Simultaneous HPLC determination with light-scattering detection of lactulose and mannitol in studies of intestinal permeability in pediatrics

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We describe a new HPLC method for the simultaneous determination of lactulose and mannitol in urine, in which cation-exchange chromatography and evaporative light-scattering detection are used. The two sugars are orally administered for the estimation of intestinal permeability in children. Samples were purified by solid phase extraction on a C₁₈ cartridge and subsequent addition of anion-exchange resin. Cellobiose may be used as an internal standard. The chromatographic separation was carried out in 16 min at a flow rate of 0.5 mL/min, using deionized water as the mobile phase. Within-run precision (CV) measured at three concentrations was 1.6–2.3% for lactulose and 1.0–1.9% for mannitol. Between-run CVs were 2.1–4.1% and 1.3–2.7% for lactulose and mannitol, respectively. Analytical recovery of both sugar probes was 97–101%. The detection limits (signal-to-noise ratio ≥ 3) were 0.82 mg/L for lactulose and 0.65 mg/L for mannitol. The lactulose/mannitol ratio in control subjects was 0.024 ± 0.006; in patients with Crohn’s and coeliac diseases in active phase, the ratios were 0.200 ± 0.082 and 0.072 ± 0.025, respectively. The method is rapid, simple, and sensitive, and suitable for determination of intestinal permeability in children.

The measurement of urinary excretion of nonmetabolized sugars has been widely used as a noninvasive method to assess mucosal integrity of the small bowel in children (1–2). This procedure is usually performed by oral administration of two sugars of different molecular size and with different absorption routes. Clear differences in the uptake of mono- or disaccharides in pathological alterations of the small intestine mucosa (3–6) have been demonstrated.

Monosaccharides such as mannitol and L-rhamnose pass through the transcellular routes of aqueous pores, reflecting the degree of absorption of small molecules (0.65 nm). Disaccharides, including lactulose and cellobiose, pass through the intercellular junctional complexes and extrusion zones at the villous tips, reflecting the permeability of large molecules (0.93 nm). The permeabilities of mono- and disaccharides are usually compared and expressed as an excretion ratio such as lactulose/mannitol or lactulose/L-rhamnose in urine samples.

This test has been used in clinical practice for the estimation of intestinal permeability in patients with coeliac disease (7–12), Crohn’s disease (13–15), atopic dermatitis (16–17), cow’s milk protein intolerance (18–19), cystic fibrosis (20–21), various acute and chronic diarrheal diseases (22–24), HIV infection (25), and jejunal bacterial overgrowth associated with immunodeficiency syndromes (26).

Lactulose and mannitol represent ideal compounds for measuring differential sugar absorption because they have a negligible affinity for the monosaccharide transport system and are passively absorbed and not metabolized before urine excretion. Intraindividual differences in gastric emptying, small intestinal transit, and urinary excretion are therefore eliminated (27).

Several enzymatic (28–29), colorimetric (30), and thin-layer chromatographic (31) methods have been developed for the determination of mannitol and lactulose. However most of them are time-consuming and do not allow a simultaneous assay of both sugars. More recently, gas chromatographic and HPLC procedures have been proposed to overcome these problems.

The gas chromatographic determinations require evaporation and derivatization of the samples before injection and limit the choice of probes (32–37).
Several HPLC procedures are available for the determination of sugars in urine. Some methods require a refractive index detector (38–40), which has the advantage of being universal. This type of detector suffers from external variations such as temperature and pressure. In addition, it is not easily adaptable for routine use, and it has poor sensitivity. Therefore pre- (41–43) or postcolumn (44) derivatization methods have been developed in the attempt to overcome these problems. Anion-exchange chromatography in conjunction with pulsed amperometric detection has been used for the rapid and simultaneous determination of sugars in urine or plasma (45–47). The high sensitivity allows injection of very diluted solutions, which leads to lower chromatographic system contamination and longer column life.

A new detector measuring the amount of light scattered from particles or droplets passing through the light beam has been introduced. The detection principles of evaporative light-scattering detectors (ELSDs) involve the following: (a) nebulization of the column effluent to form an aerosol; (b) solvent vaporization in the drift-heated tube to produce a cloud of solute droplets; and (c) detection of solute droplets in the light-scattering cell. This detector seems to be more sensitive and easy to use than the refractive index detector, and it is also compatible with gradient elution in carbohydrate analysis (48–51).

Here we describe a new, rapid, and sensitive procedure using high-performance cation-exchange chromatography and an ELSD for the determination of lactulose and mannitol in urine after oral ingestion. This procedure was applied for the estimation of intestinal permeability in children.

