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Simple and sensitive multi-sugar-probe gut permeability test by high-performance liquid chromatography with fluorescence labelling

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Abstract

Enteral intake of a mixture of inert, non-metabolic monosaccharide and disaccharide probes, followed by measurement of their urinary probe ratio, is a well known method to investigate gut permeability. However, most applications lack sensitivity, thus a large amount of especially the disaccharide lactulose has to be ingested. This may cause diarrhoea, which influences the outcome of the test.

Recently, a new fluorescent label 9-fluorenylmethyl chloroformate hydrazine (FMOC-hydrazine) was introduced, which reacts with reducing sugars to form stable and highly fluorescent single peak derivatives in organic medium. We applied this reagent to develop a sensitive measurement of reducing sugar probes in aqueous samples (e.g. urine).

The presented method has a linear response for each sugar derivative between 1 and 1250 pmol with an R^2 ranging from 0.9997 for lactulose to 0.9999 for rhamnose. The limit of detection, calculated as a signal-to-noise ratio of three, was 0.05 pmol for lactulose and 0.01 pmol for rhamnose, xylose and 3-O-methyl-D-glucose, corresponding to urine concentrations of 0.11 μmol/l for lactulose and 0.02 μmol/l for rhamnose, xylose and 3-O-methyl-D-glucose. Compared to other tests, the limit of detection is very low. This enabled a reduction in the enteral intake of the disaccharide lactulose from 6–10 g to 1.5 g, thereby minimizing the chance of introducing diarrhoea. The coefficient of variation was below 3% both in standards and urine samples. After spiking the urine with the saccharides a recovery of 102% for lactulose, 101% for rhamnose, xylose and 3-O-methyl-D-glucose was found.

In order to evaluate the presented method we compared the lactulose rhamnose ratio measured in urine of healthy human volunteers and kept the ingested dose in agreement with literature values. Furthermore, the ratio was measured after 3, 6 and 9 h to establish the minimal response time required to measure correct ratios. We found that even after 3 h the ratio was stable at a value of 0.0133 which is comparable to literature values (0.008–0.052).

Keywords: Derivatization, LC; Saccharides; Lactulose; Rhamnose; Xylose; 3-O-Methyl-D-glucose

1. Introduction

Gut permeability can be investigated by measuring the urinary monosaccharide and disaccharide ratio after the enteral intake of inert, non-metabolic probes [1,2]. Monosaccharides can be used as markers for permeation across a transcellular pathway and disaccharides for paracellular permeation. For this test the disaccharide lactulose and the monosaccharide mannitol are often used [1]. However, all methods used to measure the urinary concentrations are either laborious (gas chromatography [3]), semi-quantitative (enzymatic reaction [4]) or show lack of sensitivity (high-performance liquid chromatography (HPLC) with pulsed amperometric detection [5,9]). As a result, the required amount of ingested lactulose

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must be large, causing diarrhoea [10]. Ingestion of large amounts of lactulose can also reduce the permeation of rhamnose by 30% and while it does not effect intestinal permeability, it does reduce the sensitivity of the procedure [2]. Furthermore, small amounts of mannitol are already present in native urine, complicating the interpretation of the test results [1,4]. Therefore, recent publications suggest the use of rhamnose instead of mannitol [1].

Meanwhile, a new fluorescent label, FMOC-hydrazine [6,7] was introduced, which reacts with the ketone or aldehyde-group of reducing sugars to form stable highly fluorescent derivatives in an organic medium. This reagent also enables a simultaneous determination of 3-O-methyl-D-glucose and xylose (which are also often used as probes). The first is a marker for active intestinal mucosa transport and the second for permeation across a transcellular pathway.

The objective of our study was to develop a routine gut permeation test, by measuring urinary lactulose, rhamnose, xylose and 3-O-methyl-D-glucose concentrations with FMOC-hydrazine as fluorescent label, sensitive enough to enable reduction of the enteral intake of lactulose.

