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Rapid and Simultaneous Determination of Lactulose and Mannitol in Urine, by HPLC with Pulsed Amperometric Detection, for Use in Studies of Intestinal Permeability

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The lactulose/mannitol dual sugar absorption test is a non-invasive test of intestinal permeability. Its widespread use has been limited by the difficulties of analysis for carbohydrates in urine at low concentrations. We describe a "high-pressure" liquid-chromatographic method for determining lactulose and mannitol in urine, in which anion-exchange chromatography and pulsed amperometric detection are used. Sample preparation is simple and fast, and lactulose and mannitol and the internal standards arabinose and cellobiose are well resolved within 15 min. Analytical response of the method is linear with concentrations to 3 g/L, and one can detect as little as 0.3 mg of lactulose per liter of urine. Analytical recovery was between 90% and 107% for all sugars analyzed, and there was good agreement with results by a gas-chromatographic method ($r = 0.993$ lactulose, 0.984 mannitol). The method may potentially be applied to the study of other carbohydrates present in biological fluids at low concentrations.

Additional Keyphrases: carbohydrates · chromatography, anion-exchange · arabinose and cellobiose as internal standards

Dual sugar absorption tests have been used to assess the permeability of the small bowel mucosa, in a variety of intestinal diseases (1-3). In these tests, the permeability to nonmetabolizable mono- and disaccharides such as mannitol/lactulose or rhamnose/cellobiose is compared and expressed as the ratio of the concentrations of these saccharides in a timed urine sample. Lactulose/mannitol excretion ratios have been proposed as a useful, noninvasive test for screening and monitoring celiac disease (4).

However, the difficulty in quantifying these urinary sugars has limited the widespread use of the tests. Current methods of carbohydrate determination include thin-layer chromatography, gas-liquid chromatography, and enzymatic analysis. These techniques can be time consuming (5), and samples may require prior derivatization (6, 7). "High-pressure" liquid chromatography (HPLC) with refractive index detection does not achieve the sensitivity or specificity required, and therefore both pre- and post-column derivatization methods have been developed in an attempt to overcome these problems (8, 9).

The introduction of anion-exchange chromatography coupled with pulsed amperometric detection (PAD) (10) has made it feasible to detect carbohydrates electrochemically. The detection system consists of a gold working electrode,

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and a silver/silver chloride reference electrode, to which a series of three potentials is applied in a repeating waveform. The first potential, the "detection potential," is followed by an oxidizing potential that "cleans" the electrode surface. The final potential applied reduces the gold oxide formed back to elemental gold, so that the whole cycle may be repeated.

This technique has the advantage of greatly increased sensitivity and specificity as compared with other liquid-chromatographic methods, and derivatization is not required (11). Here we report preliminary findings of an HPLC-PAD method for the simultaneous determination of mannitol and lactulose in urine of healthy subjects.

Materials and Methods

Subjects. Eighteen healthy adult subjects, 13 men and five women (mean age 30 years), with no history of gastrointestinal disease were studied. Subjects provided a pre-test urine sample after an overnight fast, and then ingested the test solution, 10 g of lactulose and 5 g of mannitol dissolved in 50 mL of distilled water (osmolality 1350 mosmol/kg). Urine was collected for the next 6 h with 0.5 mL of 200 g/L chlorhexidine solution as preservative. The urine volume was recorded, and a 40-mL aliquot was stored at -20°C until analysis. Participants were allowed unlimited intake of fluids after the first hour of the test, to maintain an adequate urine output, and unrestricted food intake after the second hour of the test.

Sample preparation. Depending on the collection volume, urine specimens were diluted between 2.5- and 20-fold with de-ionized water. We then mixed 1 mL of diluted urine with 1 mL of the internal saccharide standards (arabinose 250 mg/L and cellobiose 25 mg/L in de-ionized water), de-salted the mixture with 0.5 g of a washed ion-exchange resin (Amberlite IR120 H and IRA400 Cl in mass proportions of 1:1.5), vortex-mixed, and centrifuged. The supernate was filtered through 0.2- μm (pore-size) disposable filters (Millipore, Milford, MA).

HPLC analysis. Fifty microliters of the filtrate was injected onto a 250×40 mm Dionex HPLC-AS6 anion-exchange column (Dionex U.K., Camberley, Surrey, U.K.) and eluted with 0.15 mol/L NaOH, at a flow rate of 1 mL/min at 20°C . Detection was by pulsed amperometric detection with a gold working electrode and silver/silver chloride reference electrode, with a detection potential of +0.05 V, an oxidation potential of +0.6 V, and a reduction potential of -0.95 V. Quantification was by peak-height analysis and peak-height ratios, with internal standardization.

