Simultaneous Quantification of Mannitol, 3-O-Methyl Glucose, and Lactulose in Urine by HPLC with Pulsed Electrochemical Detection, for Use in Studies of Intestinal Permeability

J. Anne Kynaston, Simon C. Fleming, Michael F. Laker, and Andrew D. J. Pearson

The percentage of an oral dose of mannitol, 3-O-methyl glucose, and lactulose excreted in urine is used in noninvasive investigation of active and passive intestinal mucosal transport. We developed a high-pressure liquid-chromatographic method involving anion exchange and pulsed electrochemical detection that allows the simultaneous determination of all three sugar probes in urine. Sample preparation is simple: diluting, mixing with internal standard (melibiose), and desalting. With use of a Dionex 250 × 40 mm Carboyac PA-1 column and elution with an isocratic mixture of 120 mmol/L NaOH and 0.5 mmol/L zinc acetate, all sugars were resolved within 10 min. The standard curve of the method is linear to the following concentrations: mannitol 125 mg/L, 3-O-methyl glucose 300 mg/L, and lactulose 40 mg/L. The minimal detectable concentration of lactulose is 0.4 mg/L. Analytical recovery of the sugars is between 88.0% and 99.5%. The precision of estimation (CV) ranges from 1.7% to 5.6% overall. Reference intervals were established from results for 28 healthy children. The method is adaptable for the study of carbohydrates at low concentrations in other biological fluids.

Indexing Terms: chromatography, anion-exchange, pediatric chemistry, reference interval

The measurement of the urinary excretion of orally administered nonmetabolized sugar probe molecules is well established in the noninvasive investigation of small intestinal absorption pathways and mucosal integrity in a variety of clinical situations (1). Such methods are applicable to pediatric practice, and several probe markers—including mannitol, 3-O-methyl glucose, and lactulose—have been used to assess active and passive transport in children with intestinal dysfunction (2) or pancreatic insufficiency (3) and in those receiving cytotoxic chemotherapy (4).

Mannitol, a low-molecular-mass sugar alcohol, is thought to diffuse predominantly through small water-filled pores in the enterocyte cell membrane; lactulose, a synthetic disaccharide, traverses the mucosa via intercellular pathways between tight junctions (5). Expressing the relative amounts of these substances excreted in urine as a ratio overcomes the potential influences of altered gastric emptying and transit time, intraluminal dilution, and renal impairment, because both probes are affected equally (6, 7). 3-O-Methyl glucose, a synthetic monosaccharide, is absorbed similarly to glucose and gives an index of active intestinal transport (8).

Quantification of sugars in urine demands a sensitive and selective method of detection, particularly in view of their structural similarities, the low concentration of the probe in urine, and the complex sample matrix. The use of anion-exchange HPLC with pulsed amperometric detection (9) has enabled rapid and simultaneous determination of lactulose and mannitol in urine (10). This method obviates the need for separate mono- and disaccharide analyses in thin-layer (11) and gas-liquid (GC) chromatographic (12, 13) and enzymatic techniques (14, 15). The derivatization procedures necessary for GC and HPLC with ultraviolet/visible detection (16) are not required.

However, in applying the method of Fleming et al. (10) in testing intestinal permeability in children whose urine may contain substantial quantities of lactose, we found that the co-elution of lactulose and lactose may result in errors in estimation. We have developed an HPLC method that overcomes this problem and allows the determination of mannitol, 3-O-methyl glucose, and lactulose in urine. Detection is by pulsed electrochemical detection (PED), a modification of pulsed amperometric detection that improves the signal-to-noise ratio, producing greater baseline stability. In addition, we established the reference intervals for these sugars in healthy children.

Materials and Methods

Materials

Lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) and 3-O-methyl glucose (3-O-methyl-α-D-glucopyranose) were supplied by Sigma Chemical Co., St. Louis, MO. Mannitol and melibiose (6-O-α-D-galactopyranosyl-D-glucopyranose) were supplied by BDH, Poole, UK.

HPLC-grade sodium hydroxide (500 g/L), zinc acetate (anhydrous), and Amberlite resin IR120 H⁺ were obtained from BDH.

An HPLC gradient carbohydrate analysis system with an eluent degassing module, a chromatography module (250 × 40 mm Carboyac PA-1 anion-exchange column), a quaternary gradient pump, and a pulsed electrochemical detector were purchased from Dionex UK, Camberley, Surrey, UK.

Procedures

Eluent preparation. In addition to the requirements of filtered 18 MΩ cm⁻¹ deionized water degassed with
helium to remove dissolved carbon dioxide, strict adherence to order of operations in eluent preparation is necessary to avoid zinc acetate precipitation (personal communication, A. Adam, Dionex Corp., 1992). Degas deionized water (989.4 mL) with helium for 20 min. Add 1 mL of 0.5 mol/L zinc acetate solution and thoroughly mix, then add 9.6 mL of NaOH (500 g/L) and degas for a further 10 min.