2. Experimental

2.1. Equipment

Our HPLC system consisted of a Model 2150 pump, a Model 2252 controller, a Model 2256 solvent conditioner, a Model 11300 ultrograd mixer driver panel and a Model 2155 column oven (set at 50°C), all from Pharmacia (Woerden, Netherlands). Samples of 5 μl were injected using a WISP 717plus (Millipore/Waters, Etten-Leur, Netherlands). Fluorescence was monitored with a Jasco Model 821FP fluorescence detector equipped with a xenon lamp and a 16-μl flow-cell set at an excitation wavelength of 270 nm and an emission wavelength of 315 nm (B&L systems, Zoetermeer, Netherlands). Data were collected on-line by a Model 900 interface (Perkin-Elmer/Nelson, Gouda, Netherlands) and processed by a Tandon Model MCS 486/50 personal computer (Amsterdam, Netherlands), running Model 2700 (Turbochrom: version 3.2) software (Perkin-Elmer/Nelson) under Microsoft Windows, version 3.11. The separation column was a 125×4.6 mm I.D. Bischoff Spherisorb ODS II column (3-μm particles), equipped with a 10×4.6 mm I.D. pre-column, filled with the same packing material (Applikon, Schiedam, Netherlands).

2.2. Reagents and solvents

All solutions were prepared with ultra-pure water, generated by a Super-Q system (Millipore/Waters). All chemicals used were of analytical grade (mostly from Sigma, Brunschwig Chemie, Amsterdam, Netherlands), all solvents of chromatographic grade (Janssen Chimica, Amsterdam, Netherlands).

Synthesis of FMOC-hydrazine

FMOC-hydrazine was synthesized as described previously [6], with minor changes. In short: 1 g 9-fluorenylmethyl chloroformate was dissolved in 100 ml ethanol. This solution was added dropwise under stirring to 10 ml of hydrazine monohydrate. The resulting FMOC-hydrazine precipitated within 30 min, and was collected by filtration through a 0.2-μm cellulose-acetate filter (Sartorius, Abcoude, Netherlands). The collected product was washed twice with 20 ml ice-cold ethanol to remove hydrazine traces and dried at room temperature. The dried FMOC-hydrazine was stored in an exsiccator at 4°C in the dark.

Derivatization reagent

The derivatization reagent was prepared by dissolving 5 mg FMOC-hydrazine per ml acetonitrile followed by the addition of 10 μl per ml reagent buffer (0.60 mol sodiumhydroxyde and 1.44 mol formic acid per litre water).

Standards

Aqueous sugar standards were prepared by dissolving pure sugars in water to a final concentration of 200 μmol/l, except for lactulose which was 600 μmol/l. Aliquots of 1 ml were stored at −80°C until analysis.

Eluents

Solvent A: isobutyl alcohol and isopropyl alcohol in water (6:6:88, v/v/v); solvent B: isopropyl alcohol in water (80:20, v/v).

2.3. Sample preparation

Urine samples

After an overnight fast, urine was collected from eight healthy male volunteers, aging 25 to 35 years. The sugar probes (6000 mg lactulose, 100 mg rhamnose, 50 mg xylose and 70 mg 3-O-methyl-D-glucose, dissolved in 60 ml water (osmolality 310 mosmol)) were ingested and urine was collected again in 3-h intervals. Urine volume was measured and 1-ml aliquots were stored at −80°C until analysis.

Before analyses, samples were thawed at ambient temperature. Next, 10 μl aqueous sugar standard or urine and 200 μl derivatization reagent were pipetted together in a 300-μl glass insert, mixed and placed in a 4-ml WISP vial. The resulting pH, measured directly in this mixture, both in a sample or standard, was 4.6±0.1. The vial was sealed with a screw cap and a self-sealing silicone, Teflon-coated stopper (Waters) and heated overnight (16 h) in an oven set at 65°C. The reaction mixture was allowed to cool to room temperature after which 5 μl of the clear supernatant was injected directly into the chromatographic system.

