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Novel analytical approach to a multi-sugar whole gut permeability assay

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ABSTRACT

Many pathophysiological conditions are associated with increased gastrointestinal permeability, reflecting an elevated risk of endotoxaemia, inflammation, and sepsis. Permeability tests are increasingly used in clinical practice to obtain information on gastrointestinal functioning, but tests are often restricted to the small intestine, and require large oral sugar doses. Therefore, a novel multi-sugar assay was developed, allowing assessment of whole gut permeability changes in urinary and plasma samples collected at regular intervals from 10 healthy volunteers at baseline and after intake of monosaccharides (rhamnose and erythritol) and disaccharides (sucrose, lactulose, and sucralose). Samples were analyzed by isocratic cation-exchange LC–MS. Sample preparation and detection conditions were optimized. After centrifugation, chromatographic separation was achieved on an IOA-1000 column set at 30 °C. Column effluent was mixed with ammonia for sugar-ammonium adduct formation. The lower limit of detection was 0.05 μmol/L for disaccharides and 0.1 μmol/L for monosaccharides. Linearity for each probe was between 1 and 1000 μmol/L (R^2: 0.9987–0.9999). Coefficients of variation were <5% in urine, and <9% in plasma. Recovery data were within the 90% to 110% range at all spiked concentrations. This highly sensitive novel LC–MS approach resulted in a significant decrease of the detection limit for all sugar probes, allowing a 5-fold reduction of the commonly used lactulose dose and the addition of sugar probes to also assess the gastroduodenal and colon permeability. In combination with its extended application in plasma, these features make the novel assay a promising tool in the assessment of site-specific changes in gastrointestinal permeability in clinical practice.

1. Introduction

Many pathophysiological conditions are associated with increased gastrointestinal (GI) permeability, including celiac disease [1], inflammatory bowel disease [2], and acute pancreatitis [3]. Increased intestinal permeability has been reported in patients with chronic heart failure [4] and in elite long distance runners [5], probably as a result of intestinal hypoperfusion. Increased GI permeability is an undesirable condition that renders the host more susceptible to noxious compounds present in the gut lumen, including microbes and their products [6], potentially leading to systemic endotoxaemia and sepsis. Permeability analysis is considered to provide crucial information on GI functioning, and it is increasingly used in clinical practice and research [7]. Classically, human GI permeability is assessed by the measurement of urinary excretion of an orally administered disaccharide (usually lactulose) and a monosaccharide (mannitol or L-rhamnose). The urinary excretion ratio of the two sugars is considered to be a parameter of small intestinal permeability. A common technique for determining these urinary sugars concentrations is HPLC in combination with refractive index detection [8]. However, there is room for significant analytical improvement [7]. Therefore, the aim of this study was to improve the classical sugar-based permeability test on three different levels.

First, the intake of the relatively large amount of lactulose (5 g) used in the classical sugar test can influence GI function, probably by increasing the speed of passage through the GI system, thereby affecting the sensitivity of the permeability analysis [9–11]. The lack of sensitivity and specificity of the existing methodology currently prevents the use of lower sugar doses. Increasing the analytical sensitivity was therefore our key target in this study. Secondly, the colonic degradation of the classical sugar probes restricts the usefulness of the dual sugar assay to the small intestine. In addition, the classical dual sugar test does not allow specific analysis of the upper part of the duodenum. The ideal assay includes

Abbreviations: GI, gastrointestinal; LC–MS, liquid chromatography–mass spectrometry; SSA, sulfosalicylic acid; TCA, trichloroacetic acid.

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additional probes to provide specific permeability information on the upper GI tract, i.e. the stomach and duodenal bulb, and the colon. Such an expanded sugar test mix would theoretically affect intestinal transit, if applied at the classical dose. Hence, expansion of the sugar test composition requires a further increase in analytical sensitivity to reduce the cumulative oral sugar dose, and requires a specific method of detection to assess all applied sugar probes in a single analytical run. Thirdly, the dual sugar assay is classically performed in 0–5 h urinary collections, and plasma analysis is not commonly performed. Nevertheless, frequent plasma sampling provides advantages over urinary permeability analysis, since it enables the detection of modest, transient gastrointestinal permeability changes. Plasma analysis requires a more extensive sample preparation, and a more sensitive analysis, since plasma sugar concentrations are low compared to urinary concentrations. Therefore, a novel, more sensitive approach was set up, enabling the analysis of multiple sugar probes in urine and plasma. Here, we describe this analytical method, which is based on liquid chromatography in combination with mass spectrometry (LC–MS), and the potential advantages of the novel multi-sugar permeability assay for application in clinical practice.

2. Materials and methods

The study was approved by the medical ethical committee of the Maastricht University Medical Center and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul).

2.1. Materials

All chemicals used for laboratory purposes were of analytical grade, and unless specified otherwise, purchased from Sigma–Aldrich (St. Louis, MO). Ultra pure water was generated through a Super-Q water purification system (Millipore, Billerica, MA) and used as the source of water throughout the assay.