Materials and Methods

CHEMICALS
Lactulose, mannitol, cellobiose, and chlorhexidine were obtained from Sigma Chemical Co. Amberlite IRA-400 resin (Cl⁻ form) and Suprapur calcium nitrate were obtained from Fluka and Merck, respectively. An Alltech 12-port vacuum manifold, Maxi-Clean™ C₁₈ cartridges (600 mg), and Micro-Spin™ centrifuge filters (Nylon 66, 0.2 μm) were supplied from Alltech Associates. HPLC grade methanol was purchased from Carlo Erba. Water was obtained from a MilliQ water purification system (Millipore).

SUBJECTS
Thirty healthy subjects, 13 males and 17 females (ages, 0.4–13 years; mean, 7.4 years) were enrolled as controls; 10 patients with Crohn’s ileocolitis (ages, 13.2–21 years; mean, 14.7 years) and 10 patients with coeliac disease (ages, 1.3–17.9 years; mean, 5.8 years), in the moderate or severe activity phases of their diseases, were also enrolled. The diagnosis of Crohn’s disease activity was based on the Crohn’s disease activity index (52); the diagnosis of coeliac disease was based on the European Society of Pediatric Gastroenterology criteria (53).

INTESTINAL PERMEABILITY TEST
The patients drank a solution containing 5 or 10 g of lactulose and 2 or 5 g of mannitol in 50–100 mL of deionized water (650–675 mmol/kg), according to the age (younger than or older than 12 years, respectively). Urine was collected during the next 6 h, with 1 mL of 200 g/L chlorhexidine added as preservative. One hour after the test was started, the patients were encouraged to drink; after 2 h, liberal intake of food was allowed. The total volume of urine was measured, and a 10-mL portion was stored at −20 °C. The procedure was in accordance with the Helsinki Declaration of 1975, revised in 1983.

STANDARD SOLUTIONS
Stock solutions of lactulose, mannitol, and cellobiose (internal standard) were prepared in water with concentrations of 2000, 8000, and 1500 mg/L, respectively. Stock solutions were stable for 1 month at 4 °C. Standard solutions containing 3.125, 6.25, 12.50, 25, 50, and 100 mg/L lactulose and 12.50, 25, 50, 100, 200, and 400 mg/L mannitol were prepared fresh daily in water. One hundred microliters of each sugar were added to 1.8 mL of water containing 75 mg/L cellobiose and 400 g/L Amberlite IRA-400 resin.

After the samples were vortex-mixed for 10 s and centrifuged for 2 min at 3000g, 400-μL aliquots of supernatant were centrifuged in Micro-Spin centrifuge filters (Nylon 66, 0.2 μm) for 5 min at 3000g, and 50 μL was analyzed by HPLC.

The precision of dilutions were determined by weighing appropriate samples of water 10 times at 20 °C.

HPLC APPARATUS
The model 510 pump, temperature control module, and column heater were all from Waters Associates. The model 465 autosampler and model 450-MT chromatography data system were from Kontron Instruments. The Varex MKIII ELSD was from Alltech Associates.

SAMPLE PREPARATION
Maxi-Clean C₁₈ 600-mg cartridges were conditioned with 5 mL of methanol followed by 5 mL of water. Subsequently, 2–3 mL of urine was passed through the cartridges. The first milliliter of urine was discarded, and the residual volume was collected and diluted 1:1 with water. Samples (200-μL) were diluted with 1.8 mL of water containing 75 mg/L of cellobiose, and 400 g/L of Amberlite IRA-400 resin was added. The mixture was vortex-mixed for 10 s and centrifuged for 2 min at 3000g. The supernatant was collected, and 400-μL aliquots were centrifuged in a Micro-Spin centrifuge cartridge (Nylon 66, 0.2 μm) for 5 min at 3000g. Aliquots (50 μL) were then analyzed by HPLC.
HPLC ANALYSIS
An Alltech 700 CH Carbohydrate Analysis column, (300 mm × 6.5 mm i.d.) 10-μm particle size, equipped with a Benson Carbohydrate BC-100 Ca²⁺ guard column (Alltech Associates) was used. The chromatographic separation was carried out in 16 min at a flow rate of 0.5 mL/min, using HPLC grade water as mobile phase and keeping the column temperature at 85 °C. The eluent was filtered through a 0.45-μm Millipore filter and degassed with helium before use. After optimization of the settings for the Varex MKIII ELSD, the drift tube temperature was set at 120 °C, and the carrier gas flow (air) was set at 41.67 cm³/s. The output signal attenuation and time constant of the recorder were 1/16 and 1 s, respectively.