2.4. Methods

Calibration and limit of detection

The linearity of the detector response was investigated by injection of increasing dilutions of the sugar derivatives. The response was linear over the range from 1 pmol to 1250 pmol with an R^2 ranging from 0.9997 for lactulose to 0.9999 for rhamnose. The limit of detection, determined at a signal-to-noise ratio of three, was 0.05 pmol for lactulose and 0.01 pmol for rhamnose and 3-O-methyl-D-glucose (10 μl injected). This corresponds to a urinary concentration of 0.11 μmol/l for lactulose and 0.02 μmol/l for rhamnose xylose and 3-O-methyl-D-glucose.

Precision

The coefficient of variation (C.V.) was determined by injecting 10 samples, each of aqueous standards and pooled human urine (collected over 3 h after ingestion of the sugar mixture). For the peak areas the C.V. was below 3% and for retention times it was below 0.5% both in the sugar mixture and in urine (not shown)

Analytical recovery

Spiking of the sugar probes in an urine matrix resulted in a recovery of 102% for lactulose, and 101% for rhamnose, xylose and 3-O-methyl-D-glucose.

Optimization of chromatographic conditions

The following reversed-phase stationary phases were tested: Spherisorb ODS II 3 μm (150×4.6 mm I.D.), Inertsil 5 μm (250×3.2 mm I.D.), Ultrasep 5 μm (150×4.6 mm I.D.), Cp Spher 5 μm (250×4.6 mm I.D.). The best selectivity was obtained with the Spherisorb packed column. Therefore, this column was used throughout further experiments. As mobile phase, alcohol, acetonitrile and tetrahydrofuran-based solvents were tested. Acetonitrile-based solvents could not resolve lactulose from the native present lactose while with tetrahydrofuran-based solvents the separation of xylose and native present glucose decreased. All alcohol-based solvents (methanol, ethanol, isopropyl alcohol, isobutyl alcohol) gave an acceptable separation for the probe sugars and those who are normally present in urine. However, the formic acid from the derivatization buffer interfered. When the apolarity of the alcohol increased, at an equal solvent strength, the retention of the interfering formic acid peak increased. When using a mixture of isobutyl alcohol, isopropyl alcohol and water (6:6:88, v/v/v) under isocratic conditions, the interfering formic acid eluted after the sugar derivatives (Fig. 1).

However, under these isocratic conditions the elution of the large excess of FMOC-hydrazine is time-consuming. Therefore, we regenerated the column in 6 min, using a mixture of isopropyl alcohol and water (80:20, v/v) after which re-equilibration of the column with the first solvent occurred.

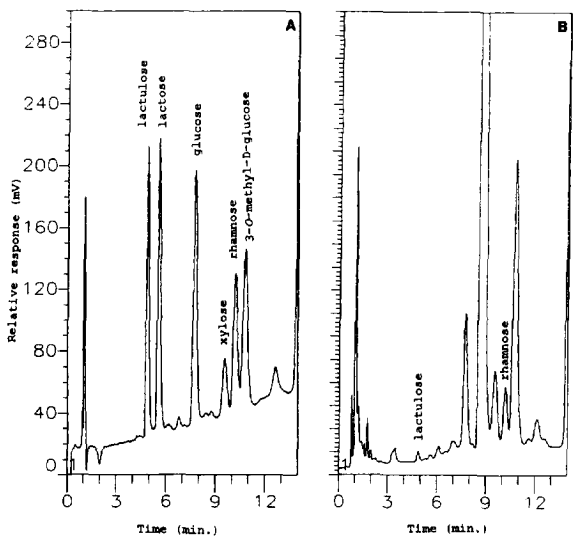

Fig. 1. (A) Chromatogram of an aqueous standard of sugars (200 μM except lactulose 600 μM, 5 μl derivatization mix injected). (B) Chromatogram of urine sample (5 μl derivatization mix injected).

