Rapid and simultaneous quantification of rhamnose, mannitol, and lactulose in urine by HPLC for estimating intestinal permeability in pediatric practice

KAZUNORI MIKI,* ROSS BUTLER, DAVID MOORE, and GEOFFREY DAVIDSON

Determination of intestinal permeability by measuring nonmetabolized sugars has been used to assess the integrity of intestinal mucosa. We have developed and validated a modified HPLC method for determining the concentration of L-rhamnose, mannitol, and lactulose in urine, using an amine-modified silica column and refractive index detection. Probe sugars are simultaneously resolved within 18 min. The calibration curve for each sugar is linear to 20 mmol/L. The minimum detectable concentration of lactulose is 0.05 mmol/L. Recovery of probe sugars is between 99.3% and 105.1%. Overall precision (CV) of estimation of probe sugars ranges from 4.2% to 6.5%. In 14 urine samples from healthy children who ingested the test solution containing 1 g of L-rhamnose, 1 g of mannitol, and 5 g of lactulose, the 5-h urinary excretion ratios of lactulose/rhamnose and lactulose/mannitol were 0.047 ± 0.018 and 0.021 ± 0.010 (mean ± SD), respectively. This method presents a rapid, convenient, and practical technique for determining intestinal permeability in clinical pediatric practice.

INDEXING TERMS: chromatography, amine-modified silica • pediatric chemistry • refractive index

The measurement of the urinary excretion of orally administered nonmetabolized sugar probes has been widely used to investigate intestinal permeability in health and disease [1–3]. Dual-sugar tests in particular have been successfully applied to the estimation of intestinal permeability in pediatric clinical practice, including use in celiac disease [4–6], Crohn disease [7–9], atopic eczema and food hypersensitivity [4, 10–12], jejunal bacterial overgrowth associated with immunodeficiency [13], cystic fibrosis [14–16], and diarrheal disease with mucosal damage [17, 18]. Smaller molecules such as mannitol and L-rhamnose are thought to permeate the mucosa mainly via the transcellular pathway (small pores), whereas larger molecules, including lactulose and cellobiose, enter through the paracellular pathway (large pores) ["pore theory"] [1]. An alternative hypothesis for the permeation of water-soluble probes is paracellular, a route regulated by the tight junctions ["paracellular theory"] [19]. In the dual-sugar test, the permeability to nonmetabolizable di- and monosaccharides (or sugar alcohol) is compared and expressed as an excretion ratio such as lactulose/mannitol or lactulose/L-rhamnose in a timed urine sample. This eliminates the potential confounding effects of gastric emptying, intestinal transit, and renal clearance [20].

Despite the obvious utility of differential sugar absorption tests, widespread application has been delayed by the complexity of the available analytical techniques. Thin-layer [21], gas–liquid [22, 23], and "high-pressure" liquid chromatography (HPLC) [24–28] are the most frequently used techniques. The first is time consuming, as is the second, which in addition requires prior derivatization. Therefore, HPLC is the most promising technique for use in a clinical laboratory, because it quickly provides precise results and is readily automated [24, 25]. We have developed a simple and reliable HPLC technique with use of the refractive index for detection. We used lactulose as the larger probe sugar and L-rhamnose and mannitol as smaller probe sugars, and evaluated their urinary excretion ratios as lactulose/L-rhamnose and lactulose/mannitol. The smaller probe sugars chosen were selected because the routes of permeation through the intestinal mucosa may be different for the two sugars: The permeation route of L-rhamnose is hypothesized to be predominantly transcellular in vivo, whereas mannitol is thought to permeate by both the transcellular and paracellular routes [1].

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Materials and Methods

Materials. We used d-xylose and mannitol (Sigma Chemical Co., St. Louis, MO) to make both calibrator and test solutions for assessment of intestinal permeability. Lactulose from E. Merck (Darmstadt, Germany) was used for making calibrator solutions and that from Janssen-Cilag (Lane Cove, NSW, Australia; as Duphalac™, Solvay-Duphar, B.V., Holland) was used for making test solutions. Thimerosal was purchased from BDH Chemical Ltd. (Poole, UK). HPLC-grade acetonitrile and distilled water were from E. Merck.

