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Simultaneous gas-chromatographic urinary measurement of sugar probes to assess intestinal permeability: use of time course analysis to optimize its use to assess regional gut permeability

Maliha Shaikh¹, Kumar Rajan², Christopher B. Forsyth^{1,3}, Robin M. Voigt¹, and Ali Keshavarzian^{1,4,5,6}

¹Department of Internal Medicine, Section of Gastroenterology, Rush University Medical Center, Chicago, IL ² Rush Institute for Healthy Aging, Chicago, IL ³ Department of Biochemistry, Rush University, Chicago, IL ⁴ Department of Pharmacology, Rush University, Chicago, IL ⁵ Department of Molecular Biophysics and Physiology, Rush University, Chicago, IL ⁶ F.C. Donders Chair, Faculty of Science, University of Utrecht – Utrecht, The Netherlands

Abstract

Background—Measurement of intestinal permeability is important in several diseases but currently several methods are employed. We sought to: (1) develop a new GC based method to measure urinary mannitol, lactulose and sucralose to assess regional and total gut permeability; (2) analyze the kinetics of these sugars in the urine to determine which ratio is useful to represent intestinal permeability; and (3) determine whether age, gender, race and BMI impact these values.

Methods—Subjects drank a cocktail of sucrose, lactulose, mannitol and sucralose and these sugars were measured in the urine at 5, 12 and 24 h with gas chromatography.

Results—Urinary mannitol exhibited significantly different kinetics than lactulose and sucralose which were similar to each other and varied little over the 24 h. No permeability differences were observed for renal function, age, race, sex, or BMI.

Conclusions—Our data do not support the use of the widely used L/M ratio as an accurate estimate of intestinal permeability. Our data support the use of: The sucralose/lactulose (S/M) ratio to measure: small intestine permeability (first 5 h); small and large intestine (first 12 hours), and total gut permeability (24 h). This was also found to be true in a Parkinson's disease model.

Keywords

intestinal permeability; leaky gut; urine; sucralose; lactulose; mannitol

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Maliha_Shaikh@rush.edu. Kumar_Rajan@rush.edu. Robin_Voigt@rush.edu. ali_keshavarzian@rush.edu. Corresponding author: Christopher B. Forsyth, PhD, 1725 W. Harrison, Suite 206 christopher_b_forsyth@rush.edu.

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1. Introduction

Recent studies have provided compelling and strong evidence for a central role for environmental factors in the pathogenesis of chronic disorders and more specifically for those diseases in which inflammation plays a key role in their pathogenesis [1-4]. The intestine is the largest interface between the environment and the body and is therefore a major gateway for environmental factors to access the body [5-7]. Indeed, one of the central and the most challenging functions of the intestine is to regulate this access path, providing entry of nutrients into the body and preventing free access to the injurious, pro-inflammatory toxins and other intestinal contents. This task is achieved through carrier mediated active absorption of nutrients by enterocytes in the small intestine and regulating passive passage of non-nutrient molecules primarily through paracellular junctions in both the small and large intestine (colon) [8,9]. The degree and nature of this passive movement of molecules across the intestinal mucosal layer depends on the structure of the intestinal mucosal membrane (intestinal epithelial layer and paracellular junctions), the physicochemical properties of the solute, and its interaction with the media that determines the level of permeability of the intestinal epithelial layer.

It is not surprising that abnormal intestinal permeability ("leaky gut") has been proposed as one of the key pathological events, not only for gastrointestinal diseases like inflammatory bowel disease [10], irritable bowel syndrome [11], celiac disease [12], colon cancer [13], and liver diseases [14,15], but also in systemic disorders like obesity and metabolic syndrome [16], diabetes [17], neurodegenerative diseases like Parkinson's disease [18], and even psychological disorders like depression, anxiety and PTSD [2,19,20], just to name a few.

Therefore, a reliable, easy to use, and safe method of assessing intestinal permeability is not only essential for basic and clinician scientists involved in elucidating the pathogenesis of these diverse disorders but also for clinical management of numerous diseases. Indeed, there are several methods that are now available and in common use. The essential characteristic of these methods is the use of inert compounds that passively move across the intestinal epithelial layer, do not entrap in the body, are not metabolized and are passively excreted in the urine. These characteristics allow for urinary concentrations of these compounds to accurately reflect intestinal barrier (permeability) function [9,21]. Examples of these probes are Cr⁵¹ EDTA [21], polyethylene glycol (PEG) [21,22] and poorly absorbed carbohydrates (sugars) [9,21]. The most common probes used are poorly absorbed sugars because they are not radioactive and can also provide information regarding the permeability in different segments of the gastrointestinal tract [9,21]. However, in spite of the widespread use by multiple investigators, there is controversy regarding the easiest and the most cost-effective method of measuring urinary sugars as well as the best means of calculating, analyzing and presenting the data. For example, there is debate whether urinary sugar should be expressed as a ratio of two sugars (differential urinary excretion ratio)[21] or simply the urinary concentration or excretion rate per dose of one sugar [9,23]. Those who favor the ratio argue that ratio values eliminate non-intestinal mucosal factors such as intestinal transit, volume of distribution of the probes, renal function and urine collection [9,21]. However, this

