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Cellobiose and lactulose coupled with mannitol and determined using ion-exchange chromatography with pulsed amperometric detection, are reliable probes for investigation of intestinal permeability

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Abstract

Lactulose/mannitol and cellobiose/mannitol tests are currently used in the investigation of intestinal permeability (IP) in many gastrointestinal diseases. The aim of this study was to produce a good technique for the determination and comparison of the above-mentioned sugar probes to overcome the problem caused by the presence of significant glycosuria in patients affected by particular metabolic disorders such as diabetes mellitus. Tests were performed in 25 healthy volunteers, using either cellobiose (Ce) (5 g) and mannitol (Ma) (2 g), or lactulose (La) (5 g) and mannitol (2 g), given as oral isosmolar loads. Sugars were recovered in urine collected for 5 h. Analysis was carried out by using anion-exchange chromatography (AEC) with pulsed amperometric detection (PAD). Baseline separation of the above carbohydrates was achieved within 13 min by using a Carbopac PA-100 column and linear gradient elution. Carbohydrate quantification was performed by an internal standard method. The calibration curve for each sugar is linear to 40 m*M*. The limit of sugar detection is 0.01 m*M*. Recovery of sugar probes is between 98.2 and 100%.The %La, %Ce, %Ma in urine were evaluated and their ratios (Ce/Ma and La/Ma) were calculated. No significant difference in IP parameters were shown (La/Ma to Ce/Ma 0.018±0.014 vs. 0.012±0.007; the attendant probability of the null hypothesis being $P=0.0714$). Ce/Ma and/or La/Ma tests result similarly reliable in the clinical investigation of IP and the described new method is also helpful in urine even with high glucose concentration, without any interference.

Keywords: Intestinal permeability; Permeability test; Lactulose; Cellobiose; Mannitol; Glucose

1. Introduction

Intestinal permeability (IP) is a measure of the intestinal *barrier* function. It represents the *passive* crossing of intestinal epithelium by water and hydro-

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soluble inert small molecules through intercellular spaces.

IP measurement is achieved by oral administration of hydrosoluble, non-toxic, non-absorbed, sugar molecular probes followed by the determination of their amounts excreted in the urine over a 5-h period. Sugar probes are usually administered in couples: a small one (5–7 Å of diameter) that, in normal conditions, should cross the epithelium in the villi domain in 15–20% of the administered amount; and a large one (10–12 Å of diameter) that crosses in the crypt domain in a very low amount (<2% of the oral dose). This coupled administration is helpful to avoid interferences due to alterations of both gastrointestinal transit (pre-mucosal factors) and urinary excretion (post-mucosal factors) [1]. In the dual sugar test, permeability to non-metabolizable di- and monosaccharides (or sugar alcohol) is expressed as the urinary excretion ratio between large probe and small probe. Normally this ratio is far less than 1.

Lactulose/mannitol (La/Ma) test is currently used in the investigation of IP in several gastrointestinal diseases such as coeliac disease [2,3], Crohn's disease [4,5], chronic diarrhoea [6] and malnutrition [7]. Cellobiose/mannitol (Ce/Ma) test has also been employed in diabetes mellitus type I patients [8] even if the use of Ce has been criticized because of the possible presence of cellobiose in both the small intestine and some foods [1,9].

So far, several methods have been proposed for the quantitative determination of urinary sugars; but none of them is without limitations. Thin-layer chromatography [10] is time consuming; colorimetric/enzymatic procedures [11] do not provide information on the composition of saccharides of biological materials and are often subjected to many interferences; gas chromatography involves the necessity of derivatization [12,13]. So far, LC with refractive index detection has been the most common method for saccharide quantification; however, this technique has a low sensibility and no selectivity [14,15].