Results
In Fig. 1, the chromatographic profile of the pre-test urine sample (Fig. 1A) from a patient with glycosuria (120 mg/L), is compared with a chromatogram of urine collected in the course of an absorption test (Fig. 1B). Lactulose, mannitol, and internal standard peaks are well resolved, with retention times of 8.88 ± 0.15 min (n = 25), 13.25 ± 0.21 min (n = 25), and 7.55 ± 0.13 min (n = 25), respectively. Sample pretreatment with 400 g/L of IRA-400 anion-exchange resin (Cl⁻ form) allowed a chromatographic profile without interfering substances at the retention time corresponding to the recorded peaks.

SAMPLE PREPARATION
Cation-exchange columns made of polystyrene divinylbenzene sulfonate resins in calcium form offer an high efficiency and selectivity for carbohydrate analysis, but their use is limited by high cost and fragility to pressure. Accurate and specific sample preparation and the use of an appropriate guard column are needed to eliminate back pressure increases caused by nonpolar compounds adsorbing to the column matrix and/or by clogged frits. Sample purification by solid phase extraction on a C₁₈ cartridge and injection of very diluted solutions minimize the risks of column contamination and allow analysis of up to 2000 injections without any variation of retention time and shape of the recorded peaks in the chromatograms. Periodic regeneration with 0.1 mol/L calcium nitrate every 200 injections is recommended.

CALIBRATION CURVES
The response of an ELSD is nonlinear because of basic light-scattering principles. Therefore, it is necessary to
prepare calibration curves at optimized instrument settings. Calibration curves were plotted using double logarithmic coordinates according to the equations below (54):

\[ y = a \times x^b \]  
\[ \text{LOG}(y) = \text{LOG}(a) + \text{LOG}(x) \times b \]

where \( y \) is the detector response, \( x \) is the sample concentration, and \( a \) and \( b \) are numerical coefficients. Calibration curves were plotted considering concentrations ranging from 3.125 to 100 mg/L (3.125, 6.25, 12.50, 25, 50, and 100 mg/L) for lactulose and from 12.50 to 400 mg/L (12.50, 25, 50, 100, 200, and 400 mg/L) for mannitol. Each point was established from an average of five determinations.

Equation curves were \( \text{LOG}(y) = -0.6347 + 1.2562 \times \text{LOG}(x) \) (\( S_{y|x} = 0.0179 \)) and \( \text{LOG}(y) = -0.7077 + 1.2733 \times \text{LOG}(x) \) (\( S_{y|x} = 0.0178 \)) for lactulose and mannitol, respectively.

When the internal standard cellobiose was used in the calibration procedure, the compounds were quantitated as peak-area ratios (sugar-to-internal standard) vs concentration, and the equation curves were \( \text{LOG}(y) = -0.6299 + 1.2567 \times \text{LOG}(x) \) (\( S_{y|x} = 0.0178 \)) and \( \text{LOG}(y) = -0.7056 + 1.2724 \times \text{LOG}(x) \) (\( S_{y|x} = 0.0178 \)) for lactulose and mannitol, respectively.

The correlation coefficient (\( r \)) was 0.999 for both sugar probes. The lower detection limits (signal-to-noise ratio = 3) were 0.82 mg/L for lactulose and 0.65 mg/L for mannitol.

**INTERFEROES**

To verify interferences by other carbohydrates, an aqueous solution containing 60 mg/L each of cellobiose, lactulose, mannitol, glucose, fructose, and galactose was analyzed by carrying out the proposed procedure. No interferences with the peaks of either sugar probe or with the internal standard peak were observed in the chromatogram (Fig. 2).

**PRECISION**

The within-day and the total (between-day) imprecision of the method were assessed by analyzing, 10 times per day for 10 days, three urine samples with different lactulose and mannitol content (Table 1).

**ACCURACY**

Recovery was determined by a standard addition technique. Urine samples were passed through the C18 cartridges, treated with Amberlite IRA-400 resin, and then supplemented with three known different amounts of
lactulose and mannitol. The percentage of recovery was obtained by measuring the sugar concentration in the samples before and after the addition. The analytical recovery ranged from 99.6% to 99.9% for lactulose and from 97.3% to 100.7% for mannitol (Table 2).

**Clinical Results**

The excretion ratio lactulose/mannitol (L/M) in the urine of healthy subjects was 0.024 ± 0.006 (range, 0.011–0.40; Table 3). The control subjects showed a mean lactulose recovery of 0.33% (range, 0.07–0.522%; SD, 0.13) and a mean mannitol recovery of 14.12% (range, 3.92–29%; SD, 6.63). Lactulose excretion was increased (range, 0.73–6.80%) in patients with active Crohn’s disease, whereas lactulose urinary recoveries of lactulose and mannitol were 0.53% (range, 0.31–0.68%) and 8.00% (range 4.71–11.72%), respectively.

**Discussion**

Small bowel diseases are difficult to investigate; jejunal and ileal anomalies often require invasive examinations because noninvasive tests are almost always nonspecific for the diagnosis of diseases involving damage of the intestinal mucosal.