For comparison, we also analyzed 18 urine samples for mannitol and lactulose by a gas-liquid chromatographic procedure (6, 7).

Results

Figure 1 shows chromatograms of a pre-test sample of urine from a healthy subject and from the same subject after having ingested the lactulose/mannitol mixture. Lactulose, mannitol, and the internal standards are well resolved within 15 min. We encountered no major interference problems and very rarely was an unidentified peak eluted at a retention time >15 min.

The standard curves for both lactulose and mannitol were linear from 0.5 to 3000 mg/L and are represented by the following regression equations: lactulose, $y = 0.212x + 0.845$ (SEE 0.145); mannitol, $y = 0.797x + 0.097$ (SEE 1.475).

By this method, the minimum detectable concentration of lactulose in urine is 0.3 mg/L.

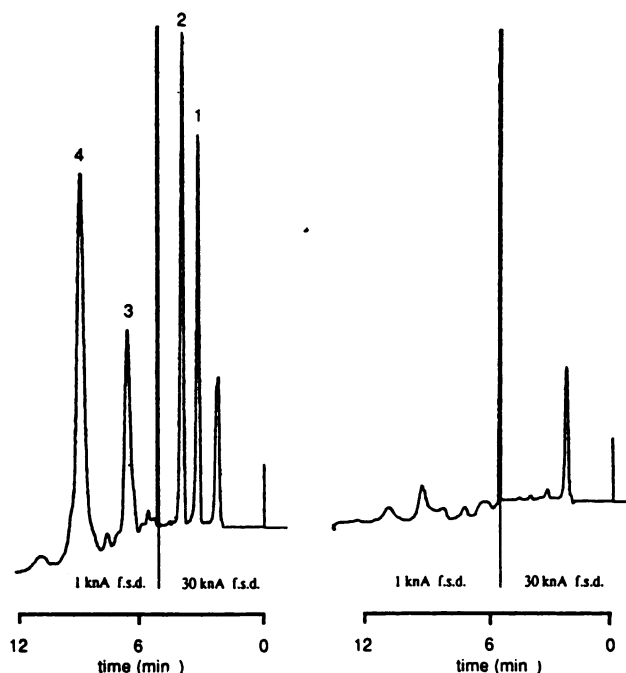


Fig. 1. (Right) Chromatogram of a pre-test urine sample from a healthy subject; (left) chromatogram of a urine sample from the same healthy subject who ingested the test mixture described in the text. 1, mannitol; 2, arabinose; 3, lactulose; 4, cellobiose. Note changes in scale, from 30 to 1 μA full-scale deflection.

Analytical recoveries of lactulose, mannitol, arabinose, and cellobiose added to a urine sample in various concentrations are given in Table 1. Between 90% and 107% of all sugars was recovered, and there was no significant difference between the recoveries of lactulose and mannitol and their respective standards.

Table 2 shows the imprecision of the assay, as assessed by the repeated measurement of lactulose and mannitol in a urine sample from a control subject, who ingested the lactulose/mannitol mixture as described.

Figure 2 compares the concentrations of lactulose and

Table 1. Analytical Recoveries of Lactulose, Mannitol, Arabinose, and Cellobiose Added to Urine

| Quantity, mg | | |
|-------------------|------------------------|-------------------------|
| Added | Recovered ^a | % recovery ^a |
| Mannitol | | |
| 62.5 | 63.7 \pm 3.4 | 101.9 \pm 5.4 |
| 125 | 122.3 \pm 9.0 | 97.8 \pm 7.4 |
| 250 | 245.8 \pm 13.3 | 98.3 \pm 3.2 |
| 500 | 488.0 \pm 13.1 | 97.6 \pm 1.6 |
| Lactulose | | |
| 0.625 | 0.62 \pm 0.013 | 99.3 \pm 2.2 |
| 1.25 | 1.26 \pm 0.038 | 100.9 \pm 3.0 |
| 2.50 | 2.47 \pm 0.089 | 98.7 \pm 3.6 |
| 5.0 | 5.01 \pm 0.34 | 100.1 \pm 6.9 |
| 10.0 | 10.4 \pm 0.60 | 104.1 \pm 5.8 |
| Arabinose | | |
| 250 | 231.0 \pm 2.38 | 92.4 \pm 1.0 |
| Cellobiose | | |
| 25 | 23.6 \pm 0.73 | 94.3 \pm 3.1 |

^a Mean \pm SD, n = 10 each.