The permeability test mix consisted of 1 g sucrose (342.3 Da; Van Gilse, Suiker Unie, Dinteloord, the Netherlands), 1 g lactulose (342.3 Da; Centrafarm B.V., Etten-Leur, The Netherlands), 1 g sucralose (397.6 Da; Brenntag Mülheim an der Ruhr, Germany), 1 g erythritol (122.1 Da; Danisco Sweeteners, Copenhagen, Denmark) and 0.5 g of L-rhamnose (rhamnose; 164.2 Da; Danisco Sweeteners, Copenhagen, Denmark), freshly dissolved in 150 mL of tap water. All products used in the test mix were intended and tested for safe oral consumption. The purity of these sugar probes was reported to be 98.0–99.9%.

2.2. Preparation of standards

Standards were prepared by dissolving analytical grade sugars into Super-Q water to a concentration of 10 mmol/L each. Next, a mixture was composed of each individual sugar to a final concentration of 500 μmol/L and aliquots were stored at −80 °C.

2.3. Sampling from healthy human volunteers

Included were 10 healthy men and women aged 18–75 years. The volunteers did not take medication in the month prior to participation, and had no history of GI disease or abdominal surgery. The volunteers were informed about the nature and risks of the experiments. Written consent was obtained 5 days before the experiments. Subjects refrained from strenuous physical activity and from oral intake of caffeine, alcohol, spicy foods and drinks, and any of the sugars used in the permeability test from 2 days before, to the morning after the test day.

Subjects were tested after an overnight fast. In short, a catheter (20 Gauge, B. Braun, Melsungen, Germany) was placed in the participant's forearm vein. Blood was collected into pre-chilled ethylenediaminetetra-acetic acid tubes (Vacucontainer, Becton Dickinson, Franklin Lakes, NJ), and kept on ice. Urine was collected by the subjects in plastic cups of 1 L, after which urinary volume was recorded and 4 mL was directly transferred to pre-chilled polypropylene tubes (Greiner Bio-One, Kremsmünster, Austria). Blood and urine samples were centrifuged within 1 h at 4 °C at 2300 × *g* for 15 min and aliquoted. Both plasma and urine were immediately stored at −80 °C until analysis. Following the collection of baseline plasma and urine samples, the test subjects ingested the test mix described above. Plasma samples were collected every 20 min and urine every hour until 5 h after intake of the test mix. Moreover, all urine produced in the 5–24 h interval after intake was collected by the subjects in a 3 L flask, which was returned to the laboratory the following morning and immediately processed as above.

2.4. Sample preparation

Due to the selectivity of the LC–MS approach, we could suffice with centrifugation of the urinary samples. Aliquots with 500 μL of urine were thawed to room temperature, transferred to 1.5 mL Eppendorf cups, centrifuged at 50,000 × *g* for 10 min at 4 °C in a high-speed centrifuge (model Biofuge Stratos, Heraeus, Hanau, Germany). Clear urine supernatant (200 μL) was transferred into a 300 μL glass insert; spring loaded in a 4 mL WISP style vial (Waters, Milford, MA) and placed into a Peltier chilled Gilson 233 XL sample processor (Gilson, Middleton, WI).

Two plasma sample preparation protocols were tested to remove plasma proteins. The first one involved the addition of sulfosalicylic acid (SSA) after which the samples were vortexed and centrifuged. In the second protocol, 300 μL of sample was transferred to Eppendorf cups containing a 3000 Da cut-off filter (Amicon Ultra 0.5 mL 3 K, Millipore) to remove the plasma proteins. The filter cups were centrifuged for 30 min at 11,000 × *g* at 4 °C, and clear plasma filtrate (200 μL) was inserted in the Gilson sample processor as described.

2.5. Optimized LC–MS procedure

Chromatographic separation was based on isocratic elution of individual sugars probes on an IOA-1000 9 μm cation-exchange column (300 mm × 7.8 mm ID; Grace, Deerfield, IL), mounted in a Mistral column oven (Separations, H.I. Ambacht, the Netherlands) at 30 °C. An aqueous solution of 20 mmol/L formic acid and 10 mmol/L trichloroacetic acid (TCA), was delivered using a Model PU-1580 HPLC pump (Jasco Easton, MD) at a flow rate of 0.225 mL/min. Samples and standards were injected using a Model 233XL sample processor with Peltier chilled sample storage compartments (10 °C), equipped with a 20 μL sample loop (Gilson, Middleton, WI). After separation, the column effluent was mixed with 30 mmol/L ammonia in 20% methanol/water (v/v) delivered by an additional Model PU 980 pump (pump B, Fig. 1) at a flow of 0.15 mL/min to allow the formation of ammonium adducts. MS detection was performed using a model LTQ XL (Thermo Fisher Scientific, Waltham, MA) equipped with an ion-Max electrospray probe. The MS was operated in positive mode. Spray voltage was 4.8 kV. Sheath and auxiliary gas were 99 and 30 units, respectively, while capillary temperature was 220 °C. The system was set to a mass range of 125–460 Da in full scan enhanced mode.