Sample preparation. Depending on the urine collection volume, dilute the samples between 10- and 40-fold with deionized water to a volume of 1 mL. After adding 1 mL of internal standard (melibiose 250 mg/L), desalt the mixture with washed hydrogen ion-exchange resin (occupying 50% of the volume), mix thoroughly, and centrifuge. Similarly prepare appropriate concentrations of analytical standards.

HPLC analysis. Inject 25 μL of supernate onto the column and elute isocratically with a solution of 120 mmol/L NaOH and 0.5 mmol/L Zn acetate at a flow rate of 1 mL/min at 20 °C. Monitor the effluent by FPD with integrated amperometry, using a detection potential of −0.01 V (0–0.5 s), an oxidation potential of +0.75 V (0.51–0.64 s), and a reduction potential of −0.75 V (0.65–0.75 s), with an integration period of 0.05 to 0.5 s.

Quantification. Peak-height analysis and peak-height ratios with internal standardization were used to quantify the results.

Comparison study. Twenty test urine samples from children being treated for acute lymphoblastic leukemia were analyzed by this method and by GC methods (12, 13). In view of the different dilution factors used in sample preparation for GC and HPLC, we compared the concentrations of sugars estimated by each method to have been present in urine before dilution. Results were compared by simple-regression analysis and Bland–Altman bias plots (17).

Reference Interval Subjects

We studied 28 healthy children (13 boys, 15 girls), ranging in age from 3.5 to 16 years (median 9 years), with no significant medical histories or gastrointestinal symptoms for ≥2 weeks. After an overnight fast, a pretest urine sample was collected, and a test solution (5 g of mannitol, 2 g of 3-O-methyl glucose, and 5 g of lactulose in 100 mL of water; osmolality 696 mmol/kg), 80 mL/m² of body surface area, was ingested. Fasting continued for a further 2 h, after which eating and drinking were allowed. All urine for 5 h from the time of ingestion was collected into a bottle containing 0.5 mL of thimerosal, 1 g/L, as preservative. The urine volume was recorded and 20 mL of it was stored at −20 °C until analysis. Reference intervals were determined as the mean ± 2 SD of these measurements.

Results

Assay Evaluation

Linearity. The linearity of the method was tested by adding known amounts of sugars to urine, while maintaining a constant concentration of internal standard. The results varied linearly for each sugar to the following concentrations: mannitol 125 mg/L, 3-O-methyl glucose 300 mg/L, and lactulose 40 mg/L. Standard curves were represented by the following equations: mannitol, \( y = 0.00622x + 0.0185 \) (SEE 0.035); 3-O-methyl glucose, \( y = 0.0021x + 0.0001 \) (SEE 0.005); and lactulose, \( y = 0.00144x + 0.002 \) (SEE 0.051).

Limit of detection. Lactulose was detectable at 0.4 mg/L in urine.

Analytical recovery. Recovery was determined by adding known amounts of sugars to urine and comparing the concentration estimated with the amount added. The results (Table 1) ranged from 89.0% to 99.5% for all sugars.

Precision. The imprecision of the assay was determined by the repeated measurement of sugars from a test urine sample. As Table 2 shows, the overall coefficients of variation (CVs) ranged from 1.8% to 5.6%.

Interferences. No nonsugar peaks were found with the same retention time as the probe markers. All sugars in urine were well resolved by 10 min, as indicated in chromatograms from a healthy child who ingested the test sugars (Figure 1). Testing sugar solutions showed a partial overlap of internal standard (melibiose 250 mg/L) with fructose at concentrations of 100 g/L. However, no fructose was detectable in the 27 prepared test urine samples.

Comparison with gas chromatography. The correlation coefficients between the two methods were mannitol, \( r = 0.98 \); 3-O-methyl glucose, \( r = 0.99 \); and lactulose, \( r = 0.92 \). Figure 2 shows for each sugar the bias plot of the difference in the concentrations estimated by GC and HPLC plotted against the mean of the concentra-

| Table 1. Analytical Recoveries of Mannitol, 3-O-Methyl Glucose, and Lactulose Added to Urine |
|---|---|---|
| Sugar | Amount added, mg/L | Measured conc, mg/L | Recovery, % |
| Mannitol | 31.25 | 28.5 ± 0.2 | 91.3 ± 0.7 |
| | 62.5 | 55.6 ± 0.8 | 89.0 ± 0.9 |
| | 125 | 111.4 ± 1.4 | 89.1 ± 1.1 |
| 3-O-Methyl glucose | 75 | 66.8 ± 0.7 | 89.1 ± 0.9 |
| | 150 | 149.2 ± 4.8 | 99.5 ± 3.1 |
| | 300 | 275.6 ± 6.0 | 91.9 ± 2.0 |
| Lactulose | 6.25 | 6.1 ± 0.4 | 97.6 ± 6.8 |
| | 12.5 | 12.1 ± 0.7 | 97.1 ± 6.0 |
| | 25 | 22.3 ± 0.4 | 89.1 ± 1.6 |
| Melibiose | 250 | 244.9 ± 2.7 | 98.0 ± 1.1 |

