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Original Communication

Validation of a GC-MS and HPLC-ELSD method to study intestinal permeability

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ABSTRACT

Disturbed intestinal permeability is assessed by quantification of orally administered sugar probes (mannitol, lactulose and sucralose). We validated a gas chromatograph-mass spectrometry (GC-MS) (mannitol, lactulose and sucralose) and a high performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD) (mannitol and lactulose) method for the analysis of the sugars in urine. After validation, both methods were applied to a human and rat pilot study. Limits of quantification were < 15 mg/L for mannitol and lactulose, and 45 mg/L, 10 mg/L and 10 mg/L for mannitol, lactulose and sucralose, respectively, measured with HPLC-ELSD and GC-MS, respectively. Using HPLC-ELSD, recoveries varied between 89.8 and 109.5% for mannitol and lactulose. GC-MS analysis resulted in a recovery between 95.8 and 121.9% for all sugar probes. Imprecision was lower than 15% for all sugars measured with both techniques. Comparison of mannitol and lactulose concentrations measured with GC-MS and HPLC-ELSD by Bland-Altman and Deming regression resulted in a good agreement. In the human and rat pilot study, the lactulose mannitol ratio, and the 24-h sucralose excretion, increased significantly after oral administration of indomethacin. We can conclude that both methods can be used to accurately quantify urinary sugar concentrations in humans and rats to study intestinal permeability.

KEYWORDS: intestinal permeability, chromatography, mannitol, lactulose, sucralose

ABBREVIATIONS

PEG, polyethylene glycol; ^{51}Cr-EDTA, ^{51}Cr-labeled ethylenediaminetetraacetic acid; LMR, lactulose over mannitol ratio; GC, gas chromatography; FID, flame ionization detector; MS, mass spectrometer; HPLC, high performance liquid chromatography; ELSD, evaporative light-scattering detector; PAD, pulsed amperometric detector; DMSO, dimethyl sulfoxide; BSTFA, bis(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; LOD, limit of detection; LOQ, limit of quantification; IDL, instrument detection limit; MDL, method detection limit; WR, within-run; BR, between-run; WD, within-day variability; CI, confidence intervals; NSAID, non-steroidal anti-inflammatory drug

INTRODUCTION

The intestinal epithelium is our largest body surface in contact with the external world and therefore constitutes a selective barrier that absorbs nutrients and protects against potentially harmful substances. Intestinal permeability is assessed non-invasively *in vivo* by quantification of the urinary excretion of orally administered macromolecules, such as polyethylene glycol (PEG)

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[1, 2], ^{51}Cr-labeled ethylenediaminetetraacetic acid (^{51}Cr-EDTA) [3, 4], sucralose [5] and disaccharides in combination with monosaccharides [6].

By far the most widely applied test is the differential sugar excretion test, in which a monosaccharide (mannitol or rhamnose) and a disaccharide (lactulose or cellobiose) are administered orally. Mono- and disaccharides have different absorption routes. Monosaccharides pass through transcellular routes, whereas disaccharides pass through paracellular junctional complexes and extrusion zones at the villous tips [7]. To exclude pre- and post-absorption variables influencing the absorption (e.g. gastric emptying rate, intestinal transit, kidney function), the intestinal permeability is expressed as the ratio of the urinary recovery of the disaccharide over that of the monosaccharide, most often the lactulose over mannitol ratio (LMR) [8]. Since lactulose and mannitol are fermented by colonic bacteria, the LMR is not useful to evaluate colonic permeability. However, LMR is a sensitive and accurate marker to estimate small bowel permeability [3].

Sucralose, an artificial sweetener formed by chlorination of sucrose, is passively absorbed across the entire gastrointestinal mucosa, mainly through the paracellular pathway. As it is not metabolized by colonic bacteria, the entire intestinal permeability can be evaluated by quantification of urinary sucralose [9].

Other probes that are not metabolized include PEG and ^{51}Cr-EDTA. However, the absorption mechanism of PEG is still unclear and seems to differ from that of sugar, making comparison difficult [10]. Similar to sucralose, ^{51}Cr-EDTA can be used to assess the intestinal permeability along the entire gut. Fractionation of the urine collections in 0-6 h and 6-24 h reflect the small intestinal and colonic permeability, respectively. ^{51}Cr-EDTA follows a similar pathway as lactulose and its excretion in a 0-6 h collection correlates well with that of lactulose [11]. Although the use of the radioactive label makes it very easy to quantify in urine (γ- or β-scintillation counting), it reduces the feasibility in daily practice [10].

Several methods have been reported to quantify sugars in urine, such as thin-layer chromatography [12], enzymatic assays [13, 14], gas chromatography coupled to a flame ionization detector (GC-FID) [15-17] or to a mass spectrometer (MS) [18], capillary electrophoresis [19], high performance liquid chromatography (HPLC) coupled to a refractive index detector [20], evaporative light-scattering detector (ELSD) [21], pulsed amperometric detector (PAD) [9, 22] and tandem mass spectrometry [23].

In this paper, we validated and compared two analytical methods (GC-MS and HPLC-ELSD) to quantify mannitol and lactulose simultaneously in urine of rats and humans. We also validated the quantification of urinary sucralose with GC-MS. Both techniques were applied to a pilot study in healthy human subjects (lactulose, mannitol and sucralose) and rats (mannitol and lactulose).

EXPERIMENTAL

Chemicals and reagents

D-(+)-cellobiose (> 99%), lactulose (> 98%) and myo-inositol (> 99%) were obtained from Fluka (Steinheim, Germany). Dimethyl sulfoxide (DMSO), hydroxylamine hydrochloride (98.3%), indomethacine (> 99%), D-mannitol (> 99%), neomycin trisulfate (99%), sucralose (> 98%) and D-turanose (98%) were purchased from Sigma Aldrich (Steinheim, Germany). Acetonitrile (99.8%) was supplied by VWR (Haasrode, Belgium), pyridine (pro analysis) by UCB (Leuven, Belgium), n-heptane (99.8%) by Fisher Scientific (Pittsbugh, PA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) by Grace (Deerfield, MA). MilliQ water was obtained from a purification system (Sartorius, Bohemia, NY).

Standard solutions for HPLC-ELSD analysis (mannitol (4 g/L), lactulose (8 g/L) and cellobiose (8 g/L)) and for GC-MS (mannitol (6 g/L), lactulose (1 g/L), inositol (6 g/L) and turanose (800 mg/L)) were prepared in demineralised water, stored at 4 °C and used within 2 months. The oxime reagent consisted of 250 mg hydroxylamine hydrochloride in 10 mL pyridine and was stored at -20 °C.

Sample collection and storage

Urine was collected in recipients to which 750 mg neomycin (human samples) or 100 µL chlorhexidin

(1 g/L) (rat samples) was added to prevent bacterial growth. The volume of the collections was recorded and aliquots were stored at -20 °C until further analysis.