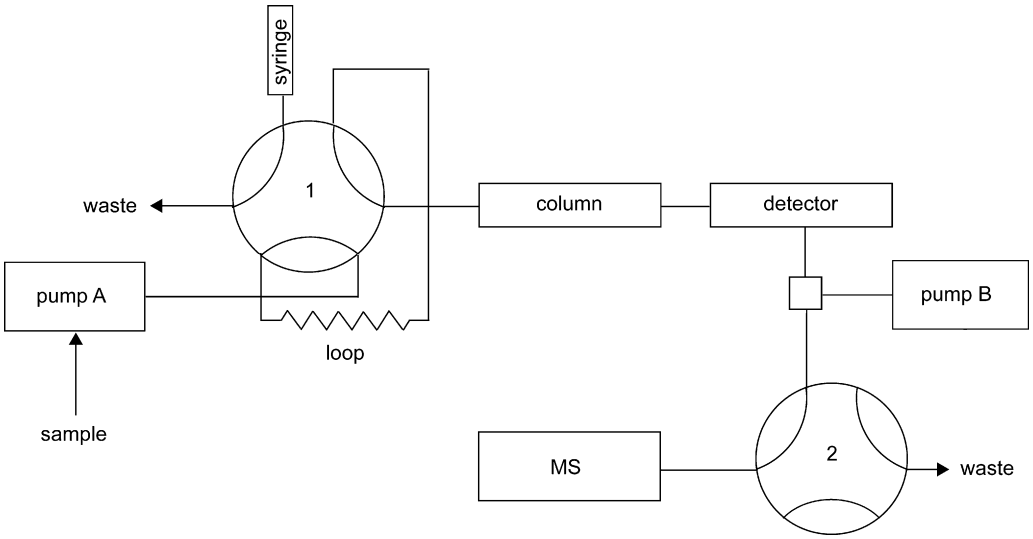

Fig. 1. Schematic set-up of the detection system. Samples are delivered by pump A to the solvent stream via valve 1, which is equipped with a 20 μL sample loop. The solvent is then directed to the cation-exchange column, mounted in a thermostatic oven. After separation by the column, the solvent goes through the detector, and is mixed with an ammonia/methanol mixture delivered by pump B to allow formation of sugar–ammonium adducts. Finally, the solvent stream is directed to the MS system through valve 2.

3. Results

3.1. Optimizing of chromatographic procedure

To obtain optimal chromatographic results, two approaches were tested. The first involved the use of straight phase chromatography using a carbohydrate ES column (Grace) as described before [12]. As we intended to apply MS for detection, ionization of sugars was a prerequisite. Adduct formation using sodium [13], iodine [14], formic acid [15], and ammonia [16] has been described. In our setting, sodium rapidly blocked the ionization probe, while iodine contaminated the ion trap considerably. Adducts formed by formic acid or ammonia showed better results, with the latter giving a more abundant response. However, formic acid and ammonium adducts are only generated at very low concentrations of organic solvent (acetonitrile), while high acetonitrile concentrations are required for straight phase gradient elution of chromatographic separation. One way to solve this inconsistency is to apply isocratic elution of the sugars, and exploit the differences in molecular weight to identify and quantify the sugars [16]. Unfortunately, physiological samples contain many endogenous isobaric sugars, which have to be separated from the exogenously applied sugar probes for their correct quantification. Consequently, we studied the application of cation-exchange chromatography to separate the sugar probes of interest from the endogenous isobaric sugars. From all columns tested, only the IOA-1000 9 μm column (300 mm × 7.8 mm ID) could sufficiently separate the sugars. Therefore, due to the required column dimensions necessary for adequate separation, we had to sacrifice analysis time. To optimize chromatographic separation, the influence of column temperature on the chromatograms was determined in the range of 10–90 °C. As expected, peak symmetry and separation of especially the monosaccharides improved at higher temperatures. Since a temperature-dependent instability has been described for disaccharides in solutions [12], we studied whether this decay of disaccharides also occurred on the column above 30 °C. In an attempt to reduce the temperature-dependent instability, we reduced the retention times by applying a shorter IOA-2000 8 μm column (150 mm × 6.5 mm ID). Unfortunately, even though this column was filled with smaller ion-exchange particles, it did not provide sufficient separation of the sugar probes, as was the case when the larger IOA-1000 column was used. Therefore, we continued to use the IOA-1000 column, and the column temperature was set to 30 °C.

To enable the analysis of plasma samples, protein had to be removed. For this purpose, two procedures were evaluated, i.e. addition of SSA and the application of cut-off filters. Addition of SSA resulted in a rapid decrease of the MS signal (Fig. 2A), whereas the application of cut-off filters resulted in a stable MS signal over time (Fig. 2B). Therefore the latter procedure was applied.

Next, we studied the influence of solvent-pH and solvent-anion. Application of a stronger acid like TCA gave a lower solvent-pH, thereby improving peak symmetry and the monosaccharide ionization response, but decreasing the disaccharide ionization response, probably due to enhanced decomposition. Application of a weaker acid, like formic acid, enhanced the disaccharide response, but strongly reduced the monosaccharide response. Therefore, we compromised to a solvent containing 20 mmol/L formic acid and 10 mmol/L TCA, resulting in satisfactory separation and peak symmetry for all target sugar probes (Fig. 3). Accumulation of buffer salts on the spray shield was minimized by flushing the electrospray source in-between runs with a mixture of methanol/water (15/85, v/v), delivered by a third pump at a flow rate of 0.4 mL/min, resulting in a stable run-to-run response for all components. Every analytical run started with analysis of the standard from the described standard batch. If the results were similar to the previous results for the standard batch, i.e. if the variation coefficient between standard results was less than 2%, sample analysis was initiated. After each set of 5 samples (either urinary or plasma), a standard was injected for external standard calibration.

