Measurement of sugar probes in serum: an alternative to urine measurement in intestinal permeability testing

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The percentage dose of lactulose and mannitol excreted in urine after oral ingestion is used as a noninvasive method of assessing small intestinal permeability. The collection of incomplete or inaccurately timed urine samples can lead to errors in estimation of sugar probe molecules. We describe an HPLC method for the simultaneous determination of lactulose and mannitol in serum after oral ingestion of test sugars. We applied the test to healthy volunteers and to subjects undergoing jejunal biopsy for suspected gluten-sensitive enteropathy. The ratio of concentrations of lactulose and mannitol in serum discriminated well between subjects with a normal biopsy and those with villous atrophy, discrimination being best at 90 min postdose. The results agree well with lactulose:mannitol ratios determined in urine (r = 0.88), and the two methods can be used interchangeably. The determination of mannitol and lactulose in serum provides an acceptable alternative to urine collection and may be particularly useful in young children. It also reduces the time spent on the investigation from 5 h to 90 min.

INDEXING TERMS: mannitol • lactulose • celiac disease • pediatric chemistry • chromatography, liquid • electrochemical detection

Determination of the urinary excretion of nonmetabolized sugar probe molecules after oral administration has been used to assess the permeability of the small bowel mucosa in various intestinal diseases [1–3]. Lactulose, mannitol, and 3-O-methyl glucose are used to assess permeation, and passive and active transport, respectively, across the small intestinal mucosa. Expressing the ratios of these sugars in a timed urine sample provides an index of intestinal permeability.

The difficulty in quantifying carbohydrates in urine and serum has limited the widespread use of these tests. The introduction of HPLC with pulsed electrochemical detection (PED) has enabled the rapid and simultaneous determination of multiple sugar probe molecules in urine [4]. Nevertheless, an inherent source of error in such tests is the ability to collect complete and accurately timed urine samples, particularly in young children and neonates.

We have therefore developed a method to measure lactulose and mannitol in serum samples after ingestion of test sugars. Here we describe the method and compare its results with urine measurements and the histology of jejunal biopsy specimens.

Materials and Methods

Lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) and melibiose (6-O-α-D-galactopyranosyl-D-glucopyranose) were supplied by Sigma Chemical Co., Poole, UK. Mannitol, sodium hydroxide (500 g/L), zinc acetate (anhydrous), Amberlite resins IR 120 H+ and IRA 400 Cl−, and 5-sulfosalicylic acid were obtained from BDH, Poole, UK.

A quaternary-gradient HPLC system with a pulsed electrochemical detector, a CarboPac PA1 (250 × 40 mm) anion-exchange column, and a PA1 guard column were supplied by Dionex UK (Camberley, Surrey, UK). The chromatographic analysis and sample preparation procedures are essentially the same as previously described with minor modifications [4, 5].

Eluent Preparation

After deionized water (968 mL, 18 Mohm cm−1) was degassed with helium for 20 min, 3 mL of 0.5 mol/L zinc acetate was added and the solution was thoroughly mixed and sparged for 5 min. To this was added 28 mL of 500 g/L sodium hydroxide with thorough mixing and sparging for a further 5 min to give a final eluent composition of 360 mmol/L NaOH and 1.5 mmol/L zinc acetate. A second eluent of deionized water was sparged for 20 min with helium. Both eluents were stored under helium at 34.5 kPa. A final eluent concentration of 120 mmol/L NaOH and 0.5 mmol/L zinc acetate was achieved by mixing eluent 1 with eluent 2 in proportions of 33:66:7 (by vol) on the chromatographic system.

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SAMPLE PREPARATION

Urine. Depending on collection volume, the urine samples were diluted between 1:2 and 1:20 with deionized water to a volume of 1 mL. Then 1 mL of internal standard (melibiose, 250 mg/L in deionized water) was added and the mixture was desalted with mixed ion-exchange resin (Amberlite IR 120 H+ and IRA 400 Cl-) taking up to one-third of the volume. Samples were centrifuged, and 25 μL of supernate was injected onto the column.

Serum. A 200-μL volume of serum was added to 200 μL of internal standard (melibiose 250 mg/L). To this, 200 μL of ice-cold 5-sulfosalicylic acid (35 g/L) was added to precipitate the plasma proteins. After standing on ice for 20 min, the samples were centrifuged at 900g for 5 min and the supernates were removed. Each supernate was desalted with the mixed ion-exchange resin and centrifuged again; 25 μL of this supernate was then injected onto the column.

CHROMATOGRAPHIC ANALYSIS

The samples were eluted with the NaOH:zinc acetate eluent at a flow rate of 1 mL/min at 25 °C. After every five serum samples, the columns were given a 5-min wash with 1 mol/L NaOH to maintain the stability of the retention times.