Recently, anion-exchange chromatography (AEC) coupled with pulsed amperometric detection (PAD) has been employed [16–19]. This technique allows a direct quantification of non-derived carbohydrates at picomole levels with minimal sample preparation and clean-up. Exchange chromatography takes advantage of the weak acid nature of carbohydrates to give highly selective separations at high pH, using a strong anion-exchange stationary phase. At high pH, carbohydrates are electrocatalytically oxidized at the surface of a gold electrode by the application of positive potential. Pulsed amperometry detects only those compounds containing functional groups oxidizable at the detection voltage employed; neutral and cationic sample components are eluted in the void volume of the column. Therefore, even if such species are oxidizable, they do not usually interfere with the analysis of the carbohydrate components of interest. Pulsed amperometry permits detection of carbohydrates in amounts even below 10 pmol with excellent signal-to-noise ratios without requiring derivation. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold electrode [20,21].

Previous studies employed this technique in IP tests to measure urinary lactulose and mannitol only in diseases which did not implicate glycosuria [16–19]. In the presence of glycosuria, in fact, the chromatographic conditions employed lead to some overlap between glucose and mannitol peaks [16]. Therefore, accurate measurement of urinary mannitol—being essential for determining the accurate value of—is possible only if no or low amounts of glucose are present in urine samples.

The aim of this work was to develop a simple, rapid and simultaneous quantification of mannitol, glucose, lactulose and cellobiose in urine specimens by LC technique with the use of amperometric detection, and to compare the results of the two mostly used IP tests: Ce/Ma and La/Ma. Moreover, urine samples with significant glycosuria were also analyzed to demonstrate the good sugar peak resolution in the presence of glucose.

2. Materials and methods

2.1. Subjects, intestinal permeability test, exclusion criteria

Twenty-five normal healthy volunteers (13 men and 12 women; mean age 28.6 years±10.3 SD) with no symptoms or signs of gastrointestinal disease and

drug-free for at least 2 weeks, were studied. They had no food allergy, cardiovascular and respiratory insufficiency, liver cirrhosis, signs of malnutrition or rheumatic diseases. None of them had undergone recent gastrointestinal surgery, smoked more than two cigarettes/day, drank more then 20 g/day of alcohol, or chronically taken non-steroidal anti-inflammatory drugs (NSAIDs). After an overnight fast, between 20.00 and 09.00 h, a pretest urine sample was collected to check for the possible presence of endogenous sugars. Then subjects drank a sugar test solution, containing 5 g of La and 2 g of Ma in 150 ml water. After 30 min, a free intake of water was encouraged to increase urine flow. Urine was collected over the following 5-h period, the total volume was recorded and 1.5-ml aliquots were stored at −20 °C until analyses. After 1 week each subject repeated the IP test by ingesting a sugar solution with 2 g of Ma and 5 g of Ce, instead of La, as large probe. For each sugar, the 5-h urinary recovery was expressed as the percentage of the ingested dose, and the ratio between the two recovery percentages (La/Ma or Ce/Ma) was calculated. This ratio is considered an index of IP.

2.2. Reagents

Standard carbohydrates were purchased from Sigma (St. Louis, MO, USA). Low carbonate 50% (v/v) sodium hydroxide was purchased from J.J. Baker (Deventer, The Netherlands).

2.3. Standard preparation

Stock analyte standards were prepared by dissolving the appropriate amount of each sugar in twice-distilled and deionized water to give a final concentration of 10 m*M* for each sugar. This stock solution was stored at −20 °C until the assay, when it was diluted to produce four working standard solutions at four different equimolar concentrations in the range from 0.01 to 0.08 m*M*, and maltotriose was added as internal standard for quantitative analysis.

2.4. Sample preparation

Urine samples to be analyzed were filtered by 0.22-μm (pore size) disposable syringe filters (Alltech, Deerfield, IL, USA) and appropriately diluted to obtain concentrations within the calibration range; afterwards, an appropriate volume of internal standard (maltotriose) solution to give a concentration of 0.04 m*M* was added. Urinary concentrations of sugar probes were calculated from the calibration curve by peak–area analysis.

