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Lactulose and mannitol intestinal permeability detected by capillary electrophoresis

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Abstract

Aim of this study was to set up a method by capillary electrophoresis to detect lactulose and mannitol in urine after an oral load, and to estimate the intestinal permeability in controls and in type I diabetes patients. The underivatized carbohydrates were monitored by indirect UV detection using sorbate, cetyltrimethylammonium bromide and LiOH as background electrolyte. Urines were purified by solid phase extraction, shaken with cation exchange resin, filtered and analysed. Carbohydrates migrated in <10 min in relation to their pK_a and M_r. Controls ($n = 33$) and patients ($n = 23$) had an excretion ratio lactulose/mannitol 0.025 (0.018–0.051) and 0.067 (0.050–0.127), respectively ($p < 0.01$, median, interquartile range).

Keywords: Mannitol; Lactulose; Intestinal permeability; Urine; Capillary electrophoresis

1. Introduction

The lactulose-mannitol (LAC-MAN) is a highly sensitive test for the screening of the diseases that affect intestinal permeability [1,2]. The test is based on the coupled oral administration of two sugar probes of different molecular weight and on the determination of their excretion rate in urine.

These two small molecules are neither hydrolysed nor actively transported across the intestinal barrier. The sugar alcohol MAN (∼5–7 Å) permeates the intestinal mucosa via a transcellular pathway through the water-filled pores on the cell membrane, whereas the disaccharide LAC (∼10–12 Å) uses a paracellular route through the intercellular junctional complexes between adjacent enterocytes and extrusion zones at the villous tip [1,3]. If the mucosal barrier is damaged as in celiac disease, Crohn's disease, atopic dermatitis, cow's milk proteins intolerance, cystic fibrosis, diarrhoea, HIV infection and diabetes, the gut is more permeable to intact sugars and proteins [3–8].

Therefore, an abnormal intestinal permeability with an unbalance in the di/mono saccharide (or sugar alcohol) excretion ratio in urine would indicate either an abnormal passive uptake of large molecules (sugars or antigens) through the paracellular route across the damaged mucosal barrier, or a reduction of the integral mucosa area with a reduced passage of the small molecules.

So far, several techniques have been proposed to set up methods for quantitative determination of sugars in urine, but all of them present some limits. Thin-layer chromatography [9] and colorimetric/enzymatic procedures [10] are time-consuming and unspecific methods, HPLC must be coupled to a refractive index, pulsed amperometric, fluorescence or evaporative light scattering detector [11–17], not always available in all analytical laboratories, while gas chromatography or gas chromatography–mass spectrometry needs a derivatization step [18–22].

Capillary electrophoresis (CE) is the technique of choice for the analysis of hydrophilic mono- and oligosaccharides carbohydrates, with an impressive number of different separation approaches, different detection modes (with or without pre-column derivatization) and different applications [23–33].

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However, despite the high separation efficiency, rapid analysis time and, more valuable, the possibility to miniaturize the sample, reagents and solvents volume [34,35], this technology has never been used, so far, to investigate intestinal permeability in clinical protocols.

Aim of this study was to test the possibility to quantify the urinary excretion of MAN and LAC by CE without the pre-column derivatization step, thus allowing the use of a basic apparatus equipped with the UV detector. The method was used to estimate the LAC-MAN intestinal permeability in a cohort of patients with type I diabetes, a disease associated with abnormal intestinal permeability [7,8], compared with healthy control subjects.

2. Materials and methods

2.1. Chemicals

All standard sugars, sorbic acid and lithium hydroxide were purchased from Sigma Chemical Co. Cetyltrimethylammonium bromide (CTAB) and HPLC grade methanol were from Fluka Chemie (Buchs, Switzerland), Amberlite IRA-400 resin (Cl^- form) (strongly basic anion exchanger, chloride form quaternary ammonium) from Aldrich (Milwaukee, WI, USA). OASIS™ HLB solid phase extraction cartridges (3 mL, 60 mg) were from Waters and Micro-SpinTM centrifuge filters (Nylon 66, 0.2 mm) were supplied from Alltech Associates. Water was obtained from a MilliQ water purification system (Millipore). The uncoated fused-silica capillary was from Beckman Coulter (Palo Alto, CA, USA). The Visiprep Solid Phase Extraction Vacuum Manifolds for samples purification was from Supelco (Bellefonte, USA).