Subjects and intestinal permeability test. We studied 14 healthy children and adolescents (10 boys and 4 girls), ranging in age from 5.3 to 16 years (median 9.2 years), with no significant medical histories or gastrointestinal symptoms for ≥2 weeks. After an overnight fast, a pretest urine sample was collected. The subjects then drank the test sugar solution, containing 5 g of lactulose, 1 g of l-xylose, and 1 g of mannitol in 100 mL of water (isotonic solution, molarity 330 mmol/L). After 30 min, a liberal intake of water was permitted, to increase urine flow. Food intake was allowed after the first 3 h. Urine was collected for a total of 5 h and stored in a container containing 0.1 mL of 10 g/L thimerosal as preservative. If the subjects had not voided within the last 30 min of the 5 h, they were instructed to collect one more void. The total volume was recorded and a 20-mL aliquot was stored at −20 °C until analysis. In each subject, total urinary excretion was calculated for each sugar, and results were expressed as the percentage of the ingested dose present in urine and as the excretion ratios lactulose/l-xylose and lactulose/ mannitol. The procedures were in accordance with the Helsinki Declaration of 1975, revised in 1983.

Sample preparation. We added 0.5 g of washed, mixed ion-exchange resin (Duolite MB 5113; BDH Chemical) to 2 mL of the thawed urine specimen for desalting. The mixture was vortex-mixed for 10 s and centrifuged for 10 min at 3000g. The resulting supernate was filtered through 0.2-μm (pore-size) disposable syringe filters (Acrodisc®; Gelman Sciences, Ann Arbor, MI).

HPLC analysis. An aliquot of the filtered supernate was injected into the manual injector (Model 7125; Rheodyne, Cotati, CA) with a 10-μL loop. The HPLC was equipped with an isocratic pump (Model SP 8810; Spectra Physics, San Jose, CA), a polymeric guard column (Direct-Connect™ Cartridge Guard Column; Alltech, Deerfield, IL), and an amine-modified silica column (Kromasil™ NH2 Column, 5-μm particle size, 250 × 4.6 mm; Alltech) and was used at ambient temperature. The mobile phase was degassed acetonitrile in distilled deionized water (70/30 by vol) at a flow rate of 1 mL/min. Detection was by a refractive index detector (LC 1240 R.I. Detector; GBC Scientific Equipment, Dandenong, Victoria, Australia) and was recorded on a linear chart recorder (Linear 1200; Alltech). Calibration curves were prepared by analyzing appropriate concentrations of each compound in distilled water and plotting the peak-height obtained at 1.0 × 10⁻⁴ refractive index unit (full-scale) sensitivity of the detector. Urinary concentrations of sugar probes were calculated from the calibration curves by peak-height analysis.

Results

Fig. 1 shows chromatograms of a pretest sample of urine from a healthy subject (left panel) and the sample from the same subject after ingestion of the test solution (right panel). L-Rhamnose,
mannitol, and lactulose are well resolved within 18 min. No major interference was seen at the retention times corresponding to the peaks of probe sugars. Two small peaks of residual materials with retention times close to those of the l-rhamnose and mannitol peaks were present in the pretest urine. However, the retention times of the residual peaks (6.6 and 9.7 min) differed enough from the retention times of l-rhamnose (6.2 min) and mannitol (9.4 min) to allow separation, although baseline resolution is not achieved. By peak-height analysis these peaks do not interfere with the assessment of the concentrations of l-rhamnose and mannitol. The peak at 5 min appeared in all urines analyzed, its retention time corresponding to that of urea.

When common sugars, including glucose, fructose, sucrose, and lactose were chromatographed individually with probe sugars in the same solution, the only overlap was between the glucose and mannitol peaks. However, as shown in Fig. 2, the glucose peak was resolved with almost baseline resolution from the mannitol peak, thus allowing quantification.

