCA), which is increased in disorders of peroxisomal biogenesis (20).

We also found that the presence of salts in urine led to significant suppression of negative-ion signals. However, as in the case of the positive-ion signals, these effects could be largely overcome by use of ratios relative to internal standards and matching the suppression in the calibrators by inclusion of salts to match the urine matrix. The analytical performance of the various anionic metabolites determined over eight consecutive batches are shown in Table 2. The linearities, interbatch variability, and analytical recoveries were somewhat poorer than those obtained in positive-ion mode. Possibly this reflects the higher salt content of the samples and the fact that a single internal standard was used, which may not adequately compensate for suppression effects in chemically disparate metabolites. Nevertheless, reasonable analytical performance, suitable for quantification, was obtained for many metabolites.

APPLICATION TO NORMAL AND ABNORMAL URINE SAMPLES

Several urine samples from control individuals and individuals with confirmed IEM were analyzed throughout the evaluation process. These results are summarized in panels A and B of Fig. 4 for the positive- and negative-ion data, respectively. Overall, 108 samples from patients with 37 different types of IEM were analyzed. In nearly all cases, clearly abnormal concentrations of diagnostic metabolites were detected for individuals with IEM. This is exemplified by individuals with methylmalonic acidemia, who had C4 dicarboxylate concentrations at least 3.5 times higher than the maximum of controls (Fig. 4B). As indicated above, the C4 dicarboxylate signal included both methylmalonate and succinate, but clearly the succinate concentrations were sufficiently low that they did not hamper the detection of the increased methylmalonate in these individuals. For those metabolites for which semiquantitative data were obtained, this did not appear to be an impediment to the accurate diagnosis of IEM associated with that metabolite. For example, despite the relatively poor analytical performance of ASA (Table 1), the large increases in this metabolite in individuals with ASA lyase deficiency (OMIM 207900) were readily detectable (Fig. 4A). In some cases, clearly abnormal profiles were obtained, but the results could not be considered specific for a single IEM. For example, individuals with tyrosinemia type 1 (OMIM 276700) had grossly increased tyrosine (Fig. 4A) and 4-hydroxyphenylpyruvate (data not shown), which are typical of this condition but are also found in tyrosinemia type 2 (OMIM 276600). In practice, the diagnosis of tyrosinemia type 1 can be confirmed by the finding of increased succinylacetone in urine. Attempts to measure succinylacetone at the concentrations likely to be encountered in patients with tyrosinemia type 1 by TMS were unsuccessful, meaning that GC-MS confirmation is required. Similarly, increased hydroxy-C5-carnitine, C5:1 glycine, and hydroxy-C5 acid were found in both holocarboxylase β-synthase deficiency (OMIM 253270) and β-ketothiolase deficiency (OMIM 203750). In this case, the inability to differentiate these two IEM reliably is attributable to the excretion of isomeric metabolites that could not be distinguished by specific fragmentation using TMS. In holocarboxylase synthase deficiency, these metabolites correspond to 3-hydroxyisovaleryl carnitine, 3-methylcrotonylglycine, and 3-hydroxyisovalerate, whereas in
Fig. 4. Excretion of urinary metabolites in controls and individuals with IEM measured by ESI-TMS.

Values are in mmol/mol of creatinine (log scale) except for metabolites marked with an *, which are in arbitrary units (see text). The vertical lines represent the range of values for controls with the median, 99th percentile, and maximum values are represented by horizontal bars. ♦ represent individuals with IEM. Positive-ion data are for 278 controls (A), and negative-ion data are for 120 controls (B). Note that cerebrospinal fluid glycine concentrations are generally preferred for confirming the diagnosis of nonketotic hyperglycinemia. DC, dicarboxyl; 3MCC, 3-methylcrotonyl-CoA carboxylase deficiency; CBS, cystathionine β-synthase; OTC, ornithine transcarbamylase deficiency.
β-ketothiolase deficiency, they correspond to 2-methyl-3-hydroxybutyrylcarnitine, tiglylglycine, and 2-methyl-3-hydroxybutyrate (21). Organic acid testing by GC-MS is therefore required to differentiate these two conditions.