2.2. CE apparatus and analytical conditions

Analyses were carried out with a P/ACE System 5500 (Beckman Coulter) controlled by System Gold 8.1 software. The instrument was set in reversed polarity mode (injection performed at the cathode) and equipped with a monochromatic UV detector set at 254 nm, which polarity was reversed and the negative offset (%) was set at 90. The bare fused-silica capillary (27 cm, 50 μm ID; 20 cm to the detector) was assembled in a Beckman cartridge (200 mm × 400 mm slit aperture). The background electrolyte (BGE) consisted of 6 mmol/L sorbic acid, 1.25 mmol/L CTAB, 50 mmol/L LiOH (pH 12.5). At the beginning of the day, the capillary was rinsed in direct mode (from the inlet to the outlet) with 1 M LiOH, 0.1 M LiOH, and the BGE (5 min each). After application of 20 kV for 10 min the capillary was ready for use. The following procedure was applied for a typical analysis: 1 min pre-rinse with the BGE (20 psi, 138 kPa), 1 s injection of the sample at pressure mode (0.5 psi, 3.4 kPa) followed by 1 s pressure injection of water. Separation was performed at −5 kV (constant voltage) over 10 min with a typical current of 29 mA within the capillary column at 20 °C. Between runs the capillary was rinsed with BGE for 0.5 min by applying high voltage (−20 KV) and then it was flushed in direct mode with 1 M LiOH, 0.1 M LiOH, and distilled water (0.5 min each).

At the end of the day the same rinsing cycle was scheduled but with a longer duration (10 min each). The capillary was stored overnight with the two ends dipped in water. For longer periods, the capillary was dried.

2.3. Standard solutions and calibration

Standard solutions of all sugars were prepared in water with concentrations 20, 10 and 2 mg/mL. Rhamnose used as internal standard (IS) was prepared 10 mg/mL. These solutions were stable for 1 week at 4 °C. For the set up of calibration curves, standards were diluted with control urine or water (final volume 1 mL) so to cover the 0.01–0.5 and 0.05–2.0 mg/mL, final concentration range for LAC and MAN, respectively. The samples were freshly prepared for each analytical run and treated as described below.

2.4. Urine sample treatment

The OASIS HLB cartridges were pre-activated by washing with 1 mL of methanol followed by 1 mL of water (1 mL/min). Urines (1 mL) were added with the IS (50 μL, 0.5 mg) and passed through the conditioned column. The eluate was collected in a tube containing 0.5 g of Amberlite HCl resin, vortexed and centrifuged. The supernatant (0.4 mL) was centrifuged in a Micro Spin Centrifuge Filter before injection in CE.

2.5. Subjects and intestinal permeability test

Healthy volunteers ($n = 33$, 24 ± 11 years) and patients with type 1 diabetes ($n = 33$, 42 ± 12 years) were investigated with the LAC-MAN test. After collection of a basal urine sample, the fasting subjects drank a solution containing 5 g of LAC and 2 g of MAN in 50 mL of water. Urine was collected during the next 5 h in the presence of 2 mL sodium azide (15 g/L) as preservative. Two hour after the test was started the patients were encouraged to drink water. The total volume of urine was measured, and a 10-mL portion was stored at −20 °C. The procedure followed was approved by the hospital's responsible committee and the patients gave informed consent.

3. Results and discussion

3.1. Validation of the CE method

The non-invasive diagnostic test to evaluate the intestinal mucosal function measures the uptake of two sugars probes (LAC and MAN) after oral administration. The availability of a CE-based method to assess the urinary concentration quickly and at low cost is therefore attractive for the clinician but few studies addressed the issue of carbohydrates in human urine (29) and none specifically investigated the quantitative determination of LAC and MAN after an oral load of the two sugar-probes.

Three factors mainly affect the analysis of carbohydrates by CE: the hydroxide concentration, the type and the concentration of the monitoring ion, and the EOF direction. Underivatized carbohydrates does not exhibit UV absorption and the most suitable

approach for their detection at low levels is to derivatize them with a fluorescent tag before analysis [32–34], or to use electrochemical detection [28]. For the set up of our analytical method, however, we chose a basic CE instrument, mostly available in a clinical chemistry laboratory, not equipped with laser induced fluorescence (LIF) or on-capillary electrodes (OCEs) specific detectors. So, we had to use a strong UV absorbing BGE able to detect the passage of sugars as a loss of absorbance or a negative peak. Various monitoring ions have been suggested including *p*-nitrophenol [24], 2,6-pyridinecarboxilic acid [36], sorbic acid [23,36] and particularly riboflavine for its low noise and baseline disturbance [23]. However, we preferred sorbate for its easy handling being riboflavine a photosensitive and instable compound to be kept in a dark and cool place.