3.2. Optimizing of MS signal

The formation of ammonium adducts was initiated by mixing the column effluent with a solution of 30 mmol/L ammonia dissolved in methanol–water (20/80, v/v), delivered to the system through a second pump that was set at a flow rate of 0.15 mL/min. The methanol concentration was crucial to obtain maximal signal intensity [16]. Alternative organic solvents such as acetonitrile or isopropanol only increased the MS signal marginally. The concentration of ammonia in such solutions was less important. An ammonia concentration of 30 mmol/L gave the best results. The

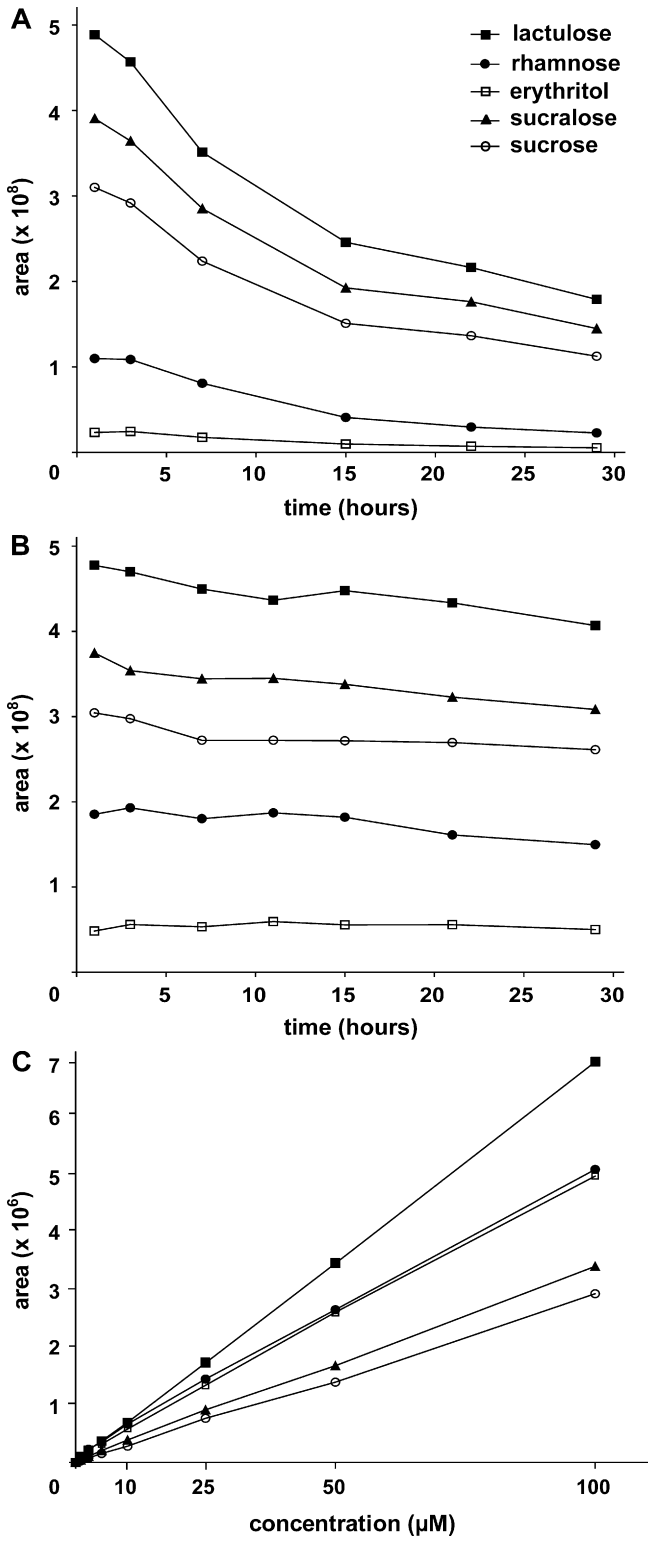

Fig. 2. Plasma deproteinization techniques and calibration curves. (A) The application of SSA for deproteinization decreases signal in time due to contamination of the MS. (B) Cut-off filters provide sufficient plasma deproteinization and stable MS signal strength. (C) Calibration curves of target sugar are linear up to 1000 μmol/L. Equations and R^2 are given in the text.

MS system was set to a high-flow situation, by infusing the sugars through a T-piece into the combined ionization solvent-column effluent solution. Maximal signal intensity was obtained with the following settings: sheath gas 99 (arbitrary) units, auxiliary gas 30 units, spray voltage 4.8 kV, heated capillary temperature 220 °C, capillary voltage 44 V and tube lens: 100 V.

3.3. Linearity and limit of detection

Aqueous standards of the sugar probes of interest were run individually as well as combined in one standard sugar solution. Combination of the sugars in one solution did not change the individual sugar responses (data not shown). The linearity of the detection method was determined by injecting aqueous standards containing all sugars ranging from 1 to 1000 μmol/L. Linear regression analysis of the calibration curves revealed the following slopes, intercepts, and correlation coefficients (R^2): Sucrose: $y = 28{,}777(338)x + 1787(13{,}779)$ with $R^2 = 0.9992$. Lactulose: $y = 68{,}418(240)x + 21{,}190(10{,}464)$ with $R^2 = 0.9999$. Rhamnose: $y = 49{,}822(745)x + 99{,}218(30{,}352)$ with $R^2 = 0.9987$. Erythritol: $y = 49{,}046(553)x + 65{,}236(22{,}535)$ with $R^2 = 0.9992$. Sucralose: $y = 33{,}383(310)x + 27{,}250(12{,}614)$ with $R^2 = 0.9995$ (data are presented as mean(SD); Fig. 2C). The limit of detection, the concentration which generates a signal-to-noise ratio of 3:1, was 0.05 μmol/L for the disaccharides and 0.1 μmol/L for the monosaccharides.