Nevertheless, in various diseases affecting the small bowel, the damaged mucosa is abnormally permeable, and the absorption of molecules may be increased through a "leak", or decreased because of diminished surface area. This may lead to increased permeability of larger molecules and/or to impaired transcellular passage of smaller molecules. Gastric emptying, intestinal transit, and renal function reduce the correlation between absorption of a single sugar and the degree of mucosal damage; therefore, the xylose test is today considered inadequate. Measurement of the urinary excretion of two or more orally administered nonmetabolized sugar probes of different sizes (mono- and disaccharides) has been established as an excellent noninvasive approach for the assessment of intestinal permeability in humans.

We present here an HPLC method that has the advantages of being rapid, precise, and accurate. The use of 400 g/L of Amberlite IRA-400 ion-exchange resin (Cl⁻ form) in sample preparation eliminates any interfering substance in the chromatograms and does not require additional cation-exchange resin purification, which is necessary for other methods (37, 40, 45).

A C₁₈ solid phase extraction cartridge was used to prevent damage and/or reduction of performance of analytical columns: we can make up to 2000 injections without any variation of retention time and shape of the recorded peaks in the chromatograms. Periodic regeneration with 0.1 mol/L calcium nitrate is, however, required every 200 injections.

The column we used, packed with a microparticulate cation-exchange gel in calcium form (38–39), is very selective for mono- and disaccharides and, compared with amino-bonded silica columns, presents the advantage of better separation of mannitol from glucose peaks, even in diabetic patients with high glycosuria (Fig. 2) (40). Moreover the cation-exchange column does not present the typical disadvantages of amine-modified silica column because of low chemical stability and the formation of Schiff bases.

Although the analytical recovery of both sugars is satisfactory, ranging from 97% to 101%, precision and accuracy of the method can be increased by the use of an internal standard. When urine samples containing <10 mg/L of lactulose and/or <25 mg/L of mannitol are analyzed, the utilization of cellobiose as an internal standard can improve the within-day and the total (between-day) precision up to 30% and can increase the analytical recovery of both sugars.
recovery of mannitol by 1% (data not shown). Cellobiose was chosen because it has a retention time different from the other eluted components and it does not occur naturally in urine. The use of ELSDs is very flexible because of the absence of baseline drift, rapid equilibration, easy automation, low influences of external variations such as temperature and pressure, and compatibility with gradient elution. The detection limits of a light-scattering detector lie between those of refractive index and pulsed amperometric detectors. In fact, in HPLC methods that use refractive index detectors, the detection limits for both sugars ranged from 10 to 20 mg/L (38–40), whereas the minimum detectable concentrations (signal-to-noise ratio = 3) in our method are 0.82 mg/L and 0.65 mg/L for lactulose and mannitol, respectively. These data are comparable with the values obtained with several HPLC methods that use pulsed amperometric detectors (45).

The disadvantage because of the absence of detector response linearity can easily be overcome by appropriate calibration with logarithmic coordinates and appropriate identification of concentrations ranges. Moreover a new calibration procedure is required every 25 sample injections.

Although the ELSD presents the advantage of being sensitive and easy to use, only one HPLC method using light-scattering detection is reported in literature (51). This method requires time-consuming sample preparation by thin-layer chromatography, and the chromatographic separation is performed with the use of an amino-bonded column. The detection limits of the method are not reported.

The excretion ratio of lactulose/mannitol in healthy control subjects (mean ± SD) is 0.024 ± 0.006; these values are comparable with those reported by other authors who use test similar protocols for the amounts of administered mannitol and lactulose and for the period of urine collection (25, 28, 37, 40).

In conclusion, this technique is rapid, sensitive, and easy to automate for the study of intestinal permeability in clinical practice.

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### Table 3. Results of the lactulose/mannitol test in control subjects and in patients with Crohn’s and coeliac diseases, both in active phase.

<table>
<thead>
<tr>
<th>Urinary recovery</th>
<th>Controls (n = 30)</th>
<th>Crohn’s disease (n = 10)</th>
<th>Coeliac disease (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactulose, %</td>
<td>0.33 ± 0.13</td>
<td>2.25 ± 2.10</td>
<td>0.53 ± 0.14</td>
</tr>
<tr>
<td>Mannitol, %</td>
<td>14.12 ± 6.63</td>
<td>11.91 ± 7.95</td>
<td>8.00 ± 0.13</td>
</tr>
<tr>
<td>L/M ratio</td>
<td>0.024 ± 0.006</td>
<td>0.200 ± 0.082</td>
<td>0.072 ± 0.025</td>
</tr>
</tbody>
</table>

### References

20. Penny DJ, Inglis CB, Boulton P, Walker-Smith JA, Basheer SM.