When 30 aliquots of a mixture of l-rhamnose, mannitol, and lactulose at a concentration of 2 mmol/L each were chromatographed, the overall CVs were 2.91%, 2.77%, and 4.80%, respectively.

As shown in Fig. 3, the calibration curves for lactulose, l-rhamnose, and mannitol were linear to 20 mmol/L and gave the following regression equations: lactulose, \( y = 17.1x - 0.85 \) (\( S_{y|x} = 0.80 \)); l-rhamnose, \( y = 9.60x - 0.53 \) (\( S_{y|x} = 0.36 \)); and mannitol, \( y = 12.9x - 0.73 \) (\( S_{y|x} = 0.53 \)).

We also tested the linearity by adding the same concentrations of sugar probes to urine, using the same sample preparation. The result for each sugar was similar to that obtained in aqueous solution, being linear to 20 mmol/L concentration and giving similar regression equations: lactulose, \( y = 16.6x + 0.36 \) (\( S_{y|x} = 0.49 \)); l-rhamnose, \( y = 9.37x - 0.12 \) (\( S_{y|x} = 0.40 \)); and mannitol, \( y = 12.6x - 0.12 \) (\( S_{y|x} = 0.44 \)).

By this method, the minimum detectable concentrations of lactulose, l-rhamnose, and mannitol in urine are 0.05, 0.1, and 0.05 mmol/L, respectively.

Analytical recovery of individual sugars was determined by adding known amounts of probe sugars to the urine collected for 5 h without ingestion of test solution, assaying as above, and comparing the measured concentrations with the estimated concentrations. The recovery of each sugar at 2 mmol/L of estimated concentration was 105.1% ± 3.1% (mean ± SD) for l-rhamnose, 100.2% ± 2.5% for mannitol, and 99.3% ± 3.4% for lactulose (n = 10 assays).

The precision of the assay was determined by repeated measurement of sugars in a urine sample from a healthy subject. As shown in Table 1, the overall CVs ranged from 4.2% to 6.5%. Over several concentrations of probe sugars in urine samples from other subjects, both within-batch and overall CVs were similar to the ranges listed in Table 1 (e.g., for respective mean concentrations of l-rhamnose, mannitol, and lactulose of 1.29, 1.69, and 0.12 mmol/L, overall CVs were 5.0%, 5.1%, and 7.1%, respectively).

### Table 1. Precision of measurement of l-rhamnose, mannitol, and lactulose in a urine sample from a healthy subject.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Within-batch (n = 10)</th>
<th>Overall (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Rhamnose</td>
<td>6.78 ± 0.13 (1.94)</td>
<td>6.78 ± 0.30 (4.47)</td>
</tr>
<tr>
<td>Mannitol</td>
<td>13.7 ± 0.18 (1.31)</td>
<td>13.8 ± 0.58 (4.19)</td>
</tr>
<tr>
<td>Lactulose</td>
<td>1.15 ± 0.03 (2.92)</td>
<td>1.18 ± 0.08 (6.50)</td>
</tr>
</tbody>
</table>
The range (and mean ± SD) of percentage of urinary excretion of each sugar in the 14 healthy subjects studied was 4.1–10.9% (6.2% ± 1.9%) for \(\alpha\)-rhamnose, 8.1–20.2% (14.0% ± 3.4%) for mannitol, and 0.11–0.67% (0.29% ± 0.14%) for lactulose. The excretion ratios of lactulose/\(\alpha\)-rhamnose and lactulose/mannitol in these subjects were 0.023–0.074 (0.047 ± 0.018) and 0.011–0.036 (0.021 ± 0.010), respectively.

