Rapid Gas-Chromatographic Assay of Lactulose and Mannitol for Estimating Intestinal Permeability

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We developed a gas-chromatographic method to determine urinary mannitol and lactulose. The procedure for purification of urine by a resin was optimized for purification of analytes and high recovery; the aliquot of resin chosen (500 mg) was kept in contact with the urine for 1 min. The recoveries of mannitol and lactulose were >85% at concentrations that include both normal and pathological values. Sugars were converted to oximes before the silylation step to avoid multiple peaks for the anomeric forms. The calibration was linear over the range 0.1–1 μg of sugar injected. Analytical recovery of the sugars ranged from 90% to 95.3% for Mannitol and from 90.4% to 95.8% for lactulose. The mean within-day imprecision (CV) was 6.2% for mannitol and 4.7% for lactulose; the between-day CV was 6.7% for mannitol and 5.1% for lactulose. A lactulose/mannitol ratio of 0.035 completely differentiated 28 normal children and 28 children with active gluten-sensitive enteropathy, whose mean ratios were 0.022 (SD 0.007) and 0.084 (SD 0.054), respectively.

Indexing Terms: celiac disease/urine/pediatric chemistry/sugars/enteropathy

The sugar intestinal permeability test is regarded as the most valuable screening test for mucosal damage of the small intestine in children (1, 2). This procedure is performed by orally administering two sugars of different molecular size and with different absorption routes and then measuring the urinary excretion. In disorders of the small intestine, transcellular permeability tends to decrease, reflecting a diminished number of mucosal cells, whereas paracellular permeability tends to increase, reflecting damaged tight junctions (3, 4). The change of the two types of intestinal permeability in opposite directions in intestinal diseases has been the rationale for using the ratio between lactulose and mannitol (L/M) urinary excretion for investigational purposes (5). This approach is based on the assumption that the two sugars are handled identically in all other physiological respects and thus intraindividual differences in gastric emptying, small intestinal transit, urinary excretion, and so forth can be eliminated (3, 6–11).

Since the first report of gas chromatography of carbohydrates (12, 13), numerous reports have appeared describing applications to the field of carbohydrate chemistry (14–21). To date, however, no reports have used the desalting of urine with different aliquots of resin for a good purification of the sample with an adequate recovery of sugars. Therefore, we developed a gas-chromatographic method for the simultaneous measurement of lactulose and mannitol in urine after desalting the samples. We have used this method to determine the sugar intestinal permeability in normal children and in children with active gluten-sensitive enteropathy (GSE).

Materials and Methods

Materials

Pyridine, hydroxylamine chlorhydrate, and sodium azide were obtained from Merck (Darmstadt, Germany). Trimethylchloorosilane and bis(trimethylsilyl)trifluoroacetamide were obtained from Supelco (Bellefonte, PA). Inositol (1, 2, 3, 5, 7, 8-hexahydroxycyclohexane), fructose (D-levulose), mannitol (mannite), and turanose (3-O-a-D-glucopyranosyl-D-fructose) were obtained from Sigma-Aldrich (Milan, Italy). D-Glucose (dextrose) and D-galactose were from Merck. Lactulose (Dia-Colon) was obtained from Vecchi e C. Piam (Genoa, Italy). Duolite MB 5113 (a mixture of Duolite C225 in the H+ form and A101 D in OH− form) was supplied by BDH (Poole, UK).

Working solutions. Oxime solution: hydroxylamine chlorhydrate in pyridine (15 g/L); silylating solution: equal volumes of trimethylchlorosilane and bis(trimethylsilyl)trifluoroacetamide; solution A: aqueous mannitol, inositol, lactulose, and turanose, 1 g/L each; solution B: aqueous mannitol and lactulose, 1 g/L each; solution C: aqueous inositol and turanose, 1 g/L each. The internal standard solution consisted of inositol (1 g/L) and turanose (0.2 g/L) in H2O (concentrations reflecting the mean content of each sugar in pathological urine).

Instrumentation

The gas chromatograph (Model 5890 A), integrator (Model 3390 A), and column [HP1; 25 m × 0.32 mm (i.d.) × 0.17 μm film thickness] were all from Hewlett-Packard, Palo Alto, CA. The injector was a split-splitless used in a split mode of 1:80. The detector operated by flame ionization detection. Temperatures were: column, 150–270°C (8°C/min); injector, 270°C; detector, 270°C. The carrier gas was nitrogen (5 mL/min); the make-up gas was nitrogen (30 mL/min).
Procedures

**Derivatization.** Known amounts (100–200 μL) of sugar solutions A, B, or C were dried in a screw-top vial at 60°C under a gentle stream of nitrogen; 100 μL of oxime solution was then added, the vial was capped, and the sample was incubated at 60°C for 20 min. After cooling the sample to room temperature, 100 μL of silylating solution was added and the incubation period was repeated.