Multiple metabolite abnormalities were apparent for many IEM; the most consistent diagnostic abnormalities are displayed in Fig. 4. Fig. 5 shows two conditions that produced multiple abnormalities and illustrates the value of including negative-ion data for comprehensive testing of IEM. It is well known that diagnosis of medium-chain acyl-CoA dehydrogenase deficiency (MCADD; OMIM 201450) by analysis of urine acylcarnitines may be unreliable unless the patient has been given carnitine (22, 23), whereas plasma/blood-spot analysis does not suffer this limitation. In contrast, urine concentrations of C6 glycine (hexanoylglycine) appear to be always increased (24). Urine concentrations of C8 carnitine were above the 99th percentile of the controls in only four of seven individuals with MCADD. In contrast, all seven individuals had obviously increased C6 glycine. It should be noted that some of these individuals were without symptoms when urine samples were obtained, and this may provide a reason for the normal concentrations of C8 carnitine. In fact, exactly these types of samples may be referred for diagnostic purposes, as obtaining urine samples during acute MCADD presentations may not be feasible because individuals may recover quickly from their presenting symptoms. If only C8 carnitine in the positive-ion mode was determined, the diagnosis of MCADD could be missed. Similar considerations apply to methylmalonic acidemia, in which C4 dicarboxylate was increased in all individuals, in contrast to C3 carnitine, which was increased in only two of seven individuals (Fig. 5).

Using 99th percentiles as cutoffs, we obtained abnormal metabolite profiles for 36 types of IEM of the total of 37 types analyzed. Three IEM individuals had metabolite concentrations below the 99th percentile. These included one of two cases of 2-aminoacidipic acidemia (OMIM 204750), one of seven cases of 4-hydroxybutyric aciduria (OMIM 271980), and a single case of Barth syndrome (X-linked cardiomyopathy; OMIM 302060). In the last case, the 3-methylglutaconate concentration was only just below the 99th percentile (Fig. 4B). It should be noted that the increases in these metabolites for these IEM may be quite subtle and challenging for existing methods of analysis, such as GC-MS (25). Overall, 105 of a total of 108 individuals with IEM (97%) had abnormal metabolite profiles.

**Discussion**

Several previous studies used ESI-TMS to analyze urine samples for IEM, but these studies used liquid chromatographic separation (26–29) before introduction into the mass spectrometer, generally detected only a limited number of metabolites, and were directed toward specific IEM. Some studies have used ESI-TMS to determine a limited number of metabolites, such as glycine conjugates (30). Other studies have been performed to provide a wider coverage of IEM, but these too have used liquid chromatography (31–33) or electrophoretic separations (34). These separation techniques and the need for re-equilibration between runs generally limit sample throughput. The high specificity conferred by the use of TMS means that complex mixtures such as urine can be analyzed without the need to separate components.

Sample preparation protocols for the analysis of amino acids and acylcarnitines in dried-blood spots by TMS are well established. The method described here for positive-ion analysis is an adaptation suitable for urine samples. Dried-blood spots are generally extracted with methanol before butylation (9), but this step was not necessary to obtain adequate extraction for urine samples. Amino acids and acylcarnitines could be successfully extracted simultaneously and butylated from the dried urine samples during incubation with n-butanol–acetyl chloride. The presence of salts can cause suppression of analyte signals during the ESI process. An added advantage of the simple one-step extraction and derivatization process presented here is that it also facilitated separation of much of the salt and protein content of the urine samples. These remained as an insoluble residue during the incubation step, and simple decantation was effective in removing them. Despite this, significant suppression of signals was still apparent as indicated by the absolute signals of the internal standards in urine samples compared with water blanks (data not shown). For this reason, calibrators were prepared in a salt-containing matrix that would approximate the suppression observed for the urine samples. This approach, combined with the use of internal standards, enabled quantification of many metabolites (Tables 1 and 2). In the case of the negative-ion analysis for organic acids and other anions, we found that sample preparation could be extremely simplified, so that samples could be merely diluted and introduced directly into the ion
source. Such rapid TMS "dilute and shoot" procedures have also been used by the pharmaceutical industry in metabolism studies (35).