assumption is valid only if the kinetics of intestinal handling of sugars are similar; otherwise ratio values could be misleading [23].

To help shed light on the debate and determine the validity of different types of analysis the aims of the current study were to: (1) develop a new GC based method to measure urinary sucralose to improve the sensitivity of the method we previously developed [24] for measurement of total gut permeability and the colonic permeability; (2) analyze the kinetics of urinary mannitol, lactulose and sucralose in healthy subjects with normal renal function to determine whether handling of these sugars by the intestine is similar to justify calculation of ratios and if so then which ratio is the most appropriate (the ones in which kinetics are similar) and; (3) determine whether age, gender, race and BMI impact values of urinary sugars (intestinal permeability) in the non-disease (healthy) state.

2. Materials and methods

2.1 Subjects

We evaluated 40 adult healthy subjects with no gastrointestinal symptoms and no systemic diseases who were not taking any regular medications and had a normal CBC and comprehensive metabolic profile. No subjects were daily alcohol drinkers and none were at risk drinkers based on NIAAA criteria (i.e., females more than 3 drinks per day on a regular basis; for men more than four drinks per day) [25]. Subjects had not taken antibiotics for the previous three months, over the counter probiotics or multivitamins for at least the previous two weeks, nor NSAID or high dose aspirin in the previous 4 weeks. Low dose (81 mg/day) aspirin was allowed. Subjects were not allowed to drink alcohol for 24 h prior to the test.

Subjects median age was 42 y (range 20–72 y). The subjects' demographic characteristics are found in Table 1. The study was approved by the Rush University Institutional Review Board (IRB). The participants all signed an informed consent, and were subsequently given sets of structured demographic questionnaires to complete in order to ensure that they fulfilled the inclusion/exclusion criteria. All questionnaire packets were labeled by a sequential patient number to maintain patient confidentiality, and served as the patient identifier for the remainder of the study.

All work for this study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and Uniform Requirements for manuscripts submitted to Biomedical journals published by the International Committee of Medical Journal Editors.

2.2 Reagents

Lactulose (4-*o*- β -D-galactopyranosyl-D-fructofuranose) was obtained from Bertek Pharmaceuticals, as brand name Kristalose. Mannitol (D-mannitol) and sucrose (α -D-glucopyranosyl- β -D-fructofuranoside) were obtained from Sigma Aldrich. Sucralose (1,6-dichloro-1,6-dideoxy- β -D-fructofuranosyl-4-chloro-4-deoxy- α -D-glucopyranoside) was supplied by Tate and Lyle. Trifluoroacetic acid ammonia, sodium borodeuteride, acetone, acetic anhydride and glacial acetic acid were purchased at their highest grade purity from Sigma Aldrich.

2.3 Gas chromatography measurement of intestinal permeability (urinary sugar probes)

Intestinal Permeability in 40 healthy subjects was measured by oral administration of a sugar cocktail (i.e., mannitol, lactulose, sucrose, and sucralose) and analysis of subsequent sugar excretion in urine. Subjects fasted overnight and after emptying their bladder they subsequently ingested a sugar mixture containing 2 grams mannitol, 7.5 g lactulose, 40 gm sucrose mixed in 8 oz. of water and 1 g sucralose in 2 capsules at 6AM, then collected urine from 0-5, 6-12, and 13-24 h. Subjects were asked to remain fasted for 2 h and then could eat normally except that they were restricted from drinking or eating any “diet” drink/or food (possibly containing sucralose) 48 h before the start of the test and throughout the 24 h of urine collection.

Urine was analyzed for sugar content using gas chromatography (GC) techniques, following the conversion of sugars and methylated sugars to their alditol acetate derivatives. The hydrolysis of glycosides and polysaccharides to reducing sugars and their conversion to alditol acetates (borohydride reduction and acetylation) is a standard method used to analyze polysaccharides containing aldoses, ketoses, deoxyaldoses, and acetamido hexoses and other related sugars [26,27].