Sorbate looked as particularly suitable, being a singly charged molecule and enabling a transfer ratio of 1:1 for negatively charged carbohydrates, so one BGE molecule is displaced by one analyte anion ensuring maximum sensibility. At 254 nm it has a high molar absorption coefficient and an electrophoretic mobility similar to that of anionic carbohydrates and it does not exhibit interactions or redox reactions with carbohydrates analytes.

Sugars are weekly acidic compounds, having pK_a from 12 to 13, so a high pH BGE is needed for their migration. Applying the CE conditions described by Plocek et al. [24] based on the use of a sodium borate at pH 10 as BGE, voltage +6 kV, the negatively charged underivatized sugars are dragged to the cathodic detection end of the capillary by the EOF while, at the same time, are attracted electrophoretically toward the anode in the opposite direction and the analysis takes considerable time (glucose $M_t \approx 120$ min). Soga and Serwe [37] studied the separation of 17 saccharides (acidic-, neutral-, amino-sugars and sugar alcohols) with 20 mmol/L 2,6-pyridinedicarboxylic acid (PDC) as BGE, with or without reversal of the EOF. Albeit they presented conditions by normal EOF able to analyze the strongly acidic sugars within 18 min, the neutral sugars migrated too fast and were poorly resolved, while the sugar alcohols could not be even detected. The reversal of the EOF slightly increased the total analysis time but improved the peak shape and the quality of the separation for every sugar component so that the authors adopted this last condition. Several cationic additives like hexadimethrine bromide (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) [25], diethylamine [38], CTAB [36] have been exploited for the dynamic coating of the capillary to achieve a reversal of the EOF and a co-EOF migration of the negatively charged sugars in a relatively short analysis time. We achieved the baseline separation of seven underivatized carbohydrates moving towards the anode in less than 10 min using sorbate-CTAB with 50 mmol/L LiOH as BGE and applying a separation voltage of −5 KV. The order of migration was in correspondence to their pK_a values, with disaccharides exhibiting a lower mobility than monosaccharides of the same acidity, due to the higher size-to-charge ratio (Fig. 1); MAN (a sugar alcohol) exhibited the longer migration time (M_t). LiOH concentration was tested from 7.7 to 75 mmol/L: below 50 mmol/L the separation of IS, glucose and LAC worsened, while by increasing the hydroxide concentration up to

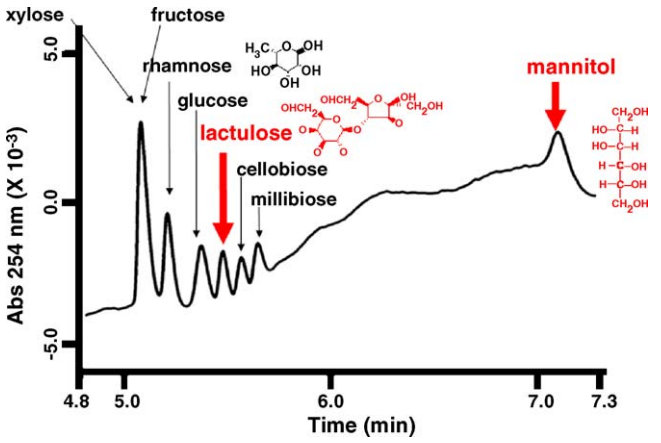

Fig. 1. Separation of a standard mixture of 7 sugars and 1 sugar alcohol (MAN) at concentration 0.125 mg/mL in water. A constant voltage of −5 kV was applied within the capillary column (27 cm, 50 μm ID; 20 cm to the detector) at 20 °C. The BGE consisted of 6 mmol/L sorbic acid, 1.25 mmol/L CTAB, 50 mmol/L LiOH (pH 12.5). Other technical conditions are reported under Materials and Methods. The chemical structures of glucose, LAC and MAN are also reported.

75 mmol/L the sensitivity, especially for MAN, decreased due to increased competition of the hydroxide ions in the displacement of the chromophore ion.