All sample preparation was carried out manually in microtiter plates. Separate injections were performed for the positive- and negative-ion analyses, and each injection had a cycle time of 2.1 min. Thus, the total instrument time was 4.2 min/sample. Using these procedures, a single operator could readily complete the analysis of 30 urine samples in 6 h. This compares very favorably with the established procedures in our laboratory (amino acids by paper electrophoresis and organic acids by GC-MS). Two operators are occupied for a similar period of time in sample preparation. This is followed by overnight runs before all results are available to be interpreted. It should also be noted that the procedures used here were largely manual and that there is certainly opportunity to scale up the process, using automatic sample dispensing if higher throughput is needed. The ESI-TMS method also detects several metabolites (e.g., formiminoglutamate and THCA) that are not detected during amino and organic acid screening by established procedures. The ESI-TMS method also requires a much smaller urine sample (40 µL vs 1200 µL), which may be important when dealing with samples from premature infants or postmortem samples.

One area of concern was that the introduction of relatively large amounts of salts into the ion source would degrade the performance of the instrument, particularly in the case of the negative-ion analysis. Under routine operating conditions, no obvious deterioration in absolute sensitivity (as determined by the absolute values of the internal standards) was apparent over the course of each batch or between batches, and other assays run on the same instrument were unaffected. It should be noted that the electrospray ion source used in the current study uses an orthogonal spraying arrangement to minimize contamination by salts. Whether other ion source designs are capable of delivering comparable performance with the sample preparation procedure outlined here will need to be determined on a case-by-case basis.

The primary aim of the present study was to develop a screening procedure capable of detecting a wide variety of IEM in urine with relatively simple sample preparation suitable for routine diagnostic work. Because of the very large increases in metabolites found in most IEM, accurate quantification of metabolites was not a major priority. Indeed, many existing methods for detecting IEM are qualitative or semiquantitative. However, during the development of the procedure it became apparent that the analytical performance for many metabolites was acceptable, with CVs less than ~15% and analytical recoveries in the range 80–120% (Tables 1 and 2), meaning that reliable quantification of these metabolites could be achieved. Some metabolites, such as ASA, homocystine, and 2-hydroxyisovalerate, had higher imprecision, and the values for these metabolites should be regarded as semiquantitative at best. Despite this limitation, obvious increases in these metabolites were apparent in individuals with ASA lyase deficiency, cystathionine β-synthase deficiency (OMIM 236200), and maple syrup urine disease (OMIM 248600), respectively (Fig. 4), indicating that the relatively poor analytical performance for these metabolites is not an impediment to detecting the large increases in these IEM. Overall, the results obtained from control and IEM individuals indicate that the ESI-TMS method has high diagnostic sensitivity, with 105 of 108 IEM individuals giving abnormal profiles. For most of these the patterns of abnormal metabolites were consistent with only one IEM, but there were some situations where the pattern indicated two or more possible IEM, e.g., tyrosinemia types 1 and 2, holocarboxylase synthase deficiency, and β-ketothiolase deficiency. Additional testing using established methods, e.g., organic acids by GC-MS, could be used to differentiate these conditions.