**Discussion**

Here we have validated a rapid and convenient HPLC method for quantifying nonmetabolized sugars in urine of healthy children. Under the chromatographic conditions described, \(\alpha\)-rhamnose, mannitol, and lactulose were resolved from each other and from other common carbohydrates present in urine. Some overlap between glucose and mannitol was observed, but the mannitol peak could still be resolved from the glucose peak. Determination of mannitol concentration can therefore be estimated by using peak-height measurement even if a urine sample contains low concentrations of glucose. When significant glucosuria is evident in a urine sample, such as that from a diabetic patient, accurate measurement of mannitol is not possible. Pretreatment of the urine by enzymatic degradation of glucose or the use of a solvent with a higher proportion of acetonitrile as the mobile phase in the assay to increase the retention time of glucose [29] can overcome this problem.

The detection limit of the method is sufficiently low to determine the very low concentration of lactulose in urine, even though the refractive index detection method [24, 26, 28] is less sensitive than pulsed amperomteric detection [25, 27].

Sample preparation with a mixed ion-exchanged resin (Duolite MB 5113) is simple and rapid. Celli et al. [30] used the same commercial resin for desalting urine in their gas-chromatographic method. They recommended using 0.5 g of resin to 1 mL of urine as an optimal ratio of resin/urine; lower ratios compromised the purification, whereas a higher ratio reduced sugar recovery. In our HPLC assay, however, 0.5 g of resin to 2 mL of urine both desalted the urine specimen and provided the highest recoveries of probe sugars. For all of the probe sugars studied, analytical recoveries were close to 100% after the desalting procedure. The slightly higher recovery of \(\alpha\)-rhamnose than of the other probe sugars may be due to enhancement of the \(\alpha\)-rhamnose peak by small amounts of residual material after the desalting procedure; this material may include \(\alpha\)-rhamnose itself, which might be excreted in the urine from the food ingested by the subject in the last 2 h of the 5-h test period.

The percentage of urinary excretion of \(\alpha\)-rhamnose in this study is lower than the mean value in healthy adult subjects given an iso-osmolar test solution [1]. Consequently, the excretion ratio of lactulose/\(\alpha\)-rhamnose we found is slightly higher than previously published data in both adults and children [1, 8, 13, 15]—perhaps because of the effect of osmotic retention or "solvent drag" of mannitol given in iso-osmolar solution [31]. The range of urinary excretion and the lactulose/mannitol ratio in healthy children is similar to published data for normal children as measured by gas–liquid chromatography [7, 9, 11, 16, 30] and HPLC. [27].

Although the ratio of lactulose/\(\alpha\)-rhamnose we determined is higher than other published data, results for both lactulose/\(\alpha\)-rhamnose and lactulose/mannitol show that either ratio provides a useful technique for assessing intestinal permeability [1]. Thus either can be used to assess the mucosal integrity of small bowel in pediatric clinical practice [2], even though \(\alpha\)-rhamnose and mannitol may be absorbed by different pathways.

Other probes such as polyethylene glycol (PEG) and \(^{51}\text{Cr}\)-labeled EDTA have been used to assess permeability in children [2, 3]. However, both PEG and \(^{51}\text{Cr}\)-labeled EDTA are unlikely to be suitable probes in children. Commercial formulations of PEGs consist of polymers of different sizes. PEG-400, for example, contains eight different sizes of molecules, ranging from 194 to 502 Da [32], and substantial variation has been observed in the proportion of each individual polymer excreted in urine after oral intake [33, 34]. This variation affects the reproducibility of results. On the contrary, the sugar permeability test is characterized by good repeatability of the test and of the laboratory assay [33]. \(^{51}\text{Cr}\)-labeled EDTA is unacceptable as the ideal probe because of its radioactivity. By contrast, sugar probes are well defined, are nonradioactive, and provide a noninvasive test that can be performed on multiple occasions in the same subject.

A further advantage of the sugar permeability test is that it allows simultaneous measurement of a range of nonmetabolized sugars such as 3-O-methyl glucose or d-xylene excreted in urine as an index of active intestinal transport [1, 27]. Thus, the sugar permeability test seems to be the most useful technique for assessing small bowel damage and dysfunction in pediatric populations.

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**References**