To determine accuracy and imprecision, a human and rat urine pool was prepared. The pools were stored in 1 mL aliquots at -20 °C.

Imprecision of the instruments and the effect of long term storage was studied using a blank human urine sample spiked with 200 mg/L mannitol, 40 mg/L lactulose and 40 mg/L sucralose and stored in 1 mL aliquots at -20 °C.

Sample preparation and analysis

Sample preparation and HPLC-ELSD analysis

The internal standard cellobiose (100 µL, 800 mg/L) was added to urine (10-400 µL) or to a standard solution (20-100 µL) containing mannitol (400 mg/L or 4 g/L) and lactulose (80 mg/L or 800 mg/L). All samples were diluted to 500 µL with demineralised water. Twenty µL of the diluted samples was analyzed by HPLC (Alliance 2695, Waters, Milford, MA) equipped with a Prevail Carbohydrate column (250 mm x 4.6 mm internal diameter, 5 µm particle size; Grace, Deerfield, MA). The chromatographic separation was carried out isocratically with 75% acetonitrile/ 25% MilliQ water for 16 minutes. The column was regenerated with 95% acetonitrile/5% MilliQ water for 5 minutes and equilibrated with 75% acetonitrile/25% MilliQ water for 5 minutes before the next analysis. The effluent was analyzed in an ELSD (ELSD 3300, Grace, Deerfield, MA) with a N_2 flow of 1.5 L/min at 40 °C and the detector signal was amplified 16 fold. Data was processed with Empower (Waters, Milford, MA).

Sample preparation and GC-MS analysis

An internal standard mixture (200 µL) containing inositol (600 mg/L) and turanose (150 mg/L) was added to 20-150 µL standard solution containing mannitol (600 mg/L or 6 g/L), lactulose (100 mg/L or 1 g/L) and sucralose (100 mg/L or 1 g/L) or to 10-400 µL urine. Samples were diluted with demineralised water to 1 mL and 125 µL of the diluted samples was dried overnight at 50 °C in a vacuum concentrator (RVC 2-18, Christ, Osterode am Harz, Germany). The sugars were converted into oximes by addition of 25 µL oxime reagent and incubated at 75 °C for 30 minutes. The samples were cooled down at -20 °C for 10 minutes and derivatized with 25 µL BSTFA + 1% TMCS for 35 minutes at 75 °C. We injected 0.5 µL into the GC-MS (Trace GC-MS, Thermofinnigan, Pittsburgh, PA) with a split ratio 1:12 and injector temperature at 250 °C. Chromatographic separation was achieved with an Rxi-5ms column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness; Restek, Bellefonte, PA) and a constant helium flow of 1 mL/min. The initial oven temperature of 100 °C was kept isothermal for 3 min, ramped to 210 °C with 30 °C/min, increased to 270 °C with 15 °C/min, subsequently to 290 °C with 30 °C/min and was held for 10 minutes. The Rxi-5ms column was conditioned at 310 °C for 10 minutes. Mass spectrometric detection was performed by electron impact in full scan mode (2 scans/s). M/z 361 was used to determine the area under the curve for lactulose, sucralose and turanose, m/z 319 and 318 were used for mannitol and inositol, respectively. Data were processed by Xcalibur (Thermo scientific, Pittsburgh, PA).

Method validation

Limit of detection (LOD) and quantification (LOQ)

LOD was defined as the lowest analyte concentration that significantly exceeds the measurement of a blank sample and was calculated as the mean of the peak to peak noise + 2SD. We distinguish the instrument detection limit (IDL) and the method detection limit (MDL). The IDL was the lowest concentration the instrument can detect, whereas MDL was the lowest concentration in samples which have gone through the entire sample preparation [24]. LOQ was defined as the lowest analyte concentration that could be quantified with a coefficient of variation (CV) $\leq$ 20% [25]. Standards in water were prepared and analyzed in triplicate as described above.

Calibration curves for HPLC-ELSD analysis

Calibration curves were constructed over a range of 0 to 2000 mg/L mannitol and over a range of 0 to 400 mg/L lactulose. To improve quantification, the curves were divided into 2 parts comprising

0-400 mg/L and 300-2000 mg/L for mannitol, and 0-80 mg/L and 40-400 mg/L for lactulose. Each calibration point was prepared in triplicate on 3 different days and analyzed. A standard curve for both mannitol and lactulose was fitted through the mean of each calibrator by polynomial regression. The equation was $y = ax^2 + bx + c$, where y represented the detector response and x the sample concentration. Within-run variability (WR) of the calibrators were calculated and expressed as CV (%). To determine the between-run variability (BR) of the calibration curves, the mean of each calibration point on day 1, day 2 and day 3 was plotted in a new calibration curve. CV was calculated for each calibrator. Accuracy was determined by calculating the relative residuals (%).

Calibration curves for GC-MS analysis

Calibration curves were constructed over a range of 0 to 975 mg/L for mannitol and 0 to 325 mg/L for lactulose and sucralose. Three aliquots of each calibration point were prepared on 3 different days and analyzed. Standard curves were fitted through the mean of each calibration point by polynomial regression according to the equation $y = ax^2 + bx + c$. WR and BR variability were calculated as described above.

Imprecision of the instruments

The spiked urine sample was analyzed at the beginning and at the end of each sample sequence. The within-day variation (WD) was calculated on 3 consecutive days and expressed as mean CV ± SD (%).

Accuracy and imprecision of the methods in the urine matrix

The accuracy of the analysis in urine was estimated by a recovery study after addition of 2 known amounts of sugar to 3 human and 3 rat pools in triplicate during 20 days. For HPLC-ELSD analysis, spike 1 contained 240 mg/L mannitol and 48 mg/L lactulose and spike 2 contained 1200 mg/L mannitol and 240 mg/L lactulose. For GC-MS analysis, spike 1 contained 150 mg/L mannitol, 50 mg/L lactulose and 50 mg/L sucralose. Spike 2 contained 450 mg/L mannitol and 150 mg/L lactulose and sucralose. The recovery was expressed in %.

Imprecision was determined by calculating the repeatability (WR variation) and reproducibility (BR variation) of the analytical methods [26]. WR variability was defined as the variability of the samples within 1 sample list and a mean WR, expressed as mean ± SD (%), and was calculated over 20 days. The BR variation over 20 days represented the variation due to changed conditions (time, operator, calibrator and chromatographic changes).

Long term stability of sugar concentrations in urine

The spiked human urine sample was analyzed regularly over a period of 15 months with HPLC-ELSD. In addition, the concentrations of mannitol and lactulose were measured after each of 13 thawing-freezing cycles.

Comparison of the concentration of mannitol and lactulose between GC-MS and HPLC-ELSD

Urinary mannitol and lactulose concentrations were analyzed in 44 human and 57 rat samples with both analytical techniques. The results were compared using Bland-Altman plots [27] and Deming regression [28].