3. Results

3.1. Derivatization.

The major problem was to establish optimal reaction conditions for aqueous solutions (e.g. urine) as the reagent hydrolyses in presence of water [5]. Using an 500-fold excess of FMOC-hydrazine the sugar derivatives reached a maximum response (Fig. 2). Once they were formed and stored at ambient temperature, they were stable for at least 2 weeks. The response of the lactulose derivative was, compared to the other sugar derivatives which were stable in a wide pH range, strongly dependent on the pH of the derivatization mixture (Fig. 3). Therefore, buffering of the reaction was required to obtain a correct ratio and a 100% recovery. Using the described buffer, the pH measured directly in the derivatization mixture was 4.6±0.1, for the samples as well as for the standards.

The reaction temperature was considered optimal

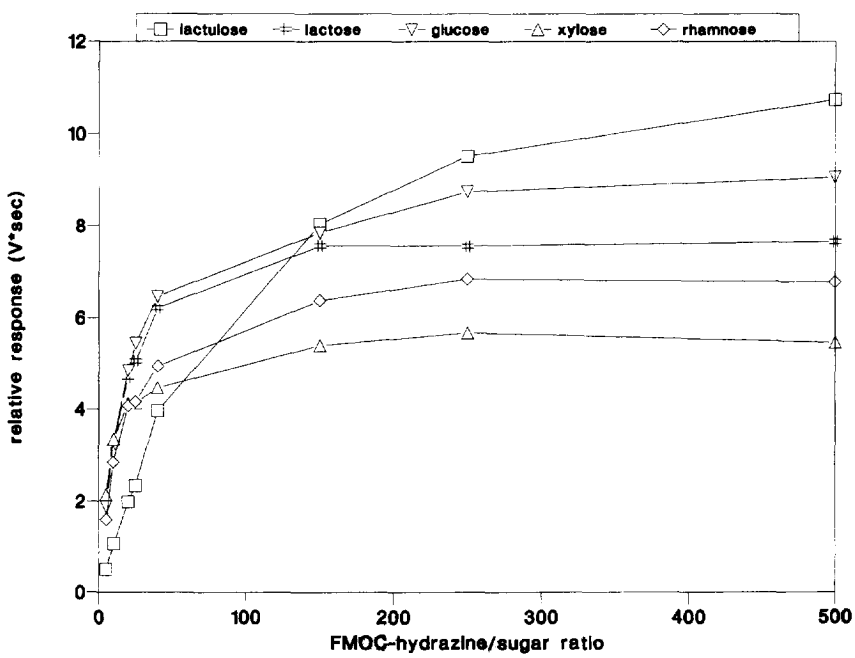

Fig. 2. Influence of FMOC-hydrazine/sugar ratio on the fluorescence response of the sugar derivatives.