| n = 10 each. |

| Table 2. Precision of Estimates of Mannitol, 3-O-Methyl Glucose, and Lactulose from a Single Test Sample |
|---|---|---|
| | Mean ± SD, mg/L (and CV, %) |
| | Within batch (n = 10) | Overall (n = 20) |
| Mannitol | 104.3 ± 0.42 (0.4) | 107.8 ± 2.70 (2.5) |
| 3-O-Methyl glucose | 226.0 ± 0.90 (0.4) | 222.6 ± 3.92 (1.76) |
| Lactulose | 3.08 ± 0.15 (4.9) | 3.23 ± 0.18 (5.6) |
The limits of agreement (defined as the mean difference ± 2SD) between GC and HPLC are acceptable, indicating that the two methods can be used interchangeably.

Reference Intervals

Reference intervals determined for the percentage of sugar excreted during the 5-h period were mannitol 5.0–22.0% (mean 13.6%), 3-O-methyl glucose 20.5–63.5% (mean 42.0%), and lactulose 0.069–0.393% (mean 0.231%). The ratio of % lactulose:% mannitol was 0.006–0.030 (mean 0.018). No correlations were found between age and the percentage of dose excreted for mannitol (r = 0.065, P = 0.743), 3-O-methyl glucose (r = 0.206, P = 0.294), or lactulose (r = 0.275, P = 0.165) or for the lactulose:mannitol ratio (r = 0.361, P = 0.064). Reference intervals obtained by GC (3, 4) in children were 9.2–22.0% (mean 16.7%) for mannitol, 30.4–64.6% (mean 48.3%) for 3-O-methyl glucose, 0.058–0.45% (mean 0.281%) for lactulose, and 0.005–0.029 (mean 0.017) for the lactulose:mannitol ratio.

Discussion

For the purposes of urinary carbohydrate determination applied to intestinal permeability studies, HPLC with anion-exchange chromatography and PED (10) offers much better sensitivity than the methods based on refractive index detection (18, 19). The HPLC-PED method described here is precise, reproducible, and highly sensitive, and the inclusion of zinc acetate in the eluent promotes the separation of lactulose and lactose—a potential problem in the pediatric age group. Sample preparation is simple and fast. Analysis time is short, with all sugar probes well resolved by 10 min; thus, many samples can be processed in one day. No interference problems were encountered in our study; however, an alternative internal standard may be needed in the rare situations of essential fructosuria and hereditary fructose intolerance.

Good correlations have been shown between this method and GC for the estimations of mannitol, 3-O-methyl glucose, and lactulose. The bias plots (Figure 2) show good limits of agreement for mannitol and 3-O-methyl glucose. The limits of agreement are a little wider for lactulose, and this may be reflected by greater imprecision in both methods for the determination of lactulose.

The overall CVs for lactulose determination by HPLC were 5.6% and by GC 5.5%, compared with 2.5% (HPLC) and 4.3% (GC) for mannitol and 1.8% (HPLC) and 4.2% (GC) for 3-O-methyl glucose. The greater imprecision in the assay of lactulose compared with those of mannitol and 3-O-methyl glucose is not altogether surprising, because lactulose concentrations in urine are 100 times lower than those of mannitol and 3-O-methyl glucose.
less than those of the other two sugars. Another explanation may relate to overlapping with the peak for sucrose, a recognized problem in GC (12). Despite the slightly wider limits of agreement for lactulose, the limits are narrow enough for the results of the HPLC and GC methods to be used interchangeably. Further evidence in support of this is shown by the fact the reference ranges we obtained by HPLC-PED were very similar to those determined by GC methods for children (2–4) and by HPLC-pulsed amperometric detection for adults (10).

In the presence of a small sample population and on the basis of one child's results, it would have been invalid to conclude that the percentage of lactulose excreted and the lactulose:mannitol ratio were dependent on the age of a child. A larger study would be necessary to address this question; however, except for neonates and elderly people, mucosal permeability has not been reported to vary with age in healthy individuals (18).

We found that in all the children monitored by both methods, the values obtained by GC and HPLC ran in parallel over the treatment period. At no time were there discrepancies between the HPLC and GC results (i.e., one being normal and the other abnormal), and no child had overt clinical evidence of malabsorption. Therefore, we believe that both methods are accurate in predicting changes in intestinal permeability.

An additional advantage of this method of carbohydrate analysis is that it allows flexibility in the simultaneous measurement of a range of sugars present in urine, and is adaptable for the study of carbohydrates at low concentrations in other biological fluids.

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References