2.5. Spiked urines

A pool of blank urines was divided into two aliquots. Known amounts of four of the analyzed sugars were added to each aliquot in order to obtain two spiked urine samples: the first with 0.02 m*M* and the second with 0.2 m*M* of each sugar. To determine the inter-assay variation, the two spiked urine samples were run 40 times within 30 days. To determine the intra-assay variation, each spiked urine sample was run 20 times; this protocol was repeated three times.

2.6. Chromatographic instrumentation

AEC-PAD was performed on a Dionex (Sunnyvale, CA, USA) Model DX-500 with a eluent degassing device module, a gradient pump module GP 40 and a ED40 pulsed amperometric detector with a gold working electrode. Sample injection was via an autosampler module AS-50 Dionex equipped with a 25-μl peek sample loop with a rheodyne valve. Carbohydrate separation was carried out by a Carbopac PA-100 pellicular anion-exchange resin (4.6 I.D.×250 mm, Dionex) connected to a Carbopac PA-100 guard column (4.6 I.D.×50 mm, Dionex) at room temperature.

2.7. Chromatographic condition for AEC-PAD analysis

All eluents were degassed on line by covering them with helium to prevent absorption of atmospheric carbon dioxide and subsequent production of carbonate, which would act as a displacing ion and shorten retention times. The elution was carried out with the following binary gradient: NaOH 160 m*M* (eluent A) and NaOAc 300 m*M*+NaOH 160 m*M*

(eluent B), (time=0–6 min 100% eluent A; time=13 min 53.2% eluent A, 48.6% eluent B; the flow-rate was 1 ml/min for total cycle time.

Carbohydrate components were detected in the eluent by the ED-40 module using a gold electrode and triple-pulse potential. The electrode was maintained at the following potentials and durations: sampling $E_1=0.05$ V ($t_1=0.40$ s); oxidation $E_2=0.75$ V ($t_2=0.41$ s); reduction $E_3=-0.15$ V ($t_3=0.61$ s).

Twenty-five μl of each of the four standard solutions were used to calibrate the LC system at the beginning of the measurements and every 10 injections (10 samples) to correct the loss of sensitivity due to the accumulation of the materials on the electrodes; i.e., value of the intra-assay variation.

To achieve highly reproducible retention time the column was then equilibrated for 10 min with the eluent in order to reset initial conditions. In order to determine the reproducibility of the retention times, the mean value and the relative standard deviation (RSD) of the retention time were calculated from chromatograms obtained from four repeated injections of a working standard solution containing equimolar amounts of the above carbohydrates. The inter-assay variation was determined for each of the four working solution, comparing the results obtained in 40 determinations along 30 days.

2.8. Statistical analysis

IP values are expressed as means±SD; differences between means were evaluated by Student's *t*-test; the attendant probability for the null hypothesis is also indicated.

Calibration graphs were plotted based on the linear regression analysis of the peak area ratios. Regression analyses were employed to determine the correlation coefficients of the standard sugars. The data were analyzed for intra- and inter-assay precision, dilution linearity of the standard concentrations, percentage of sample recovery, specificity of separation of the tested sugars and detection limit.

3. Results

3.1. Separation of sugars and precision of the AEC-PAD method

Fig. 1 reports the typical aspect of separation of Ma, Gluc, Ce, La and maltotriose standards by the AEC-PAD method. The employed column exhibits rapid mass transfer, high pH stability and excellent mechanical stability permitting back pressure for long time.