Pilot study

Twenty-one healthy subjects (12 men, age 21 ± 0 years) without a history of gastrointestinal disorders and not taking any medication except for oral contraceptives, participated in this study. The study protocol was approved by the Ethics Committee of the University of Leuven in accordance with the declaration of Helsinki. All subjects gave informed consent before initiation of the study. Each volunteer underwent a test at baseline and a test after intake of indomethacin separated by at least 1 week. On the day before each test, the volunteers did not consume any dairy products. For both tests, the volunteers drank 150 mL water, containing 5 g lactulose, 2 g mannitol and 5 g sucralose after a 6-h fasting state. For the second test, the volunteers took 75 mg of indomethacin 16 h before the test and 50 mg indomethacin 4 h before drinking the test solution. Urine was collected for 24 h in 4 fractions: 0-2, 2-4, 4-6 and 6-24 h. Volunteers were allowed to drink water, but not to eat during the first 4 hours of the collection period.

In a second study, intestinal permeability was studied in 9 male Wistar rats (Janvier, Le Genest Saint Isle, France). The protocol was approved by the animal Ethics Committee of the University of Leuven. After a 1-hour fasting period, indomethacin (10 mg/kg) in 200 µL DMSO (n = 5) or vehicle (n = 4) was administered by oral gavage. Five hours later, 2 mL of water containing 120 mg lactulose and 80 mg mannitol was administered by oral gavage, followed by a 6 h urine collection. During the test, rats were placed in metabolic cages with free access to water.

Statistical analysis was performed using SPSS 17.0 (SPSS Inc.). As the data were not normally distributed (Shapiro-Wilks), data are presented as median and interquartile range. Data were compared by non-parametric tests. For paired comparisons, a Wilcoxon test was performed. Non-paired comparisons were performed by applying a Mann-Whitney test. The level of statistical significance was set at $p < 0.05$.

RESULTS

Under the described chromatographic conditions, retention times of mannitol, lactulose and cellobiose on HPLC-ELSD were 8.1, 11.5 and 14.6 minutes, respectively (Figure 1). Sucralose eluted after 3.6 min and was not separated from interfering compounds. Therefore, sucralose was not quantified on HPLC. During GC-MS analysis, mannitol, inositol and sucralose eluted at 10.1, 11.1 and 15.1 minutes, respectively. Lactulose and turanose eluted as double peaks due to tautomeric forms of this reducing sugars [29] with retention times 16.3-16.4 and 17.5-17.6 minutes, respectively (Figure 2).

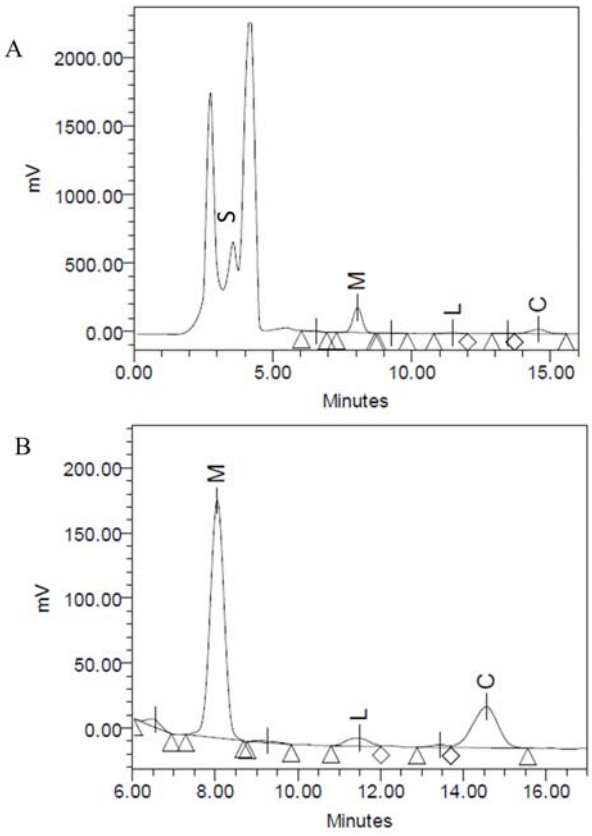

Figure 1. Representative HPLC-ELSD chromatogram of a human urine sample after oral intake of mannitol (M), lactulose (L) and sucralose (S) with internal standard cellobiose (C).

Method validation

LOD and LOQ

The IDL of mannitol and lactulose measured with HPLC-ELSD amounted to 0.12 and 0.10 mg/dL, respectively, and the MDL to 6.3 mg/L and 5.0 mg/L, respectively, whereas the LOQ was 12.5 mg/L and 15.0 mg/L, respectively.

The IDL of mannitol, lactulose and sucralose of the GC-MS was 0.04 mg/L, 0.05 mg/L and 0.10 mg/L, respectively, whereas the MDL amounted to 1.5 mg/L, 2.0 mg/L and 4.0 mg/L, respectively. The LOQ was 45 mg/L, 10.0 mg/L and 10 mg/L for mannitol, lactulose and sucralose, respectively.

Calibration curves

The mean calibration curves for HPLC-ELSD analysis of mannitol were $y = 1.33\ 10^{-5}x^2 + 2.98\ 10^{-3}x - 9.62\ 10^{-3}$, $R^2 = 0.999$ (0-400 mg/L) and $y = 8.84\ 10^{-6}x^2 + 2.51\ 10^{-3}x - 2.27\ 10^{-4}$, $R^2 = 0.991$ (300-2000 mg/L). For lactulose, calibration curves $y = 1.70\ 10^{-5}x^2 + 9.45\ 10^{-2}x - 4.60$, $R^2 = 0.996$ (0-80 mg/L) and $y = 8.11\ 10^{-6}x^2 - 2.93\ 10^{-3}x - 2.93\ 10^{-2}$, $R^2 = 0.997$ (4-400 mg/L) were obtained. Accuracy, WR and BR variability for all calibrators for both mannitol and lactulose were < 13% (Table 1).