The main application of the ESI-TMS urine screening method is likely to be as an initial test for suspected IEM. As such, it could be used to test all urine samples, with conventional tests such amino acid analysis and organic acid analysis by GC-MS reserved for confirmation of abnormal ESI-TMS results (e.g., distinguishing isomers) or samples with a high index of suspicion of a disorder that may not be adequately detected by ESI-TMS. The ability to incorporate metabolites into the ESI-TMS procedure that are not usually measured, such as guanidinoacetae, THCA, and formiminoglutamate, increases the range of IEM that can be potentially detected over that of conventional methods. We obtained abnormal results for 36 different types of IEM, but 50 or more IEM could potentially be diagnosed by use of the panel of metabolites listed in Tables 1 and 2. It should be stressed that the ESI-TMS method is not a replacement for conventional methods of amino acid and organic acid analysis by GC-MS and plasma/blood spot aecarnitine analysis by ESI-TMS; these methods are still required for confirmation, distinguishing isomers, and detecting subtle increases in some metabolites that might not be apparent by ESI-TMS of urine, such as occurs in Barth syndrome. However, in our laboratory <2% of urine samples submitted for testing are found to have an IEM, a rate that is typical for many other biochemical genetics laboratories. If ESI-TMS is used as an initial screening method and the conventional methods are used to confirm positive samples or to test samples with a high index of suspicion, this will greatly reduce the workload for these labor-intensive methods.

There are several areas in which the performance of the ESI-TMS method could be enhanced. For this study, we arbitrarily chose to use 99th percentiles as cutoffs. In a routine setting, this may lead to an unacceptable proportion of positive results that would require additional confirmatory testing. However, it is clear from Fig. 4 that for many metabolites these cutoffs could be set to a higher percentile, thus decreasing the overall false-positive rate. Age-related reference intervals could also help to improve
the diagnostic sensitivity. It was also apparent that interferences might be present in the signals of some metabolites, as the reference intervals determined for these metabolites were somewhat higher than those quoted in the literature. For example, the 99th percentile for tyrosine in the controls was 101 mmol/mol of creatinine compared with a quoted reference range of <55 mmol/mol of creatinine (36). If these interferences can be identified, then it may be possible to include an additional MRM function to measure them and apply a correction factor for the metabolite, further improving diagnostic sensitivity. Additional internal standards in the negative-ion profiles should also improve the quantification of some metabolites, e.g., C4:1 dicarboxylate.

Although several isomeric metabolites could be distinguished from each other by specific single MRM functions, there were examples where this could not be achieved, e.g., methylmalonate and succinate. In this case, it would be possible to include an additional MRM function unique for succinate, and a suitable correction could be applied to the methylmalonate-plus-succinate signal. This approach will clearly become impractical to apply in cases where more than two isomeric metabolites contribute to the same MRM signal, e.g., an increased C5 carboxylate signal could be attributable to glutarate, ethylmalonate, or methysuccinate. In this situation, GC-MS analysis would be required to separate these isomers. However, it should be noted that all three of these metabolites are markers for IEM, so that any increase in the C5 carboxylate signal is highly significant and unlikely to be the result of interferences unrelated to IEM. Greater experience with a wider range of IEM and controls in different physiologic states will lead to better knowledge of the most appropriate MRM functions to use in these situations.

One of the main challenges of the present study was to devise a panel of diagnostic metabolites that would detect as wide a range of IEM as possible with maximal diagnostic sensitivity. Although the metabolites listed in Tables 1 and 2 are quite successful in achieving this, it is likely that further improvements in diagnostic sensitivity could be achieved. As noted previously, many IEM produce multiple metabolite abnormalities, and inclusion of additional metabolites may help in this regard. This is merely a matter of determining the appropriate MRM function and establishing suitable analytical performance in a urine matrix. Nearly all metabolites studied were amenable to this approach, the two exceptions being oxalate, which gave irreproducible results (possibly the result of complexing with cations), and succinylacetone. However, there is a practical limit to the number of metabolites that can be added because each additional MRM channel decreases the overall dwell time for each metabolite; a stage will eventually be reached when the ion statistics become unreliable. This was not an impediment in the present study. It is likely that up to 50 metabolites could be readily analyzed in both positive- and negative-ion modes. Further experience with greater numbers of samples will no doubt lead to some modification of the panel of metabolites that are optimal for the diagnosis of IEM. Along these lines, we have obtained encouraging initial results with urate, sialic acid, and some purine and pyrimidine metabolites.

References