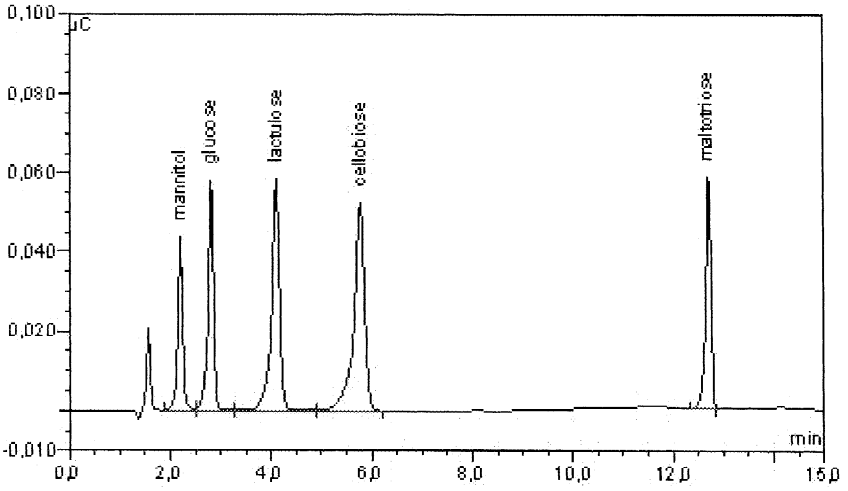

Fig. 1. Typical chromatogram of a standard mixture of sugars (0.5 m*M* of each sugar) obtained using Carbopac PA-100 column; eluent NaOH 160 m*M* and NaOAc 300 m*M* flow-rate 1 ml/min; ED-40 detector; room temperature.

Table 1
Intra- and inter-assay reproducibility of retention times (min) for the standard solution of sugars: chromatographic conditions as in Fig. 1

Sugar	Intra-assay		Inter-assay	
	Retention time mean (min)	RSD (%)	Retention time mean (min)	RSD (%)
Mannitol	2.18	0.7	2.17	0.7
Glucose	2.83	0.5	2.80	0.6
Lactulose	4.02	0.05	3.89	1.3
Cellobiose	5.05	0.05	5.42	1.4
Maltotriose	12.67	0.2	12.91	0.4

Mean retention times of the standard sugars are shown in Table 1. Relative standard deviations (RSD) of intra-assay determinations (12 samples) were less than 0.7%; relative standard deviation (RSD) of the inter-assay (40 samples) resulted less than 1.4% for the retention times.

3.2. Dilutions linear variation and percentage of standard samples recovery

The linear variation was evaluated as the correlation coefficient obtained from regression analysis of eight points of each standard sugar used at the following concentrations: 0.01, 0.02, 0.04, 0.06, 0.08, 4, 20, 40 m*M*. Each calibration point was replicated four times. The calibration curves for Ma, Gluc, La, Ce and maltotriose were linear up to 40 m*M* and gave the following regression equation: cellobiose $y = 0.3396x - 3E\text{-}06$; lactulose $y = 0.2697x - 0.0001$; maltotriose $y = 0.2317x + 0.0002$; glucose $y = 0.2278x + 0.0002$; mannitol $y = 0.155x - 7E\text{-}05$. The correlation coefficients were $r^2 = 99.5$ for mannitol, $r^2 = 99.9$ for glucose, $r^2 = 99.7$ for lactulose, $r^2 = 99.7$ for cellobiose, $r^2 = 99.8$ for maltotriose. The detection limit was 0.01 m*M* for all sugars and was defined as the amount (expressed in m*M*) of each sugar of diluted sample giving a signal-to-noise ratio = 2.

3.3. Validation of the permeability tests using mannitol, glucose, lactulose, and cellobiose

The precision and the accuracy of the method were determined by spiking two urine samples with known amounts of analyzed carbohydrates as indicated in Section 2. The results obtained are reported in Table 2. Intra-assay variation recovery percentage of the standard samples ranged from 98 to 100% (Table 2) and inter-assay variation ranged from 97.6 to 100% (Table 2). Relative standard deviation (RSD) of intra-assay variation, resulted less than 1.5% for the recovery of sugar samples.