**Internal standard calibration.** Inositol and turanose were used as internal standards for the quantitative determination of mannitol and lactulose, respectively. Investigation of the response factors (RF) over a wide range of concentrations (0.05–1.5 g/L) gave the following results:

\[
\text{Inositol/mannitol RF} = 1.03 \pm 0.025 \\
\text{Turanose/lactulose RF} = 1.05 \pm 0.041
\]

**Urinary purification.** Many methods described do not mention urine purification before derivatization and gas-chromatographic analysis. However, our preliminary tests showed that gas-chromatographic patterns with retention times close to those of investigated sugars. We therefore used Duolite MB 5113 as supplied by BDH to desalt and to purify urine samples. We evaluated the use of different amounts of resin to achieve both the best purification and the greatest recoveries of sugars. To 1-mL aliquots of sugar-free urine we added 125, 250, 500, 750, or 1000 mg of resin, shaking these for 1 min in a capped vial. We then transferred 200 μL of the supernates to screw-top vials and dried the samples at 60°C under a gentle stream of nitrogen. After derivatization, the samples were analyzed by gas chromatography.

To assess recovery, we mixed 1-mL aliquots of sugar-free urine with 1 mL of solution B and treated the mixtures with the scalar quantities of resin previously described. We then added 100 μL of solution C to 100 μL of the supernates, and dried and processed the aqueous samples as described above.

**Statistical analysis.** Statistical analysis was performed with the Mann–Whitney U-test for nonparametric data.

Clinical Studies

**Patients.** To evaluate the procedure described, we studied 56 children of both sexes: 28 control children (12 boys and 16 girls), ages 2–15 years (mean 9, SD 5.3 years), without evidence of gastrointestinal or systemic disease; and 28 patients (10 boys and 18 girls) with active GSE, ages 3–16 years (mean 10, SD 4.7 years). In each case the diagnosis was based on the European Society of Pediatric Gastroenterology criteria (22).

**L/M test.** The subjects followed a diet free of mannitol, lactulose, mannose, and fructose for 24 h before the test (17, 23–25). After an overnight fast, the subjects voided a pretest urine sample and then ingested (0.55 mL/kg body weight) a solution containing 18.2 g of mannitol and 18.2 g of lactulose in 100 mL of deionized water. We used a hypertonic solution (1500 mosmol/L) because hypertonicity increases the absorption of intact disaccharides (26–28) and enhances the sensitivity of the test (26).

After 2 h from the beginning of test, the subjects were encouraged to drink ~100 mL of water to ensure adequate urine production. All urines voided in the subsequent 5 h were collected with sodium azide as a preservative. The volume was recorded and an aliquot was kept at -20°C for subsequent analysis.

After the frozen urine aliquots had reached room temperature, 1 mL of urine was shaken with 500 mg of resin; 100 μL of this supernate was then added to 100 μL of internal standard solution, dried, and processed as described above.

**Results**

**Analytical Performance**

**Linearity.** The linearity of the method was tested for mannitol, inositol, lactulose, and turanose, inositol and turanose being tested for their suitability as internal standards. After derivatizing 10, 20, 50, and 100 μL of solution A, we injected 2 μL of each sample twice directly into the gas chromatograph. This procedure was repeated three times. A linear response (Fig. 1) was obtained in the range 0.1–1 μg of sugars injected, as shown: mannitol, \( y = 173924x - 750714 \) \((r = 0.999, S_{xy} = 0.016)\); inositol, \( y = 173541x - 603267 \) \((r = 0.999, S_{xy} = 0.012)\); lactulose, \( y = 114810x - 154469 \) \((r = 0.999, S_{xy} = 0.012)\).
Table 1. Mannitol and lactulose recoveries from 1 mL of sugar-supplemented urine samples treated with scalar quantities of Duolite MB 5113 resin.

<table>
<thead>
<tr>
<th>Resin, mg</th>
<th>Mannitol Recovery %</th>
<th>Lactulose Recovery %</th>
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<tbody>
<tr>
<td>125</td>
<td>94.8 ± 4.7</td>
<td>96.0 ± 5.0</td>
</tr>
<tr>
<td>250</td>
<td>94.0 ± 5.1</td>
<td>93.2 ± 6.1</td>
</tr>
<tr>
<td>500</td>
<td>91.5 ± 4.3</td>
<td>92.5 ± 5.2</td>
</tr>
<tr>
<td>750</td>
<td>86.2 ± 5.8</td>
<td>85.1 ± 6.7</td>
</tr>
<tr>
<td>1000</td>
<td>82.5 ± 5.4</td>
<td>83.3 ± 6.4</td>
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</tbody>
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* Recoveries are the arithmetic mean ± SD of four trials.