In Fig. 2 is reported the profile of a control urine before and after the addition of LAC, MAN and IS. No peaks were present at the M_t of the IS and of LAC, while only occasionally in some samples the presence of endogenous MAN was found before the test, possibly derived from the diet. In these cases the basal value was subtracted from the MAN excreted after the exogenous administration. A big peak (salt or proteins) migrating just prior to the sugar-probes did not impaired the analysis, although slightly delayed their M_t of the carbohydrates.

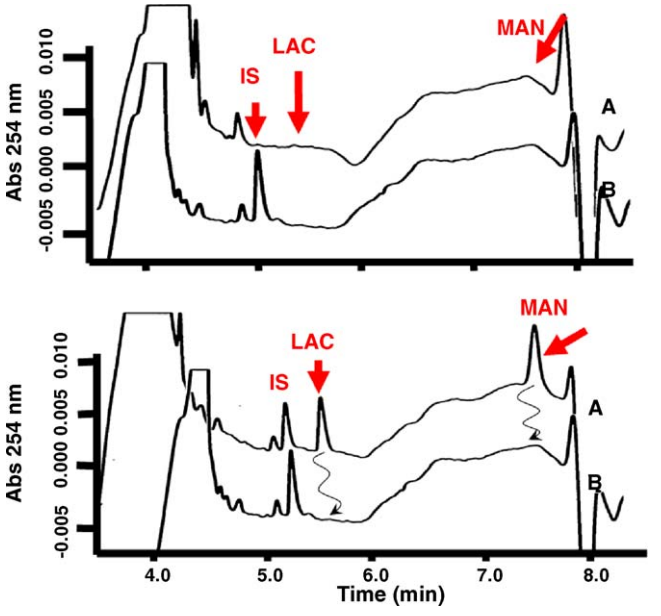

Fig. 2. Upper panel: blank urine sample without (A) or with addition of IS (B). Lower panel: blank urine sample added with the IS (A) and enriched with LAC and MAN (1 mg/mL) (B).

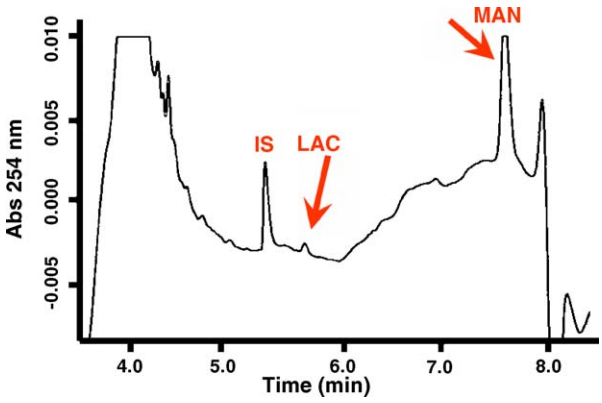

Fig. 3. Urine of a diabetes patient collected in the 5-h time-range after the sugars administration. The peaks of MAN and LAC correspond to a urinary concentration of 0.42 and 0.036 mg/mL, respectively.

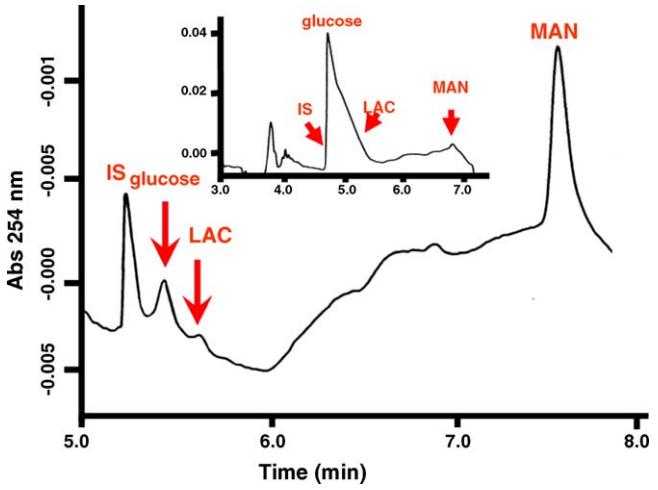

Fig. 4. Diabetic patients with glycosuria 0.17 mg/mL. Higher glucose concentrations impaired the LAC quantification: in the inset a sample with a glucose concentration of 8.4 mg/mL.