The mean calibration curves of the GC-MS analysis for mannitol, lactulose and sucralose were $y = 9.43\ 10^{-8}x^2 + 1.45\ 10^{-3}x - 4.62\ 10^{-3}$, $R^2 = 0.991$; $y = -4.71\ 10^{-6}x^2 + 3.73\ 10^{-3}x - 2.22\ 10^{-2}$,

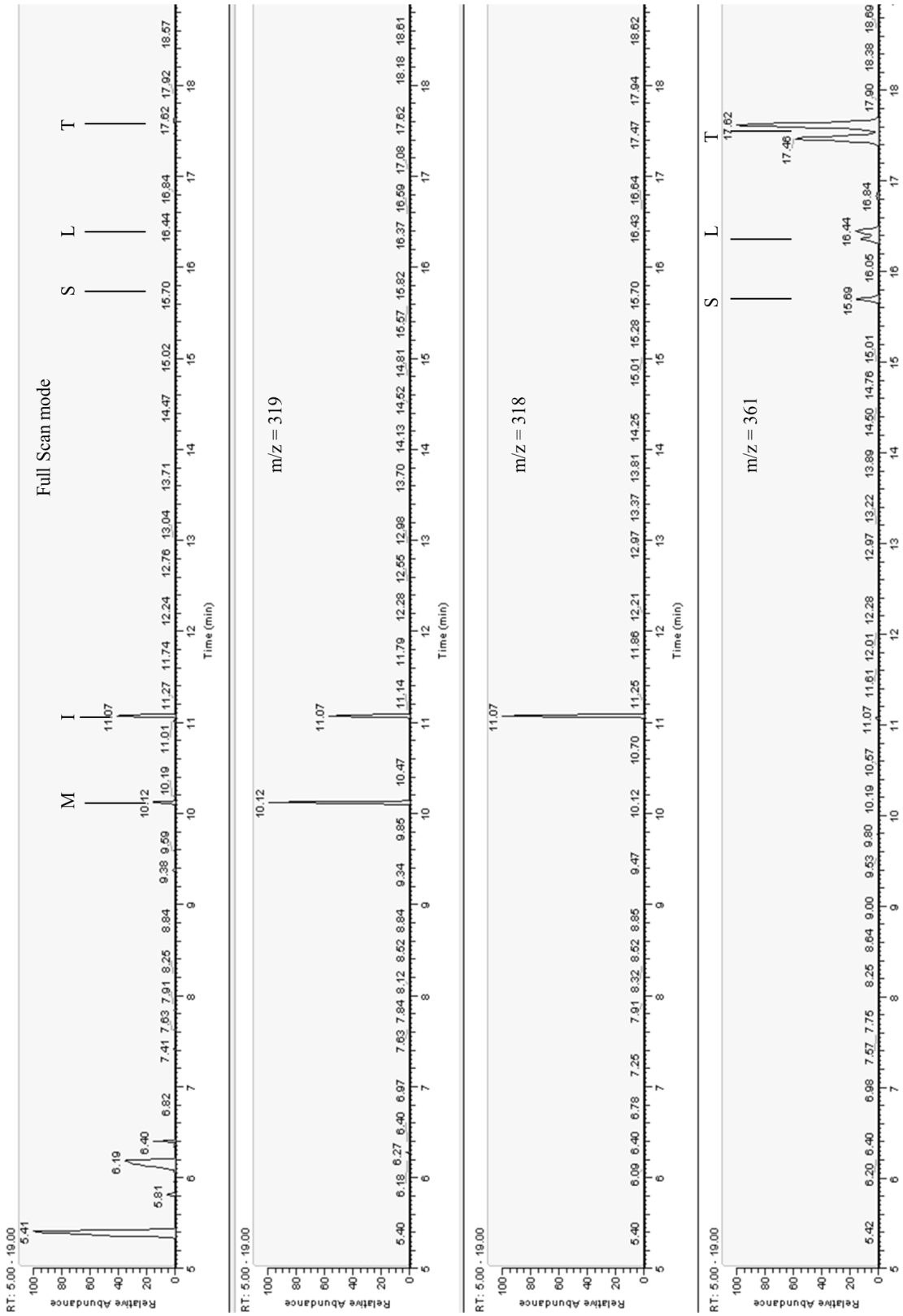

Figure 2. GC-MS chromatogram of a healthy subject after oral intake of mannitol (M), lactulose (L) and sucralose (S) with internal standards, inositol (I) and turanose (T), in full scan mode (A) and in selected ion mode (B-C-D). M/z 319 was used to detect mannitol (B), m/z 318 to detect inositol (C) and m/z 361 was used to detect lactulose, sucralose and turanose (D).

Table 1. Accuracy (ACC), within-run (WR) and between-run (BR) variations of HPLC-ELSD and GC-MS analysis in standard solutions.

		Calibrators of GC-MS (mg/L)							Calibrators of HPLC-ELSD (mg/L)						
Mannitol		**75**	**150**	**225**	**300**	**525**	**750**	**975**	**100**	**200**	**300**	**400**	**800**	**1400**	**2000**
Day 1	ACC (%)	-10.7	-1.0	-6.8	-0.7	5.3	-0.3	-12.1	-2.5	-0.6	0.8	-0.5	-5.1	2.3	-1.0
	WR (%)	6.3	5.9	11.1	6.7	2.1	0.9	1.2	6.9	4.5	1.5	1.1	1.0	0.3	1.3
Day 2	ACC (%)	-4.2	-18.7	-8.7	1.7	4.4	0.2	-6.3	-0.3	-0.2	0.2	-0.7	-7.7	-3.0	-8.0
	WR (%)	14.2	7.7	4.3	6.5	8.3	9.4	1.3	1.4	1.1	0	1.2	2.7	1.4	0.8
Day 3	ACC (%)	11.1	-7.1	2.4	2.6	4.8	9.2	-0.8	-5.1	-1.0	1.5	0.2	-4.1	1.8	-0.4
	WR (%)	2.7	9.5	1.6	3.2	2.1	2.3	0.6	1.4	0.8	1.6	1.5	1.7	1.2	1.9
	BR (%)	18.9	8.9	10.0	9.6	2.9	5.2	4.3	6.8	3.2	2.7	2.0	1.5	1.4	3.3
Lactulose		**25**	**50**	**75**	**100**	**175**	**250**	**325**	**20**	**40**	**60**	**80**	**160**	**280**	**400**
Day 1	ACC (%)	-6.8	-6.3	-7.9	-5.6	2.4	3.9	-0.2	-7.6	3.4	-1.5	-1.2	-1.1	0.4	-0.6
	WR (%)	4.3	1.4	2.7	3.6	2.1	0.8	5.1	6.2	9.1	3.4	2.9	0.5	1.9	1.8
Day 2	ACC (%)	-4.4	-6.9	-2.9	-1.3	0.0	1.2	-1.4	9.8	-1.0	-1.9	0.8	1.4	-0.5	0.3
	WR (%)	4.6	1.8	2.4	5.8	3.4	1.5	0.3	6.5	4.5	3.8	3.5	2.9	1.4	0.4
Day 3	ACC (%)	-5.3	-6.9	-3.7	-3.6	-0.5	1.9	2.3	-4.1	-2.2	2.7	-1.1	0.7	-0.3	0.0
	WR (%)	1.3	1.3	2.4	1.1	0.6	0.4	2.3	1.1	7.1	3.4	0.2	0.5	1.7	0.4
	BR (%)	15.4	6.9	8.8	7.1	1.7	1.5	4.0	12.9	10.8	5.7	6.6	3.2	5.0	2.9
Sucralose		**25**	**50**	**75**	**100**	**175**	**250**	**325**							
Day 1	ACC (%)	1.3	-14.3	-6.2	-8.6	-1.0	-1.7	-3.5							
	WR (%)	6.1	2.4	3.9	1.1	2.4	3.8	5.4							
Day 2	ACC (%)	-4.6	-4.6	-1.2	-0.3	0.2	0.8	-0.3							
	WR (%)	1.7	1.5	1.7	2.5	2.0	0.3	0.8							
Day 3	ACC (%)	-11.9	-10.3	-7.9	-3.1	4.7	4.3	6.9							
	WR (%)	0.8	1.0	3.5	3.1	2.3	1.6	2.4							
	BR (%)	14.9	0.6	3.7	2.1	6.6	6.0	7.8							

Table 2. Imprecision and recovery of unspiked, spike 1 and spike 2 concentrations of the sugars in a urine matrix on 20 consecutive days.