To investigate potential matrix interference on the slope of the calibration curves, different concentrations of sugars were spiked to plasma and urine samples, after which samples were analyzed. The data shown in Table 1, revealed that the slopes of the calibration curves were very similar in different matrices. Hence, external calibration in aqueous solution can be applied safely. Internal standard application was not applied.

3.4. Coefficient of variation and recovery

To determine the coefficient of variation, a urine and plasma sample (obtained 1 h after the oral intake of the sugar test mix) were analyzed in an alternating fashion with a 250 μmol/L standard in 10-fold. Coefficients of variation ranged from 2.4% for sucralose to 4.5% for rhamnose in the standard, from 1.6% for rhamnose to 4.3% for sucrose in urine and from 1.2% for rhamnose to 8.5% for lactulose in plasma (Table 2). Recovery data were obtained by spiking aliquots of a plasma pool prepared from aliquots of fasted volunteers, with aqueous standards with increasing sugar concentrations. Calculated recoveries were never below 90% or above 110%, even at the lowest spiked concentration (Table 3). In fact, 67% of the measurements showed a recovery between 95% and 105%. To investigate the intra-assay accuracy, aliquots of plasma and urine specimens collected after 1 h intervals after ingestion of the test mix by a healthy volunteer were measured in 6-fold. The results were plotted in time (Fig. 4). Both standards and samples were stored at 4 °C during the analytical process, and a stable response was observed for at least 24 h at this temperature.

3.5. Background sugar concentrations in plasma and urine

The optimized chromatography enabled the separation of all sugar probes. In addition, a number of components were found in both urine and plasma samples, with molecular masses similar to the target sugars. While most of these components eluted from the column at different time points, some of the components had elution times close to our sugar probes, thereby interfering with the quantification of the sugar probes. Most components could be identified by injecting a range of sugar components in the chromatographic column and compare the chromatograms of these sugars with the chromatograms of the interfering components (Fig. 3B and C).

In addition, we observed baseline sugar concentrations both in plasma and urine samples of individuals who had been deprived