Fig. 2 shows a typical chromatogram of urine from a healthy volunteer before (Fig. 2a) and after La/Ma (Fig. 2b) or Ce/Ma test (Fig. 2c) administra-

Table 2
Analysis of two spiked urine samples

Sugar	Amount added (m*M*)	Found (m*M*)	Intra-assay		Found (m*M*)	Inter-assay	
			Recovery (%)	RSD (%)		Recovery (%)	RSD (%)
Mannitol	0.02	0.0199	99.8	1.18	0.02	100	1.3
	0.2	0.196	98	1.15	0.196	98	1.7
Glucose	0.02	0.0198	99.9	1.23	0.0197	98.5	2.05
	0.2	0.195	97.5	1.12	0.197	98.5	1.7
Lactulose	0.02	0.0199	99.7	1.15	0.0199	99.5	1.6
	0.2	0.199	99.9	1.10	0.2	100	1.6
Cellobiose	0.02	0.0196	98.2	1.45	0.0195	97.5	2.8
	0.2	0.199	99.5	1.37	0.199	99.5	2.2
Maltotriose	0.02	0.020	100	1.27	0.0198	99.1	0.9
	0.2	0.199	99.5	1.26	0.199	99.5	0.9

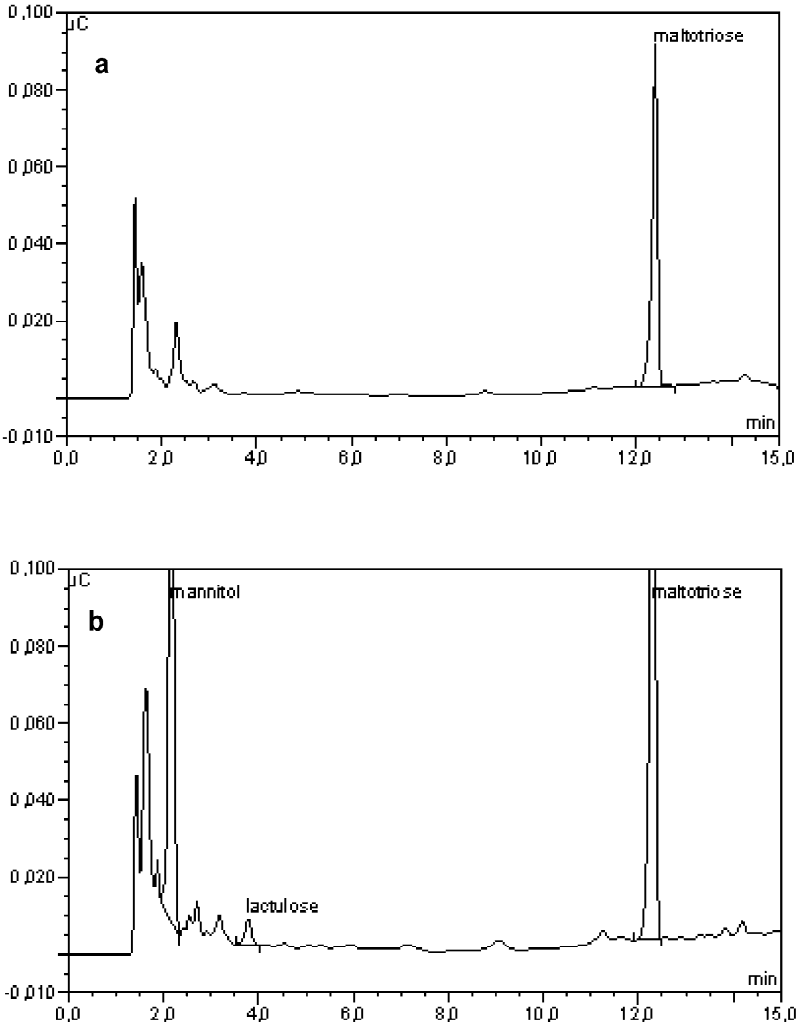

Fig. 2. Chromatogram of blank urine (a); urine after La/Ma test (b); urine after Ce/Ma test.

tion. The absence of investigated sugars in blank urine (Fig. 2a) can be observed; test sugar peaks are well resolved in chromatogram also in a urine sample with a significant glycosuria (Fig. 3), where the separation of mannitol and glucose peaks is clear.