	GC-MS (n = 20)										
	Unspiked			Spike 1[a]				Spike 2[b]			
	c	BR	WR	c	BR	WR	Recovery	c	BR	WR	Recovery
	mg/L	%	%	mg/L	%	%	%	mg/L	%	%	%
Mannitol	mean ± SD		mean ± SD	mean ± SD		mean ± SD	mean ± SD	mean ± SD		mean ± SD	mean ± SD
Human 1	293 ± 29	9.9	5.9 ± 4.7	468 ± 50	10.7	5.5 ± 3.7	116.5 ± 18.3	749 ± 71	9.4	3.2 ± 2.9	99.4 ± 13.5
Human 2	301 ± 42	14.1	7.3 ± 5.3	795 ± 57	7.1	4.9 ± 3.8	121.9 ± 17.3	795 ± 57	7.1	3.0 ± 3.1	109.8 ± 7.5
Human 3	578 ± 39	6.8	5.1 ± 3.5	578 ± 39	6.8	4.6 ± 5.4	109.5 ± 16.1	841 ± 56	6.7	2.9 ± 3.4	94.9 ± 12.2
				mean recovery human urine			*116.0 ± 6.0*				*101.4* ± 7.6
Rat 1	222 ± 33	14.7	5.0 ± 3.7	398 ± 40	10.1	5.4 ± 6.3	117.3 ± 13.9	695 ± 73	10.5	3.6 ± 3.5	107.8 ± 13.0
Rat 2	420 ± 41	9.8	4.4 ± 3.3	590 ± 46	7.8	4.4 ± 3.6	112.8 ± 20.3	859 ± 69	8.0	2.3 ± 1.8	97.6 ± 12.5
Rat 3	396 ± 41	10.3	6.4 ± 3.8	571 ± 52	9.1	3.0 ± 1.9	116.7 ± 17.8	849 ± 75	8.9	2.1 ± 1.9	100.6 ± 12.3
				mean recovery rat urine			*115.6 ± 2.4*				*102.0* ± 5.3
Lactulose											
Human 1	13 ± 4	n.a.[e]	n.a.[e]	62 ± 6	10.3	3.7 ± 3.0	96.0 ± 11.9	167 ± 14	8.6	3.2 ± 2.1	102.5 ± 9.9
Human 2	9 ± 4	n.a.[e]	n.a.[e]	57 ± 6	10.5	4.6 ± 4.1	96.5 ± 10.1	165 ± 10	5.8	2.9 ± 2.2	103.8 ± 6.6
Human 3	4 ± 9	n.a.[e]	n.a.[e]	64 ± 9	14.1	5.8 ± 5.7	100.7 ± 12.1	174 ± 12	6.7	1.9 ± 1.4	107.2 ± 7.0
				mean recovery human urine			*97.7 ± 2.6*				*104.5* ± 2.4
Rat 1	78 ± 6	7.3	3.5 ± 2.7	133 ± 1	5.5	3.4 ± 2.6	108.1 ± 10.2	231 ± 15	6.6	2.3 ± 2.4	101.4 ± 9.3
Rat 2	176 ± 8	4.7	2.7 ± 1.8	226 ± 1	4.4	2.2 ± 1.6	101.7 ± 18.2	320 ± 16	4.9	2.0 ± 2.4	95.8 ± 11.1
Rat 3	133 ± 8	6.3	3.7 ± 2.0	188 ± 1	4.6	2.3 ± 1.8	109.8 ± 127	287 ± 14	4.9	1.8 ± 1.4	102.8 ± 9.0
				mean recovery rat urine			*106.5 ± 4.3*				*100.0 ± 3.7*
Sucralose											
Human 1	54 ± 7	13.9	5.2 ± 4.1	111 ± 1	11.1	4.3 ± 2.3	112.8 ± 11.3	213 ± 16	7.6	2.3 ± 1.5	105.5 ± 10.8
Human 2	50 ± 7	13.5	7.3 ± 5.3	105 ± 1	8.6	4.9 ± 3.8	108.7 ± 7.6	204 ± 13	6.5	3.0 ± 3.1	102.4 ± 9.9
Human 3	150 ± 12	7.8	3.1 ± 1.9	199 ± 1	4.8	2.0 ± 1.3	103.1 ± 14.0	291 ± 22	7.6	1.5 ± 1.2	96.7 ± 16.6
				mean recovery human urine			*108.2 ± 4.9*				*101.5 ± 4.5*
Rat 1	50 ± 6	11.6	4.6 ± 4.0	10.2 ± 0.9	9.0	4.0 ± 2.1	106.7 ± 13.6	20.3 ± 1.7	8.3	2.3 ± 1.6	102.4 ± 11.5
Rat 2	3 ± 3	n.a.[e]	n.a.[e]	5.4 ± 0.7	12.8	3.7 ± 3.5	100.7 ± 13.7	15.3 ± 0.9	6.1	2.9 ± 2.9	99.9 ± 6.2
Rat 3	67 ± 06	8.9	3.9 ± 2.4	11.9 ± 0.7	5.6	2.9 ± 2.4	102.6 ± 9.1	21.6 ± 1.2	5.6	2.3 ± 1.9	99.3 ± 10.1
				mean recovery rat urine			*103.3 ± 3.1*				*100.5 ± 1.6*

Table 2 continued..