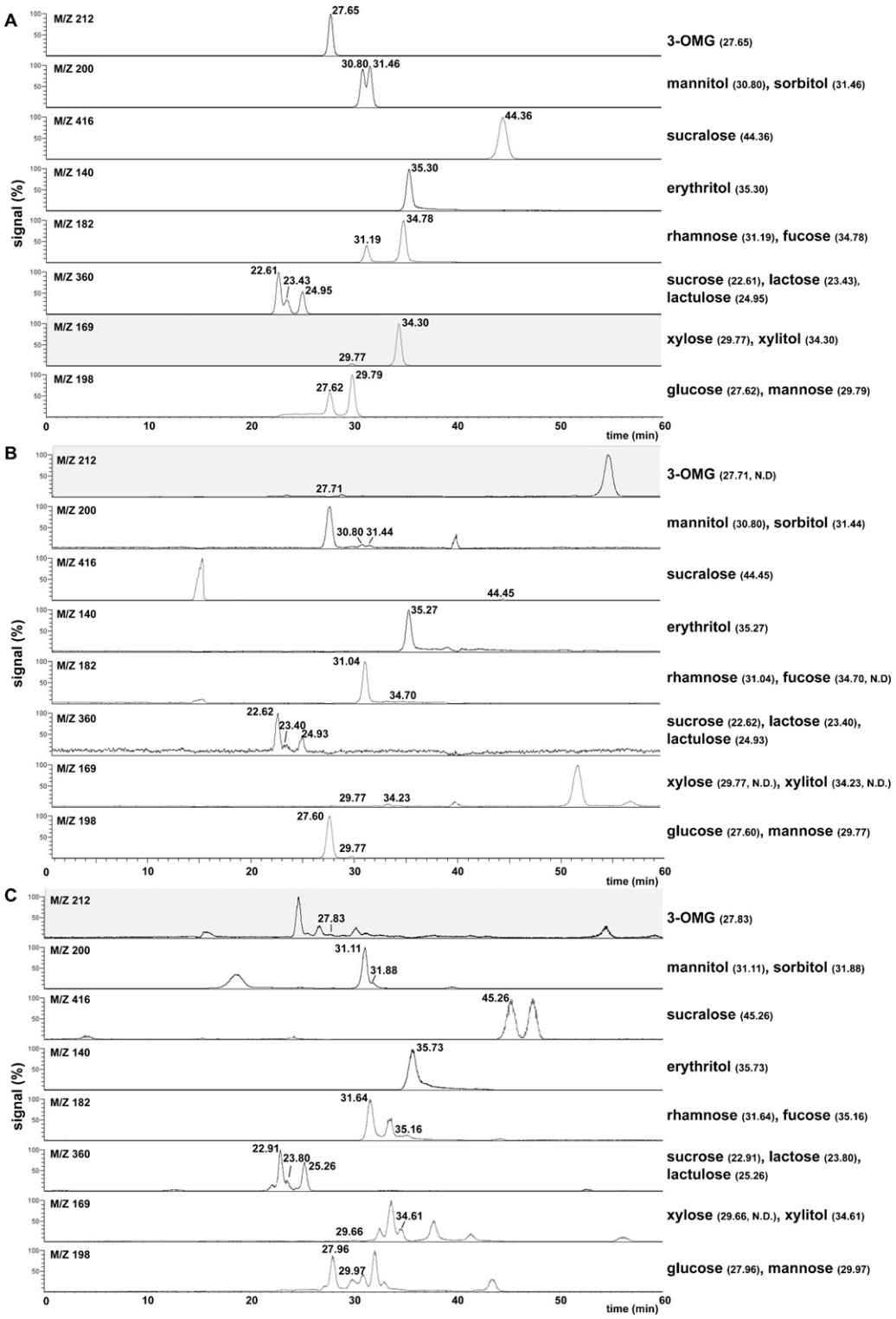

Fig. 3. The molecular mass-based separation of sugar probes is obtained by chromatography. (A) Aqueous standard (250 μmol/L). (B) Plasma sample obtained 1 h after oral intake of the sugar test mix. (C) Urine sample obtained 1 h after oral intake of the sugar test mix. For each panel, component names and mass to charge ratios of the monitored $[M + NH_4]^+$ ions (m/z values) are provided (N.D.; not detectable).

from food and drinks for 10 h. To investigate the relevance of these baseline levels in relation to the increase in concentration caused by oral intake of the test mix, we determined inter-individual and intra-individual differences in 10 volunteers, measured at 4 different days (Supplementary Tables). No plasma baseline concentrations of the disaccharides lactulose and sucralose were found, while we did observe baseline concentrations of monosaccharide rhamnose in plasma. These concentrations nearly doubled 1 h after intake of the test mix. In urine, baseline levels ranged from undetectable for lactulose to 724 μmol/L for erythritol (Supplementary Table 1). However, these high baseline concentration for erythritol was low compared to the 21,867 μmol/L measured on average 1 h after intake of the test mix (data not shown). Therefore, the reported baseline erythritol levels did not interfere with the

Table 1
Comparison of calibration curve equations of sugar permeability probes spiked in water, plasma and urine.*

	Aqueous	Plasma	Urine
Sucrose	$y=(2.9\times10^4)x+1.8\times10^3$	$y=(2.9\times10^4)x+2.5\times10^5$	$y=(2.8\times10^4)x+2.5\times10^5$
Lactulose	$y=(6.8\times10^4)x+2.1\times10^4$	$y=(7.0\times10^4)x+6.4\times10^3$	$y=(6.8\times10^4)x+1.5\times10^4$
Rhamnose	$y=(5.0\times10^4)x+9.9\times10^4$	$y=(5.2\times10^4)x+2.0\times10^6$	$y=(5.2\times10^4)x+2.0\times10^6$
Sucralose	$y=(3.3\times10^4)x+2.7\times10^4$	$y=(3.0\times10^4)x+2.9\times10^4$	$y=(3.1\times10^4)x+3.7\times10^4$
Erythritol	$y=(4.9\times10^4)x+6.5\times10^4$	$y=(5.2\times10^4)x+3.9\times10^5$	$y=(5.1\times10^4)x+5.0\times10^7$

* Plasma and urine were obtained from 10 fasted, healthy volunteers. Samples were pooled, and spiked with the sugar probes.

Table 2
Coefficient of variation (CV) of sugar concentration measurements in aqueous standard, urine*, and plasma.*

	Sucrose		Lactulose		Rhamnose		Sucralose		Erythritol	
	Conc. (μmol/L)	CV (%)	Conc. (μmol/L)	CV (%)	Conc. (μmol/L)	CV (%)	Conc. (μmol/L)	CV (%)	Conc. (μmol/L)	CV (%)
Standard	250.0	3.1	250.0	2.5	250.0	4.5	250.0	2.4	250.0	4.4
Urine	28.2	4.3	6.5	3.2	234.4	1.6	25.7	2.6	4199.0	3.8
Plasma	0.3	5.0	0.3	8.5	102.1	1.2	0.2	3.0	117.7	2.7

* Samples were obtained from a healthy volunteer 1 h after oral intake of the sugar test mix.

erythritol measurements necessary for colon permeability assessment (Fig. 4).

4. Discussion

Various intestinal and systemic diseases are associated with increased GI permeability. Permeability analysis can help to clarify its role in disease. Moreover, for evaluation of interventions directed at preventing increased GI permeability or ameliorating the integrity of the GI epithelium, a sensitive analysis of GI permeability is essential. To improve GI permeability analysis, we developed a new method to measure multiple permeability sugar probes in both plasma and urine.

The new method had to meet three goals: (1) increase the analytical sensitivity, allowing smaller quantities of the sugar probes to be used, (2) measure the classical and additional sugar probes simultaneously in one run, enabling permeability assessment of the stomach, and the small and large intestine, and (3) allow the analysis of the applied sugar probes in both urine and plasma.