3.4. Evaluation of IP in healthy volunteers

In 25 healthy volunteers, La/Ma urinary ratio did not significantly differ from Ce/Ma urinary ratio (0.018 ± 0.014 vs. 0.012 ± 0.007; the attendant probability of the null hypothesis being $P = 0.0714$). Moreover, mannitol excretion percentage did not significantly differ between tests. Urinary La excretion percentage, instead, was significantly lower than urinary Ce excretion percentage (0.1868 ± 0.14 vs. 0.335 ± 0.25; the attendant probability of the null hypothesis being $P = 0.0096$) (Fig. 4).

4. Discussion

Here we validated a rapid and convenient AEC-PAD method for quantifying non-metabolised sugars

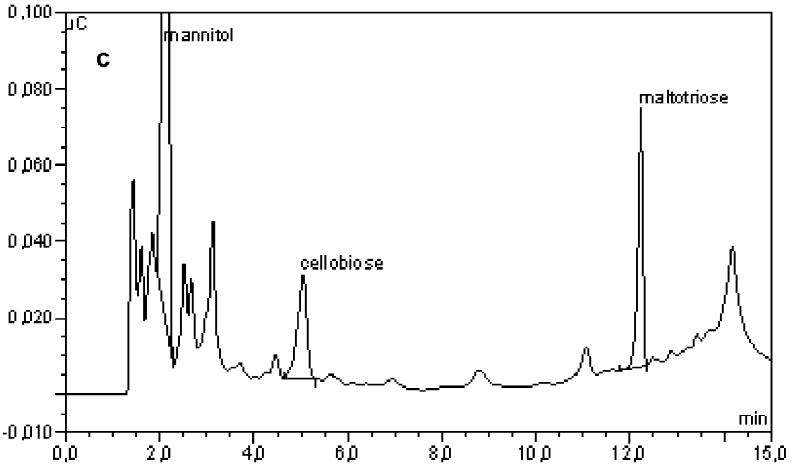

Fig. 2. (*continued*)

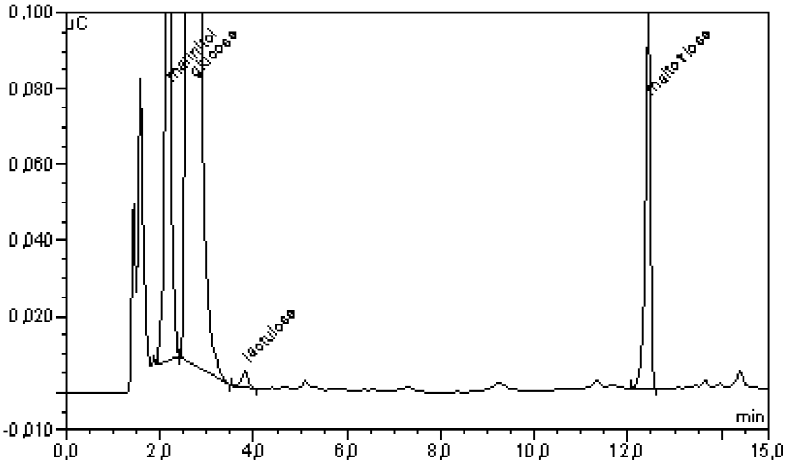

Fig. 3. Typical chromatogram of urine sample in a diabetic patient.

in urine. Under the chromatographic conditions described, Ma, La, Ce and Gluc are well resolved from each other and from other common carbohydrates possibly present in urine. The good separation of glucose peak from that of mannitol, differently from the previous assay method [15] and without any pretreatment of the urine sample allows us to propose this procedure for IP determination also in diabetic patients. Differently from Fleming et al. [16,19], our method does not require desalting of samples because the alkaline mobile phase, associated to triple-pulse potential and to the ED-40 detector, avoids salt interferences. The lack of desalting procedure of samples is an obvious advantage of our method since it improves the analytical recovery. Moreover, the different column and the different mobile phase that we employed allow to obtain a method with improved precision and accuracy and