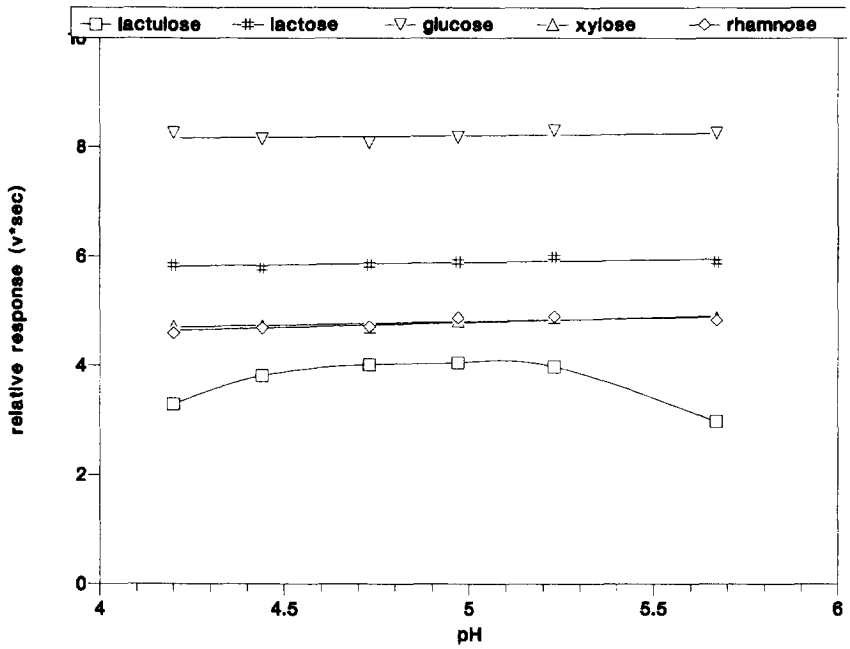

Fig. 3. Influence of pH on the fluorescence response of the derivatization mix.

at 65°C [5]. To determine the optimal reaction time at this temperature, the fluorescence response of the formed derivatives was studied in time (Fig. 4). With exception of lactulose and 3-O-methyl-D-glucose derivatives, maximal fluorescence was reached after 3 h. These two sugars reached their maximum after 16 h. Because the fluorescence of the other sugar derivatives decreased only minimal in this time, the optimal reaction time was considered 16 h.

3.2. Sugar ratio

Using the optimized chromatographic conditions, the urinary sugar concentrations of one overnight fasted normal healthy male human volunteer were studied every hour after ingestion of the sugar mixture. The concentrations of lactulose and rhamnose were well detectable, already one hour after ingestion of the sugar mixture (not shown).

Next, urine was collected and volume was measured from eight overnight fasted healthy male human volunteers (age 26 to 35) in 3-h periods after ingestion of the sugar mixture. The mean lactulose/rhamnose ratios (L/R ratio) after 3, 6 and 9 h urine collection time are given in Table 1. The L/R ratio was calculated as:

$$\frac{[\text{lactulose}]_{\text{urine}}}{[\text{rhamnose}]_{\text{urine}}} \cdot \frac{[\text{rhamnose}]_{\text{mixture}}}{[\text{lactulose}]_{\text{mixture}}} \quad (1)$$

4. Discussion

Up until now the measurement of the gut permeability was studied by ingestion of large amounts of lactulose (6–10 g). This was necessary because the normal gut permeability of lactulose is low and a detectable concentration can only be obtained when a large amount of lactulose is ingested. This large amount reduced the reliability of the measured lactulose/rhamnose ratio as described [1,2,10]. Therefore, when the detection limit of the analytical determination can be reduced, the amount of ingested lactulose can be reduced and the reliability of the ratio can be improved.

The new technique presented here has a short sample preparation, is simple and no clean-up procedures are required. It uses fluorescent detection, thus its sensitivity is high. Our urinary limit of