Increased analytical sensitivity was obtained by combining isocratic cation-exchange chromatography in combination with MS. Adduct formation, necessary for MS detection, was studied using ammonia and formic acid. Sugar adduct synthesis was much more abundant after addition of ammonia, and therefore sugar–ammonium adducts were analyzed with MS. This approach allowed quantification of all sugar probes of interest in both urine and plasma in one run, with a detection limit of 0.05 μmol/L for disaccharides and 0.10 μmol/L for monosaccharides. These lower limits of detection are significantly lower than previously reported [17,18], including a recent report of Camilleri et al. who found detection limits of 4.98 μmol/L (1.7 μg/mL) for disaccharide lactulose and 1.92 μmol/L (0.035 μg/mL) for monosaccharide mannitol using normal phase LC–MS for permeability analysis in urine [18]. In our study, the increased analytic sensitivity allowed a 5-fold reduction of the oral lactulose dose. The latter made it possible to complement the original dual sugar test mix with additional sugar probes, enabling the assessment of the small and large intestine in one analytical run, with a lower total oral sugar load. Additional sugar probes were selected to assess gastroduodenal (sucrose) and colon permeability (sucralose and erythritol), in addition to small intestinal permeability (lactulose and rhamnose). The disaccharide sucrose is considered a marker of gastroduodenal permeability, since it is short-lived in the upper part of the duodenum due to hydrolysis by sucrase [19]. The disaccharide sucralose and the sugar alcohol erythritol are not degraded by human colonic bacteria [20,21], allowing their use as markers of colonic and total GI permeability. Alternatively, assessment of samples (either urine or plasma) collected more frequently than the classical 0–5 h urinary collection, might reflect permeability of specific parts of the gastrointestinal tract [18]. Considering that sugar probes first appear in plasma after permeation through the mucosal epithelium, we aimed to perform plasma-based permeability analysis next to the classical urinary approach. Analysis of plasma samples provided additional analytical challenges. In urine, the ingested sugar probes accumulate, resulting in high urinary sugar concentrations compared to plasma. Another important difference between urine and plasma is the necessity to remove plasma proteins. While at all times, sample preparation was kept as simple as possible to avoid prolongation of the turn-around time, it was necessary to remove these plasma proteins that would otherwise clog the chromatographic column. Whereas conventional plasma deproteinization with SSA resulted in a decrease of the MS signal over time due to contamination of the MS, the application of 3000 Da cut-off filters proved to be a good and fast alternative. Analysis of frequently sampled plasma samples allows the detection of short-lived changes in GI permeability. Furthermore, combined urinary and plasma analysis might help in the clinical interpretation and understanding of increased or decreased transepithelial permeation of probes.

Table 3
Recovery of sugar permeability probes in spiked plasma.*

Spike (μmol/L)	Sucrose	Lactulose	Rhamnose	Sucralose	Erythritol
1.0	90.1	90.1	107.2	93.9	97.1
2.5	97.0	97.0	107.8	94.0	100.8
5.0	103.9	103.9	106.3	90.7	101.2
10.0	101.6	101.6	102.0	93.2	105.0
25.0	100.4	100.4	99.9	90.6	105.3
50.0	102.9	102.9	98.9	90.7	106.6
100.0	99.1	99.1	104.9	91.8	105.3

* Plasma was obtained from 10 fasted, healthy volunteers. Plasma was pooled, and spiked with the sugar probes. Recovery (%) is presented.