	HPLC-ELSD (n = 20)										
	Unspiked			Spike 1[c]				Spike 2[d]			
Mannitol	**c (mg/L) mean ± SD**	**BR % **	**WR % mean ± SD**	**c (mg/L) mean ± SD**	**BR %**	**WR % mean ± SD**	**Recovery (%) mean ± SD**	**c (mg/L) mean ± SD**	**BR %**	**WR % mean ± SD**	**Recovery (%) mean ± SD**
Human 1	273 ± 15	5.6	2.3 ± 1.4	511 ± 25	4.9	1.4 ± 0.9	99.1 ± 6.7	1503 ± 71	4.7	2.0 ± 1.0	102.5 ± 5.0
Human 2	560 ± 32	5.8	2.1 ± 1.0	767 ± 44	5.7	1.8 ± 0.7	99.6 ± 10.7	1795 ± 63	3.5	2.1 ± 1.9	102.5 ± 4.5
Human 3	349 ± 22	6.4	2.4 ± 1.7	561 ± 28	5.0	1.5 ± 0.9	89.8 ± 6.4	1478 ± 82	5.5	2.1 ± 1.3	94.9 ± 5.4
				mean recovery human urine			*96.1 ± 5.5*				*99.9 ± 4.4*
Rat 1	172 ± 6	3.4	2.7 ± 1.8	435 ± 10	2.2	1.2 ± 0.8	109.5 ± 4.3	1458 ± 52	3.5	1.7 ± 1.6	107.2 ± 4.1
Rat 2	332 ± 17	5.0	1.8 ± 0.9	573 ± 29	5.1	1.4 ± 0.7	100.5 ± 9.3	1609 ± 87	5.4	1.7 ± 1.5	106.4 ± 6.4
Rat 3	640 ± 38	6.0	3.4 ± 3.0	864 ± 72	8.3	1.9 ± 1.0	99.0 ± 8.9	1771 ± 89	5.0	3.4 ± 3.1	94.9 ± 5.7
				mean recovery rat urine			*103.0 ± 4.4*				*103.2 ± 7.2*
Lactulose											
Human 1	8 ± 5	n.a.[e]	n.a.[e]	58 ± 4	7.2	3.7 ± 2.4	105.9 ± 10.0	255 ± 12	4.7	2.0 ± 1.1	103.2 ± 6.3
Human 2	8 ± 6	n.a.[e]	n.a.[e]	61 ± 7	10.8	4.3 ± 2.0	109.0 ± 8.5	253 ± 11	4.2	3.6 ± 4.1	102.1 ± 4.0
Human 3	5 ± 5	n.a.[e]	n.a.[e]	53 ± 4	8.3	4.4 ± 2.6	99.8 ± 10.3	241 ± 14	5.6	1.6 ± 0.7	98.5 ± 6.7
				mean recovery human urine			*105.0 ± 4.8*				*101.3 ± 2.5*
Rat 1	81 ± 6	7.6	3.2 ± 2.1	130 ± 6	4.9	2.2 ± 1.3	101.1 ± 6.9	327 ± 9	2.8	1.8 ± 3.3	102.1 ± 3.8
Rat 2	181 ± 9	5.1	2.2 ± 1.1	232 ± 08	3.4	1.7 ± 0.9	105.3 ± 10.8	428 ± 15	3.5	1.7 ± 1.0	103.6 ± 7.5
Rat 3	269 ± 11	4.2	2.8 ± 2.0	317 ± 10	3.1	1.6 ± 1.7	99.7 ± 10.9	505 ± 25	5.0	2.0 ± 1.8	98.5 ± 10.0
				mean recovery rat urine			*101.3 ± 3.8*				*101.3 ± 2.9*

[a] spike 1 (GC-MS) contained 150 mg/L mannitol, 50 mg/L lactulose and 50 mg/L sucralose.
[b] spike 2 (GC-MS) contained 450 mg/L mannitol, 150 mg/L lactulose and 150 mg/L sucralose.
[c] spike 1 (HPLC-ELSD) contained 240 mg/L mannitol and 48 mg/L lactulose.
[d] spike 2 (HPLC-ELSD) contained 1200 mg/L mannitol and 240 mg/L lactulose.
[e] n.a.: not applicable, concentrations below LOQ.

$R^2 = 0.998$ and $y = 8.90\ 10^{-7}x^2 + 5.57\ 10^{-4}x - 2.15\ 10^{-3}$, $R^2 = 0.993$. For the HPLC-ELSD, accuracy and WR variabilities were < 10% for mannitol and lactulose, whereas accuracy and WR variations were < 20% for mannitol, < 8% for lactulose and < 15% for sucralose analyzed with GC-MS. (Table 1). BR variability was < 19% for the lowest calibrator and < 11% for the other calibrators.

Accuracy and imprecision of urine samples

WD variation for the HPLC-ELSD was 1.9 ± 0.7% and 3.3 ± 1.8% for mannitol and lactulose, respectively, and 1.5 ± 1.0%, 2.2 ± 1.5% and 3.1 ± 1.0% for mannitol, lactulose and sucralose analyzed with GC-MS.

The accuracy, WR and BR variation in the urine matrix are shown in Table 2. As the lactulose concentration in the unspiked human samples was below the LOQ, WR and BR variabilities were not calculated for those samples. Recoveries for all sugars on HPLC-ELSD varied between 89.8 and 109.5%. For GC-MS, recoveries between 96.0 and 112.8% were obtained except for the lowest mannitol spike (between 109.5 and 121.9%). Mean WR variations for mannitol and lactulose analyzed with HPLC-ELSD were < 5% and BR variations were < 11%. The mean WR variations of the GC-MS analysis for all sugars were < 8%, whereas BR variations were < 15%.

Long term stability of the sugars in urine

The results of the stability tests are shown in Figure 3. After 15 months storage and 13 freezing-thawing cycles, the relative errors to the known concentrations are < 10% for mannitol and lactulose.

Comparison of the concentration of mannitol and lactulose between GC-MS and HPLC-ELSD

Deming regression of the human data resulted in the following equations: y = 0.99x - 14 with 95% confidence intervals (CI) for the slope and intercept of 0.87 to 1.12 and -129 to 101, respectively, for mannitol and y = 1.20x - 13 with 95% CI for the slope and intercept of 1.03 to 1.38 and -19.19 to -6.33 for lactulose. Deming regression of the rat samples resulted in a correlation for mannitol concentrations of y = 1.07x + 2 with 95% CI of 0.93 to 1.22 for the slope and -26.05 to 30.43 for the intercept; and for lactulose concentrations of y = 1.09x - 5 with 95% CI of 1.01 to 1.18 for the intercept and -10.03 and -0.45 for the slope. Since the difference between GC-MS and HPLC-ELCD values was proportional to the mean, Bland-Altman plots were constructed using log-transformed values [27]. The plots revealed a mean bias of -23 mg/L and -1 mg/L for human urinary mannitol and lactulose, respectively. In rat urine, a mean bias of -19 mg/L and 6 mg/L for mannitol and lactulose was obtained (Figure 4).