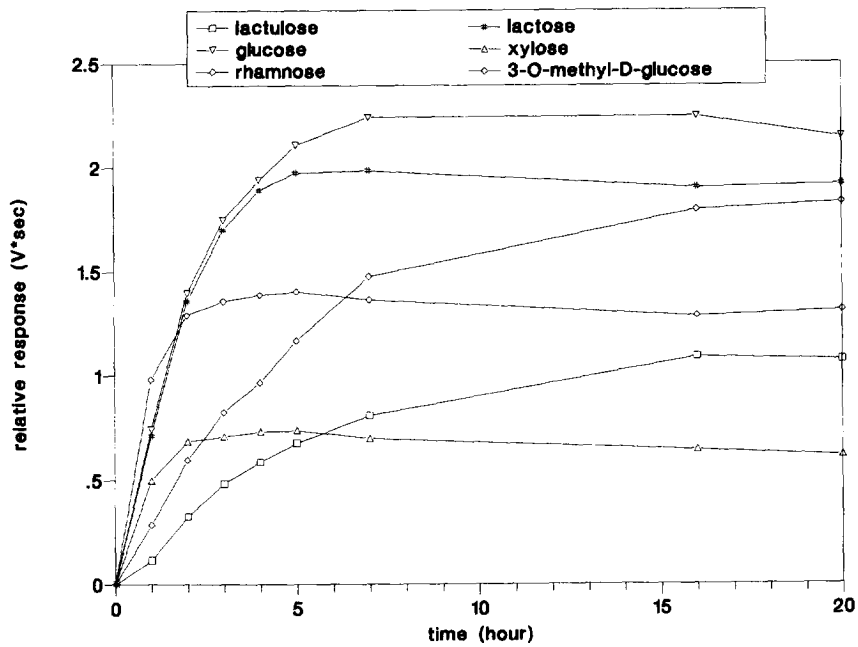

Fig. 4. Influence of reaction time on the fluorescence response at 65°C at a FMOC-hydrazine/sugar ratio of 500.

detection (0.11 μM) is very low compared to enzymatic reactions (73 μM [4]), and other HPLC methods (0.88 μM [5] and 1.2 μM [9]). The lowest lactulose concentration found, 3 h after ingestion of 6 g (a normal literature dose), was 4.3 μM. Given our detection limit, the amount of lactulose ingested could easily be reduced while still a detectable response could be obtained. Therefore, we now perform the studies with 1.5 g of lactulose.

We obtained a 100% recovery, which is very important for measuring a correct ratio. This is in contrast to the low fluctuating results of previous studies, recoveries ranging from 62% to 80% [8], 90% to 107% [3], 89% to 99.5% [9]. However, a good explanation for this discrepancy is lacking.

The mean urinary sugar ratio studied in fractions collected in 3-, 6- and 9-h periods (Table 1), were well within the normal literature range reported (0.008–0.052 [1]). The ratios were stable, although the absolute urinary concentrations changed in time. Therefore, a 3-h collection period in a healthy human seemed to be sufficient to measure a reliable L/R ratio.

In conclusion, we used FMOC-hydrazine as label for reducing sugars and adapted the derivatization method and the chromatography [6,7] to enable measurement in aqueous samples (e.g. urine). Thereby, a simple and reliable routine test for determining the gut permeability was introduced which allows the use of lactulose, rhamnose, xylose and 3-O-methyl-D-glucose sugar probes and only needs standard laboratory equipment.

Table 1
Urinary lactulose/rhamnose ratio of human volunteers after different collection periods (ingestion: 6000 mg lactulose and 100 mg rhamnose)

	Collection time (mean±S.E.M., $n=8$)		
	0–3 h	0–6 h	0–9 h
L/R ratio	0.0133±0.0018	0.0144±0.0017	0.0133±0.0015

References

[1] S. Travis and I. Menzies, Clin. Sci., 82 (1992) 471.
[2] I. Bjarnason, A. Macpherson and D. Hollander, Gastroenterology, 108 (1995) 1566.
[3] G. Jansen, F. Muskiet, H. Schierbeek, R. Berger and W. van der Silk, Clin. Chim. Acta, 157 (1986) 277.
[4] A. Northrop, P. Lunn and R. Behrens, Clin. Chim. Acta, 187 (1990) 79.
[5] C. Fleming, S. Kapembwa, F. Laker, G. Leven and G. Griffin, Clin. Sci., 36 (1990) 797.
[6] R. Zhang, Y. Cao and M. Hearn, Anal. Biochem., 195 (1991) 160.
[7] R. Zhang, Z. Zhang, Y. Hidaka and Y. Shimonishi, J. Chromatogr., 646 (1993) 45.
[8] T. Delahunty and D. Hollander, Clin. Chem., 32 (1986) 1542.
[9] J. Kynaston, S. Fleming, M. Laker and A. Pearson, Clin. Chem., 39 (1993) 453.
[10] H. Conn, in Hepatic Encephalopathy, MEDI-ED Press, East Lansing, Michigan, 1988, p. 199.