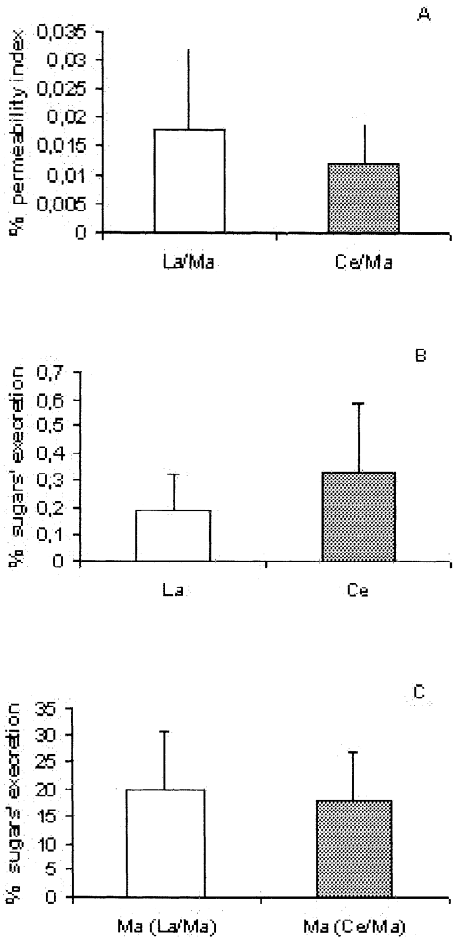

Fig. 4. La/Ma and Ce/Ma test in volunteers. (A) La/Ma vs. Ce/Ma no significant differences in permeability index (IP) is observed in volunteers. (B) Percent large probe excretion showing significant difference. (C) Percent mannitol excretion with no significant difference between the two test.

with considerably lower signal to noise ratio in respect to Fleming et al. [16,19]. The detection limit of the method is very low because pulsed amperometric detection is very sensitive [16–18]; one order of magnitude higher compared to refractive index detection [15]. Sample preparation is simple and analysis time is very short (only 13 min).

In our normal subjects the range of urinary excretion of single probes and that of La/Ma and Ce/Ma ratios are similar to already published data obtained by HPLC [15,17,18] and gas chromatography [22]. Moreover, our findings show that the results of the Ce/Ma dual sugar test overlap those of the La/Ma test.

This allows to exclude a significant cellobiose intestinal activity and seems to settle the controversy about the use of La/Ma as a better test than Ce/Ma test. The Ce/Ma test appears more convenient because Ce is a less expensive sugar and has less laxative effects. Other proposed probes, such as polyethyleneglycol (PEG) and ^{51}Cr-labeled EDTA, are unlikely to be suitable: commercial formulation PEGs consist of polymers of different sizes [23–26], and a substantial variation has been observed in the proportion of each individual polymer excreted in urine after oral intake [26,27], affecting the reproducibility of results; ^{51}Cr-labeled EDTA is unacceptable as an ideal probe because of its radioactivity. In contrast, sugar probes are well defined, non-radioactive, and provide a non-invasive test that can be performed many times in the same subject. The sugar IP tests seem to be the most useful technique to assess small bowel damage and dysfunction. From the laboratory point of view, our method allows 50 permeability tests to be analyzed per day, even more if automatic injection facilities are available and at minimal cost for consumables.

This new method, improving both sample preparation and LC conditions, enables separation of Ma from Glu in urine even with significant glycosuria, thus it can allow IP urine tests as screening for small intestinal disorders in conditions such as diabetes, other than malnutrition, food allergy, etc. This technique could easily be adapted to assay other simultaneous intestinal marker carbohydrates (mono- and oligosaccharides), such as xylose, a marker of active intestinal transport in urine.

5. Notation

IP, intestinal permeability
La, lactulose
Ma, mannitol
Ce, cellobiose

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