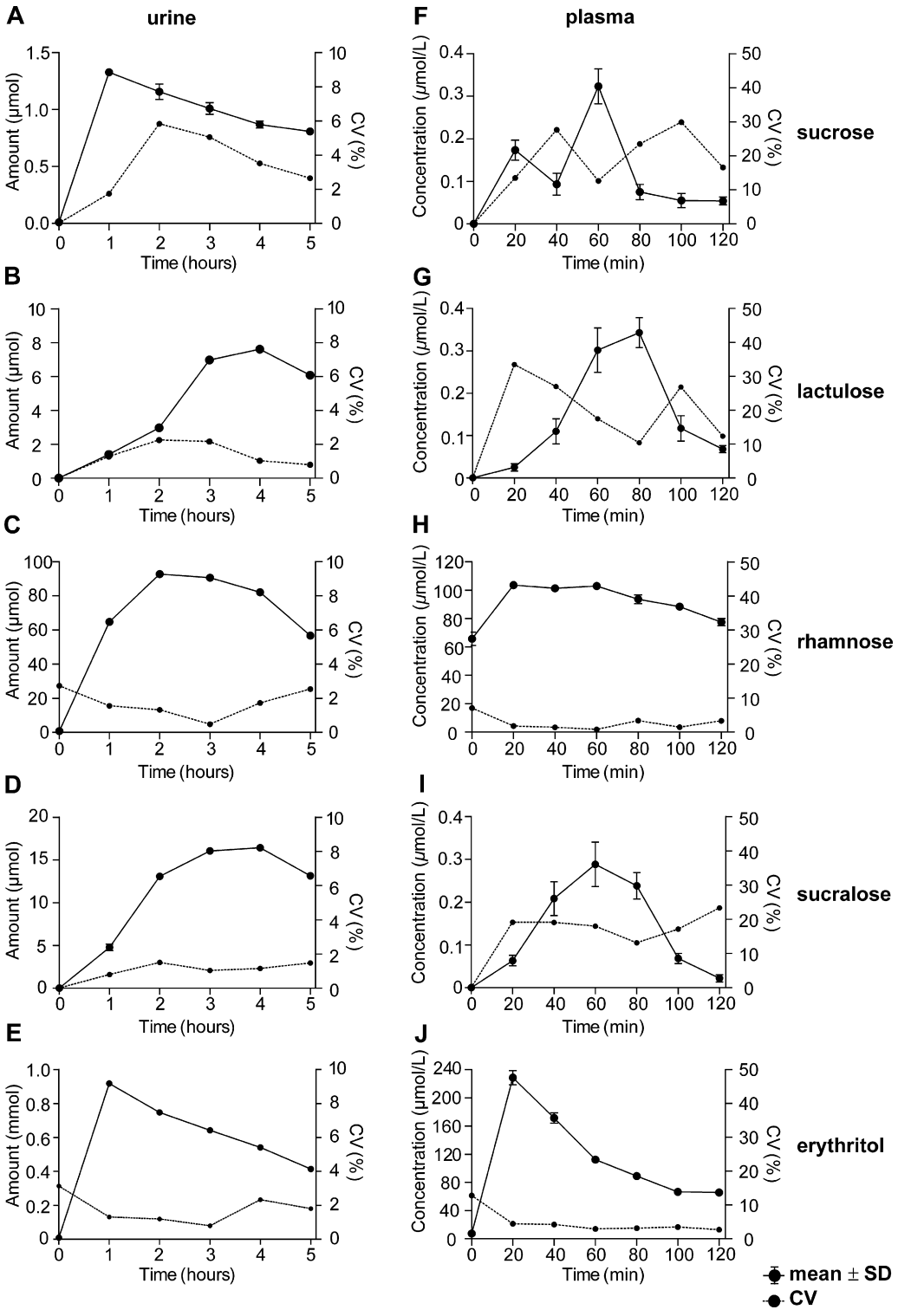

Fig. 4. Amount and intra-assay variation of sugar permeability probes over time in urine and plasma after oral intake of the probes. Amount of sugar probes in urine over time, given as mean and standard deviation (bold lines). In a majority of cases, the standard deviation is too small to indicate. Dotted lines indicate the intra-assay coefficient of variation (CV) from a 6-fold measurement. Urinary excretion of sucrose (A), lactulose (B), rhamnose (C), sucralose (D) and erythritol (E). Plasma concentration of sucrose (F), lactulose (G), rhamnose (H), sucralose (I) and erythritol (J).

Analysis of baseline plasma samples revealed MS signals at the *m*/*z* values of rhamnose and erythritol, strongly suggesting the presence of these monosaccharides in plasma of individuals who had been deprived from food and drinks for 10 h. Such baseline concentrations of sugars and sugar alcohols have been reported in serum and plasma of fasted individuals [22–24]. These results suggest that monosaccharides may be present in the daily human diet. Small amounts of erythritol have been found in some fruits and red wine for example [25], and mannitol can be found in seaweed and other plants [26], and rhamnose naturally occurs in bound form in a variety of plants. Since baseline concentrations of both monosaccharides increased substantially after intake of the probes, these

baseline concentrations did not interfere with permeability analysis. Nevertheless, we propose to always collect a baseline sample of each test subject, so that baseline endogenous sugar concentrations can be subtracted from the measured concentrations, thereby giving an accurate reflection of the increase in sugar levels after oral intake of the sugar test mix. The presence of the baseline sugar concentrations implies that a further reduction of the oral sugar dose would compromise correct measurement of raised plasma concentrations after intake of the sugar test mix, even though the high analytical sensitivity of the current assay would allow such a reduction. In urine, baseline sugar concentrations did not interfere with the analysis of the sugar permeability probes.

Analysis of both plasma and urine revealed the presence of components with identical molecular masses as the target sugars. While most of these products had other retention times than the sugar probes of interest, some components for instance co-eluted with the sucrose peak, thereby hampering accurate measurement of sucrose at the applied oral dose of 1 g. We assume that the application of more sucrose in the test drink would increase the sucrose levels after intake to a higher level, thereby reducing the interfering effect of other components. However, a larger sucrose dose would also increase the osmolarity of our test drink, and since our aim was to improve the sensitivity of the permeability analysis without affecting the GI mucosa through a sugar overload, we chose not to increase the amount of sucrose in our test drink.

The newly developed assay enables the detection of small changes in sugar concentrations in both urine and plasma, allowing accurate assessment of small and large intestinal permeability. The lactulose/rhamnose (L/R) ratio is used as a parameter of small intestinal permeability, while the sucralose/erythritol (S/E) ratio determined in 24 h urinary collections reflects total GI permeability. Moreover, the combination of the L/R and S/E ratio might enable specific assessment of colon permeability. Further study is necessary to determine the most suitable time frames to collect urine and plasma samples for the assessment of site-specific gastrointestinal permeability. Furthermore, we are performing validation studies to evaluate the usefulness of our novel approach to assess changes in GI permeability in healthy individuals with and without an oral challenge with indomethacin, after which we plan to conduct further studies to test the applicability of the test in clinical practice. Especially for application in clinical practice, timing of sampling is important to obtain accurate and site-specific information.

In conclusion, we developed a novel analytical approach, based on isocratic cation-exchange LC–MS. This technique enables highly sensitive detection of sugar probes allowing a 5-fold reduction of the oral lactulose dose, and the addition of probes to assess site-specific changes in GI permeability. This assay, applicable to urine and plasma samples, may prove useful in clinical practice for screening, prognosis estimation, and treatment decisions in conditions with suspected gut dysfunction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.08.002.

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