Table 2. Precision of Estimations of Lactulose and Mannitol, and of Their Ratio in Urine from a Single Control Subject^a

| | Within batch (n = 10) | Overall (n = 30) |
|---------------|-----------------------|---------------------|
| Mannitol, mg | 458.8 ± 14.6 (3.17) | 462.7 ± 22.2 (4.80) |
| Lactulose, mg | 48.4 ± 3.63 (7.50) | 47.9 ± 5.7 (12.1) |
| L:M ratio | 0.041 ± 0.0018 (4.4) | 0.039 ± 0.002 (5.1) |

^a Mean ± SD, CV(%) in parentheses.

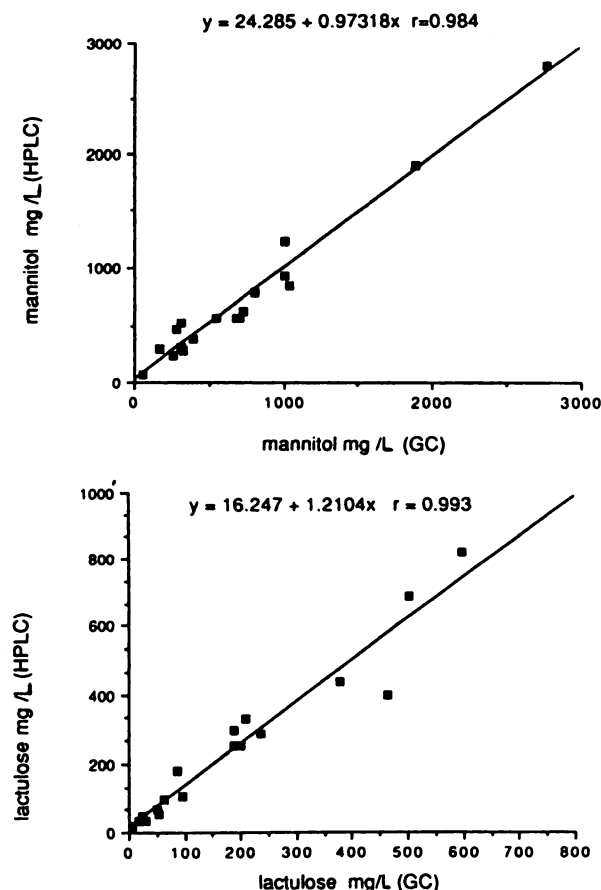


Fig. 2. Comparison of lactulose and mannitol concentrations in 18 urine samples as determined by gas-liquid chromatography (GC) and HPLC-PAD

mannitol in 18 urine samples, as determined by gas-liquid chromatography, with that of the HPLC-PAD method. As shown, the correlation for both sugars is good.

The mean percentage excretion of lactulose in the 18 healthy subjects studied was 0.33% ($\pm 0.04\%$, SEM) of the administered dose, the mean excretion of mannitol was 11.88% ($\pm 0.84\%$), and the mean ratio for percentage lactulose:percentage mannitol was 0.027 (± 0.003).

Discussion

The HPLC-PAD method described here overcomes many of the problems in the analysis of low concentrations of carbohydrates in biological fluids hitherto encountered. Under the chromatographic conditions described, lactulose and mannitol were well separated from each other and

from other carbohydrates in urine. With the use of pulsed amperometric detection, sensitivity is high and is comparable with that of thin-layer chromatography (5). Sample preparation is both simple and fast and, for all of the carbohydrates studied, analytical recovery is close to 100% after the de-salting procedure.

There is good agreement between the results measured by gas-liquid chromatography and by the HPLC-PAD method. The agreement is particularly good for mannitol, but the lactulose determination shows a slight positive bias in favor of the HPLC-PAD method. Because only a small sample population was studied, this bias may just reflect greater imprecision in the measurement of lactulose by both methods. Other possible explanations include minor interference from other carbohydrates in urine, in a few urine samples (because pre-test samples were not available for all of the subjects tested), or possibly incomplete recovery of the anomers of lactulose as measured by gas-liquid chromatography.

Nonetheless, the reference range we obtained for our 18 healthy subjects is very similar to published data of Pearson et al. (2), who report a mean % lactulose excretion = 0.25% (range 0.065–0.45%), a mean mannitol excretion = 14% (range 8.5–24.5%), and a mean % lactulose:% mannitol ratio = 0.018 (range 0.005–0.028).

With rapid sample preparation combined with a short analysis time, one can analyze at least 30 samples within one day. Addition of automatic injection could increase this further. Moreover, the range of saccharides and sugar alcohols potentially detectable by this technique is wide. We expect that the method can be applied to the study of other carbohydrates in biological fluids at low concentrations.

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