Analytes were detected with a pulsed electrochemical detector using a gold working electrode and silver/silver chloride reference electrode. The detection potential was −0.01 V (0–0.5 s), the oxidation potential was +0.75 V (0.51–0.64 s), the reduction potential was −0.75 V (0.65–0.75 s), and the integration period was 0.05 to 0.5 s. Peak heights were measured with internal standardization.

SUBJECTS AND PROTOCOL

Ten healthy Caucasian subjects, seven men and three women [mean age 34.2 ± 6.6 (SD) years] with no history of gastrointestinal disease, acted as control subjects. The study subjects were 28 Caucasians, 16 women and 12 men (mean age 41.6 ± 14.5 years), who were undergoing investigation for suspected gluten-sensitive enteropathy (celiac disease). All of the study subjects underwent a Crosby capsule jejunal biopsy on the same day as the intestinal permeability test. All subjects gave informed consent, and the study had the approval of the Ethical Committee of Glasgow Royal Infirmary. For ethical reasons, none of the control subjects underwent intestinal biopsy.

All subjects were fasted overnight (10 h), being allowed only water to drink. On the morning of the test the subjects provided a pretest urine and blood sample. They then drank the test solution, which contained 10 g of lactulose and 5 g of mannitol dissolved in 300 mL of water (osmolality 696 mmol/kg). Blood samples were collected from a forearm vein at 30, 60, 90, and 120 min postingestion. Serum was separated from the blood samples within 30 min, removed, and stored at −20 °C until analysis. Urine was collected over a 5-h period into a container with 0.5 mL of thimerosal (1 g/L) as preservative. A 20-mL aliquot of this urine was taken and stored at −20 °C until analysis.

All subjects avoided ingesting alcohol or nonsteroidal anti-inflammatory drugs for at least 24 h before the test.

Jejunal biopsies were fixed in formal saline (formaldehyde 100 g/L, NaCl 150 mmol/L) and assessed by a consultant histopathologist unaware of the intestinal permeability test result.

STATISTICAL ANALYSIS

Results for the subject groups followed a normal distribution, so we used parametric methods to compare the groups. Results are expressed as mean ± SD, and subjects groups were compared by one-way analysis of variance (ANOVA).

RESULTS

The analytical validity of the analysis of mannitol and lactulose in serum and urine has been described previously [4, 5]. The assay is linear to 125 mg/L for mannitol and 40 mg/L for lactulose, with a detection limit of 0.4 mg/L in urine and serum. Analytical recovery ranged between 89% and 105% for all analytes. Overall CVs ranged between 1.8% and 8.5% at various analyte concentrations.

CONTROL SUBJECTS

Mean urinary excretion of mannitol was 11.8% (SD ± 6.2%) and lactulose 0.15% (±0.09%), lactulose:mannitol with a mean (L:M) ratio of 0.02(±0.014). Mean serum concentrations and their respective ratios at 30, 60, 90, and 120 min postingestion are shown in Table 1.

GASTROINTESTINAL SUBJECTS

All 28 subjects underwent a jejunal biopsy procedure, but in 2 the procedure failed and no biopsy was obtained. Six subjects were graded as having total villous atrophy (flat biopsy), with an increase in interepithelial lymphocytes consistent with a diagnosis of celiac disease. One further subject had slight villous blunting with an increase in interepithelial lymphocytes. The 10th subject with an abnormal biopsy showed mild shortening of villi with a patchy increase in interepithelial lymphocytes. The remaining 18 subjects all had normal biopsy results. Therefore,

| Table 1. Serum and urine concentrations of sugar probes and calculated lactulose:mannitol ratio at various times after ingestion by control subjects (n = 10). |
|-------------------|-----------------|-----------------|---------------|
| Time postingestion* | Mannitolb | Lactuloseb | L:M ratio |
| Urine Serum | Mean (± SD) | Mean (± SD) | Mean (± SD) |
| 0 | 5.3 (2.53) | NDc | — |
| 30 | 66.9 (20.2) | 0.91 (0.55) | 0.014 (0.005) |
| 60 | 105.7 (33.8) | 1.27 (0.72) | 0.013 (0.005) |
| 90 | 116.1 (37.5) | 1.40 (0.57) | 0.013 (0.006) |
| 120 | 109.5 (36.2) | 1.90 (0.66) | 0.020 (0.008) |

* After ingestion of 10 g of lactulose and 5 g of mannitol; time is 5 h for urine, minutes for serum.

b Units are % excretion for urine and mg/L for serum.

c Not detected.
we grouped the subjects on histological evidence and compared serum and urine sugar measurements on this basis. Because only two subjects had minor histological changes, these were not considered separately but were grouped with the subjects having abnormal biopsy results.

The 18 subjects with normal biopsy results had a urine mannitol excretion of 12.6% (±4.6%), a lactulose excretion of 0.27% (±0.13%), and an L:M ratio of 0.021 (±0.013), similar to that of the control subjects. The eight subjects with abnormal biopsy results had urine excretion of mannitol of 9.0% (±3.4%), lactulose 0.65% (±0.26%), and L:M ratio 0.146 (±0.10). The L:M ratio was significantly higher than for the control subjects and those subjects with normal biopsy results (P = 0.001).