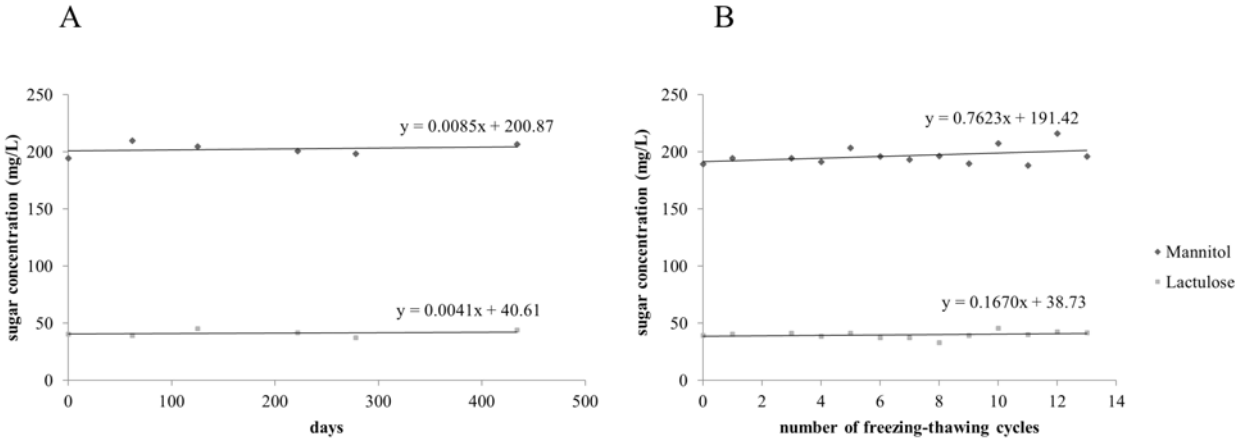

Figure 3. Stability of mannitol and lactulose after long-term storage at -20 °C (A) and after several freezing-thawing cycles (B).

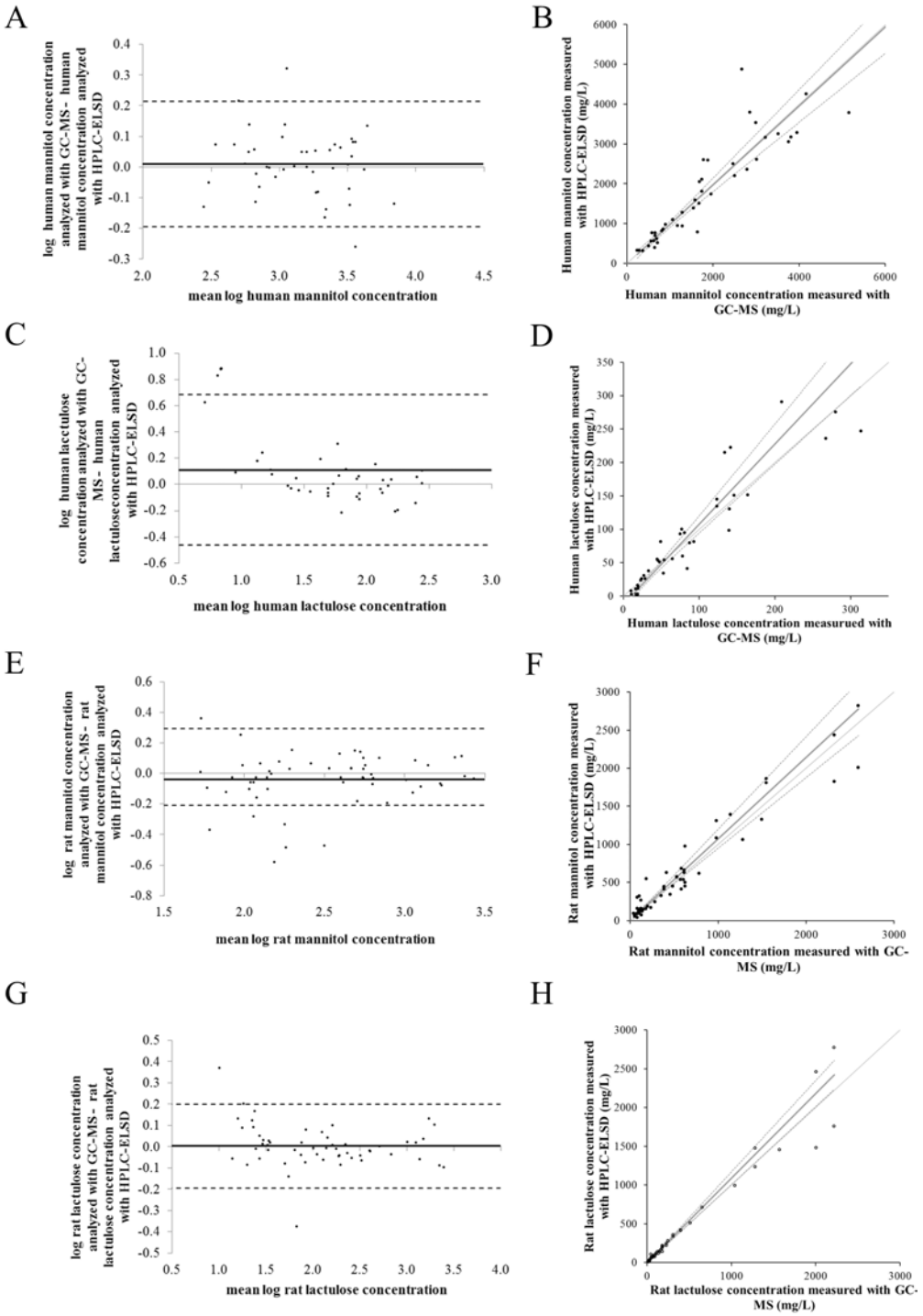

Figure 4. Comparison of human and rat urinary mannitol and lactulose concentrations obtained from HPLC-ELSD and GC-MS. Bland-Altman plots with the method difference expressed as absolute human urinary mannitol (A) concentration, human lactulose concentration (C), mannitol concentration in rat (E) and lactulose concentration in rat (G). Solid line represents the mean bias and the dashed lines represent the 96% limits of agreement (B-D-F-H). Deming regression for mannitol (B) and lactulose (D) in human subjects; and for mannitol (F) and lactulose (G) in rats with the grey solid line representing the line of identity, the solid black line representing the correlation and the dashed lines representing the 95% confidence interval.