Table 2. Analytical recoveries of mannitol and lactulose added to urine.

<table>
<thead>
<tr>
<th>Added amount, g/L</th>
<th>Mannitol</th>
<th>Lactulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>90.0 ± 5.6</td>
<td>91.7 ± 6.1</td>
</tr>
<tr>
<td>0.10</td>
<td>92.0 ± 4.3</td>
<td>90.4 ± 4.8</td>
</tr>
<tr>
<td>0.50</td>
<td>91.6 ± 4.4</td>
<td>92.3 ± 5.0</td>
</tr>
<tr>
<td>1.00</td>
<td>93.6 ± 3.9</td>
<td>94.5 ± 4.4</td>
</tr>
<tr>
<td>2.50</td>
<td>95.3 ± 4.0</td>
<td>95.8 ± 5.3</td>
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</table>

Urine and resin. We found that the purification step was not critically time dependent.

Precision. One urine sample of a patient with active GSE was analyzed four times per day for 5 days. The mean within-day imprecision (CV) for mannitol was 6.2%, for lactulose 4.7%. The between-day CV was 6.7% for mannitol and 5.1% for lactulose.

Accuracy. The accuracy of the method, calculated by testing recoveries in sugar-free urine samples mixed with known amounts of mannitol and lactulose (between 0.02 and 2.5 g/L urine), is summarized in Table 2.

Clinical Results

The subjects' results are expressed as the percentage of mannitol and lactulose recovered in the urine samples and as the L/M recovery ratio. The control subjects showed a mean mannitol recovery of 15.61% (SD 5.8%) and a mean lactulose recovery of 0.28% (SD 0.04%). In contrast, the GSE patients showed a mean recovery of 8.72% (SD 3.5%) and of 0.73% (SD 0.5%) for mannitol and lactulose, respectively. The mean L/M recovery ratio value was 0.022 (SD 0.007) in the control subjects and 0.084 (SD 0.054) in the patients (Table 3).

Chromatograms of urine samples from a GSE patient taken before and 5 h after receiving the mannitol-lactulose solution are shown in Fig. 4.

The differences between mean lactulose and mannitol urinary recovery and mean L/M in GSE children compared with controls were highly statistically significant (P < 0.001). Moreover, in all 28 GSE patients, L/M was >0.035, whereas in the controls, all L/M values were <0.035. In contrast, by receiver-operating characteristic curve analysis, neither urinary lactulose nor urinary mannitol alone provided sensitivities >80% at a specificity of 80% (data not shown).

Discussion

Ratios of L/M excretion are used to assess the intestinal permeability in various diseases and trauma

Table 3. Results of the lactulose/mannitol test.

<table>
<thead>
<tr>
<th>Urinary recovery</th>
<th>Controls</th>
<th>Active GSE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol, %</td>
<td>15.61 ± 5.8</td>
<td>8.72 ± 3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lactulose, %</td>
<td>0.28 ± 0.04</td>
<td>0.73 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>L/M ratio</td>
<td>0.022 ± 0.007</td>
<td>0.084 ± 0.054</td>
<td>&lt;0.001</td>
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</table>

n = 28 each.
conditions. Several methods have been used for determining urinary sugars: thin-layer chromatography (29), colorimetric/enzymatic procedure (30), gas chromatography (2, 14, 16, 17), and HPLC (31–33).

Our method represents a further development of the gas-chromatographic methods previously described, the main modification being an improved purification of urine. Using a Duolite MB 5113 resin yielded chromatograms free of interferences.

The best results for quantifying the sugars were obtained by using 500 mg of resin per milliliter of urine, relatively less than amounts previously described in the literature (2, 14, 16, 31, 32, 34). We found that lower amounts were insufficient for good purification, whereas greater amounts greatly reduced the quantitative recovery of sugars. The recovery efficiency of 500 mg of resin per milliliter of urine was tested for a wide range of sugar concentrations (0.02–2.5 g/L). Recoveries were nearly 90% for each sugar, as described in Results.

We also found that 1 min of contact time between resin and urine was sufficient even if a longer period did not affect the recovery of sugars. This seems to be in contrast with a previous report (2, 14).

As recommended by Shippee et al. (35), we converted sugars to the relative oximes before the silylating process. The oxime derivatives are essential to avoid the presence of multiple gas-chromatographic peaks due to the anomeric forms of sugars.

We conclude that the present technique is an improvement on the sensitivity and selectivity of some previous methods. Furthermore, the reliability and brevity of this procedure encourage its use in routine investigations of intestinal diseases.

References
29. Menzies IS, Mount JN, Wheeler MJ. Quantitative estimation