Results for sugar probes at 60, 90, and 120 min postingestion in subjects with normal and abnormal biopsies are shown in Table 2. Mannitol concentrations in serum were significantly lower at 60, 90, and 120 min in subjects with an abnormal biopsy result compared with controls and subjects with normal biopsy results (P = 0.01). Lactulose concentrations in serum were higher in the abnormal biopsy group than both other groups at 90 and 120 min postingestion (P = 0.03), and the resulting serum L:M ratio was significantly higher at 60, 90, and 120 min: P = 0.002 at 60 min, and P = 0.001 at 90 min.

Fig. 1 shows the L:M ratio in urine and serum from all the subjects at various times, indicating that the best discrimination among the groups occurred at 90 min post sugar ingestion (P = 0.001). The two subjects with only minor histological changes on their biopsy specimens had L:M ratios at 60 and 90 min that were within the reference range of the healthy subjects.

The correlation between the L:M ratios in urine and serum (at 90 min) was good (r = 0.88). Fig. 2, which depicts a bias plot for the L:M ratio in serum at 90-min and 5-h urine samples, shows that the limits of agreement between the two methods (mean ± 2SD) are acceptable; i.e., the two methods can be used interchangeably.

**Table 2. Serum concentrations and urinary excretion of mannitol and lactulose and their respective ratios after ingestion in 26 subjects being investigated for gluten-sensitive enteropathy.**

<table>
<thead>
<tr>
<th>Time postingestion*</th>
<th>Mannitol*</th>
<th>Lactulose*</th>
<th>L:M ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal biopsy results (n = 18)</td>
<td></td>
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<tr>
<td>Urine</td>
<td>12.6 (4.6)</td>
<td>0.27 (0.13)</td>
<td>0.021 (0.013)</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.6 (1.56)</td>
<td>NDc</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>60.7 (20.1)</td>
<td>1.29 (0.68)</td>
<td>0.024 (0.012)</td>
</tr>
<tr>
<td>90</td>
<td>53.6 (22.5)</td>
<td>1.58 (0.88)</td>
<td>0.028 (0.016)</td>
</tr>
<tr>
<td>120</td>
<td>51.2 (21.7)</td>
<td>1.72 (1.06)</td>
<td>0.037 (0.021)</td>
</tr>
<tr>
<td>Abnormal biopsy results (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>9.02 (3.4)</td>
<td>0.65 (0.26)</td>
<td>0.146 (0.10)f</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.0 (3.4)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>27.8 (17.9)e 2.0 (±1.3)</td>
<td>0.109 (0.08)</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>27.6 (14.7)e 4.9 (±2.7)e 0.229 (0.186)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>18.3 (16.6)e 3.0 (1.8)e 0.201 (0.162)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* After ingestion of 10 g of lactulose and 5 g of mannitol; time is 5 h for urine, minutes for serum.

* Units are % excretion for urine and mg/L for serum.

* Not detected.

* and † Significantly different from control group and subjects with normal biopsy results: ‡ P = 0.01, † P = 0.03, ‡ P = 0.002, and † P = 0.001.

**Fig. 1.** Lactulose:mannitol ratios in urine and serum (60 and 90 min postingestion) in control subjects, subjects with normal intestinal biopsy results, and subjects with abnormal intestinal biopsy results. Horizontal bars represent the mean ratio in each group. Controls (O), abnormal biopsy results (●), and normal biopsy results (○).

**Fig. 2.** Bias plot between urine and serum lactulose:mannitol ratios at 90 min postingestion.

**Discussion**

Measurement of sugars in urine after oral loading is well established in the noninvasive investigation of small intestinal absorption pathways in both adults and children [1–4]. HPLC with PED provides a sensitive and specific method for the analysis of sugars in urine and serum.

One major source of error in this type of investigation lies in the ability to provide an accurate urine collection, especially in young children [6]. The use of HPLC with PED to determine
probe markers in serum at various time points after oral ingestion allows the calculation of an L:M ratio without the need to resort to urine collection. The ratio of concentrations of sugars in serum discriminates clearly between subjects with normal mucosal architecture and those with villous atrophy. Moreover, the ratio of sugars in serum correlates well with the 5-h urine ratio, and results of the two methods can be used interchangeably.

Serum L:M ratios distinguished between subjects with villous atrophy and those with normal biopsies at 60, 90, and 120 min postingestion, with the ratio at 90 min giving the best discrimination. However, subjects with only minor histological changes on biopsy can have an L:M ratio within the reference range of normal in both serum and urine.

The results presented here show that measurement of lactulose and mannitol in serum at 90 min postoral loading provides an acceptable alternative to urine collection, and may be particularly useful in young children or neonates. It also has the advantage of reducing the time taken for the investigation from 5 h to 90 min.

References