3.2. Quantification of LAC and MAN excretion in controls and diabetic patients

For the set up of calibration curves, MAN- and glucose-free control urines were always used. The two sugar-probes were quantitated as sugar-to-IS ratio versus urine concentration (mg/mL) and the equation curves were $y = 1.194x - 0.07$ ($r^2 = 0.996$) and $y = 1.270x + 0.006$ ($r^2 = 0.995$) for MAN and LAC, respectively. It is to point out that the response factor for both sugars, particularly for MAN, is strongly dependent on the analytical condition, i.e. pH, sorbate concentration, electric field, which in turn affect the peak shape, the resolution and the baseline disturbance. Slopes of 10 calibration curves in urine prepared and analysed independently in a 3-year period showed a CV% of 5.9 and 14% for LAC and MAN, respectively. The within-day imprecision assessed by analysing 10 times per day a urine sample enriched with 0.1 mg/mL of LAC and MAN showed a CV% of 4.5 and 7.5%, respectively. The recovery (>97% for both sugars) was determined by adding LAC and MAN to a urine sample before or after the pre-analytical purification steps. The lower detection limit in urine matrix for both sugars (signal-to-noise-ratio = 3) was 0.010 mg/mL. In Fig. 3 the analysis of a urinary sample collected from a diabetes patient after the test is reported. Despite the detection limit was about 15 times higher than that reported by Marsilio et al. [15] with HPLC and light scattering detection (0.82 and 0.65 mg/L for LAC and MAN, respectively), the CE method in the present set up was suitable to assess the urinary concentration of LAC and MAN after administration of the two sugar probes in 33 control subjects and 23 patients with type I diabetes. In controls, LAC concentration ranged from 0.01 to 0.15 mg/mL, while MAN ranged from 0.5 to 10 mg/mL, depending on the diuresis. This corresponded to a total excretion of 9.5 (5.7–13.6) mg and 321 (243–458) mg (median, interquartile range) for LAC and MAN, respectively. By contrast, patients with type I diabetes ($n = 23$) showed a significantly higher LAC excretion of 15.9 (12.8–23.7) mg associated to an impaired MAN excretion 220.1 (159.8–354.4) mg. This resulted to an excretion ratio LAC/MAN of 0.067 (0.050–0.127) in diabetes patients versus 0.025 (0.018–0.051) in controls ($p < 0.01$ diabetes versus controls, Mann–Whitney rank sum test).

As the present method was aimed to evaluate intestinal permeability in patients with diabetes, we tested the possible interference of glycosuria on LAC determination by adding increasing amounts of glucose to the urine of a control subject collected after administration of the two sugar probes. Fig. 4 clearly depicts the glucose influence on LAC quantification. The LAC peak could be integrated up to a glucose concentration of 0.17 mg/mL, although the considerably wavy baseline could affect the analytical precision; when glycosuria exceeded this value, LAC became immeasurable. Ten patients of the 33 investigated, showed glycosuria above 1 mg/mL and were not considered for the analysis. This emphasizes the need for a more tight blood glucose control during the test in order to avoid interference by glycosuria. As an alternative, the urine sample could be cleared of glucose before the CE analysis (chromatography, enzymatic treatment).

3.3. Conclusions

In conclusion, the CE method here proposed proved to be a simple, rapid and inexpensive method, useful for the analysis of low-molecular-mass underivatized sugars in urine. Compared to HPLC methods based on the use of particular detectors [15] and/or expensive and critical columns dedicated to carbohydrates analysis, the CE procedure requires a basic instrument equipped with a fused-silica capillary and exploits a dynamic coating with a common surfactant (CTAB). Provided that glucose excretion is taken under control, this assay may be used as a diagnostic test to quickly detect alterations of sugars absorption in diabetes patients and in much other different kind of pathologies related to alteration of intestinal permeability. Although the CE method in the present set-up provided reliable information on intestinal permeability in the studied groups, we feel useful to improve sensitivity, especially for LAC determination, in order to apply this method to the study of different cohort of patients

and different pathologies. It has been reported that sensitivity can be gained by using Na-sorbate instead of Li-sorbate in the BGE [23]. In this case, however, the heat dissipation system and capillary thermostatting become crucial. Our next aim is to transfer this analysis to a CE instrument of new generation (ProteomeLab 800, Beckman), specifically designed for the analysis of proteins, and to evaluate its performance for the analysis of low-molecular weight carbohydrates in terms of sensitivity of the detector, low-frequency noise, drift and baseline stability.

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