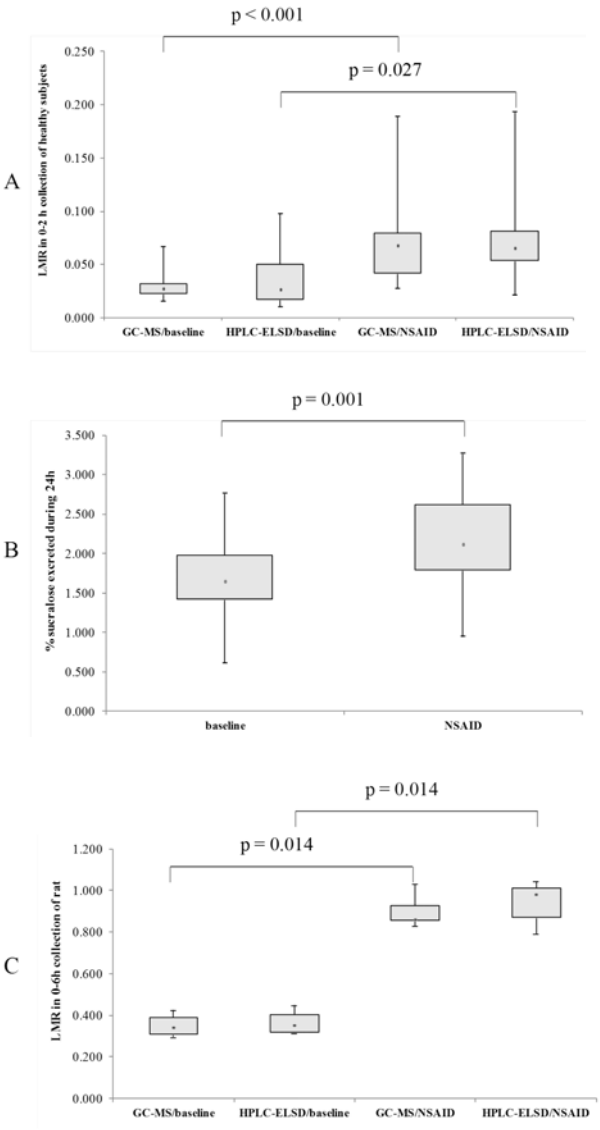

Figure 5. Gastrointestinal permeability in humans (0-2 h collection) (A, B) and rats (0-6 h collection) (C), expressed as LMR (measured with GC-MS and HPLC-ELSD) (A, C) and % sucralose excretion (B) (measured by GC-MS) with and without oral administration of indomethacin.

Pilot study

LMR and the amount of sucralose were measured in all collections, but only data of the relevant collections (0-2 h for LMR in humans, 0-6 h for LMR in rats and 0-24 h for sucralose in humans), were shown (Figure 5). Oral administration of indomethacin to healthy volunteers and rats resulted in a significantly increased excretion of sucralose and LMR ratio in healthy subjects and rats.

DISCUSSION

Disturbed intestinal permeability is an important marker of the intestinal barrier integrity and has been implicated in the pathogenesis of inflammatory bowel diseases such as Crohn's disease and celiac disease. More recently, it has been shown that intestinal barrier function is affected by obesity in animal models [30] as well as in obese patients [31]. Sugar probes are the most widely used to estimate intestinal permeability [6] and are most often quantified using GC or HPLC analyses. The choice for a GC-based or LC-based method is often not accounted for and probably depends in most cases on the availability of the instrument. In this study, we validated both a GC-MS method for the simultaneous quantification of mannitol, lactulose and sucralose and an HPLC-ELSD method to quantify mannitol and lactulose and compared the performance of both methods. To allow GC analysis, sugars need to be oximized and derivatised prior to injection [7, 15, 16], whereas no derivatization is required for HPLC analysis [19, 21, 22]. Several papers report desalting of the urine samples using ion exchange resins such as Amberlite prior to HPLC analysis. In our hands, desalting did not afford any benefit with regard to LOD or LOQ, lifetime of the column or frequency of maintenance of the system. Therefore, we omitted the desalting step from the HPLC method during validation. The IDL and LOQ obtained with both methods were similar and in line with the LOD and LOQ values reported in literature. Marsilio *et al.* also used an HPLC-ELSD method, yet with different column and chromatographic conditions, to quantify mannitol and lactulose and reported LOD values of 0.6 mg/L and 0.8 mg/L for mannitol and lactulose, respectively, which is approximately 10 times lower than the lowest sugar concentration we could detect. However, LOQ values of 10 mg/L and 30 mg/L for mannitol and lactulose, respectively, were in the same range as our values. Methods that use GC with FID detection, report LOD ranges varying between 0.5 and 5 mg/L, and LOQ values that are at least tenfold higher [7, 15, 16].

Whereas urine from healthy humans is largely free of protein, urine from rats (and other animal species) contains milligram amounts per mL of low-molecular weight proteins, so-called major urinary proteins or mups, which might have a function in pheromone communication [32]. As these proteins are able to bind small chemicals, they might interfere during sample preparation and/or chromatographic analysis. Therefore, accuracy and imprecision of the analytical methods were evaluated both in human and rat urine. Recoveries were similar for rat and human urine implicating that it was not necessary to add a deproteinisation step in the rat urine sample preparation. Both methods are accurate (recoveries ranged between 96 and 116%) and results are in line with previously published values (between 90 and 122%) [7, 15, 16, 19, 21, 22, 33]. Imprecision was examined by calculating WR and BR variability. The imprecision was slightly higher for GC-MS as compared to HPLC-ELSD, which is likely due to the more extensive sample preparation. Nevertheless, the imprecision remained below 10%.

Although numerous GC-based as well as HPLC-based methods have been described for analysis of urinary sugar concentration, only Fleming *et al.* briefly compared both methods. They found a good correlation between lactulose and mannitol concentrations measured in 18 urine samples using both HPLC-PAD and GC-FID. In the present study, Deming regression resulted in a slope of nearly 1, and a mean bias close to 0 suggesting no proportional or fixed bias between both methods. Bland-Altman analysis resulted in the same conclusions suggesting that our data show a good agreement between both techniques to quantify urinary mannitol and lactulose.

Finally, we applied both methods to a human and rat pilot study, where subjects performed a basal permeability test and a second test after intake of indomethacin, a non-steroidal anti-inflammatory drug (NSAID) known to increase intestinal permeability [34]. A potential mechanism is the reduced synthesis of mucosal prostaglandin due to inhibition of cyclooxygenase. In addition, NSAID uncouple mitochondrial oxidative phosphorylation after absorption in the intestinal cells resulting in loss of intercellular integrity and increased permeability [35]. We observed a statistically significant increase in intestinal permeability, measured as sucralose excretion and LMR, in human subjects and a statistically significant increase of LMR in rats after intake of indomethacin, which confirms earlier published data [36, 37]. Human 24-h sucralose excretions in basal conditions (1.5% of administered dose) are comparable to previously published values [15, 19, 38]. Unfortunately, LMR is difficult to compare with published data, due to differences in urine collections and dosage of mannitol and lactulose. Published LMR in humans range between 0.013 and 0.027 whereas LMR in our study was 0.025. In rats, LMR is tenfold higher, confirming previously reported values [39]. Analysis with both GC-MS and HPLC-ELSD allowed to adequately detect the increased permeability induced by indomethacin.

CONCLUSION

From this extended validation study, we conclude that mannitol and lactulose can reliably be quantified in a single analysis by either GC-MS or HPLC-ELSD. Both methods can be applied to analysis of human and rat urine without the need for an additional deproteinisation step. Analysis by GC-MS offers the advantage that sucralose can be measured in the same run. In contrast, the HPLC-ELSD method requires less sample preparation and is less time-consuming.

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