Briefly, 100 µl of urine with 1 mg of myo-inositol as internal standard in each sample is used. The standard tube contains 1 mg each of mannitol, sucralose, lactulose, sucrose and myo-inositol. The test sample as well as the standards are dried under a steady stream of nitrogen. Sample hydrolysis is completed by adding 250 µl of 2 M TFA to the dry sample, the tubes are capped, and heated at 121°C for 1 h. The acid is removed under a slow stream of nitrogen. Traces of acid are also removed by addition of isopropanol 2x200 µl, followed by blowing to dryness with nitrogen. For the reduction process, the hydrolyzed sample is dissolved in 100 µl of 1M ammonium hydroxide. Five hundred microliters of DMSO containing 20 mg/ml of sodium borodeuteride is added to the mixture, and kept for 90 min at 40°C. Glacial acetic acid is added slowly. O-acetylation is achieved by adding 100 µl of 1-methylimidazole, 0.5ml of acetic anhydride is added and the samples kept at room temperature for 10 min. A mixture of 4 ml water/1 ml methylene chloride is added to the solution and vortexed. The bottom layer is removed to clean the tube, a process that is repeated one more time. The methylene chloride layer is washed with 4 ml of water, and repeated a second time. The methylene chloride layer is removed and the sample is blown to dryness. The final residue is dissolved in 0.5 ml acetone and the sample is ready for analysis by GC.

Gas chromatography was performed using an Agilent 6890 GC equipped with a flame ionization detector. The column used was DB-225MS, which was 30 m × 250 µm ID with a 0.25 film thickness. The detector temperature was 300°C and the injector temperature was 240°C. The initial column temperature of 100°C was held for 2 min and then increased at a rate of 10°C/min to 180°C which was held for 2 min and then at a rate of 4°C /min to 240°C which was maintained for 15 min. The total run time was 42 min. The data was analyzed as a percent excretion of the oral dose.

2.4 Data analysis and statistics

Since subjects were allowed to consume sucrose-containing food after the initial 5 h urine collection, statistical analysis and measurement of sugars in the urine after 5 h were limited to lactulose, mannitol and sucralose. Data for urinary sucrose are limited to 5 h urine.

The descriptive statistics for urinary markers were computed using mean and SD at each time interval (0-5, 5-12, and 12-24 h). These overall means were then graphically represented using dot plots with time on the x-axis and percent excretion of the oral dose on the y-axis. The means were also marked with error bars. A bar chart for 24 h percent excretion of the oral dose was used to compare males vs. females, BMI groups (<30 vs. 30 y), age (<65 vs. 65 y), and smokers (yes vs. no). was used to denote urinary markers for continuous variables and percentages for categorical variables. Spearman's rank correlation coefficient was used to assess the correlation of 5-h urinary markers with age, BMI, and other markers. The urinary markers were log-transformed to model the medians rather than the means, since the distribution of markers were skewed. We used a linear mixed-effects regression model with a log-transformed outcome to account for subject specific heterogeneity in absorption and excretion of the markers. Indicators for 5, 12, and 24-h were included in the model and their interactions with age groups, BMI groups, and gender were used to test if the excretion rates were different between these subgroups. We used an unstructured covariance matrix to account for subject-specific correlation over the observation time points. We computed the cumulative excretion of the markers over the 24-h period and examined how the cumulative excretion varied over the first 5 h then between 5-12 h followed by 12-24 h. In addition to each marker, we also looked at sucralose/lactulose, sucralose/mannitol and lactulose/mannitol ratios to examine if the ratios showed similar excretion rates over the 24-h window. We also estimated the tertiles for each marker at 5-h for sucralose, 12-h for mannitol and lactulose, and examined if the marker trajectories were different between the tertiles.

3. Results

3.1 Patient Characteristics

Subject characteristics are listed in Table 1. A total of 40 healthy subjects completed the study: 16 males and 24 females. The mean age was 42.3 years (range 20-72 y), the mean BMI was 29.3, and mean creatinine clearance was 91.7 ml/min (normal 88-137ml/min).

3.2 GC based method of measuring urinary sugars

Linearity, Precision and Reproducibility. Linearity (moles sugar probe vs. peak area) of the sugar probe measurement was determined by adding a known concentration of the 4 sugar probes and the internal standard (myo-inositol). Fig. 1 shows the calibration curve for each sugar probe. The response factor for each probe, RF= ratio of sugar peak/total sugar administered. The RRF = the RF for each sugar /RF myo-inositol internal control. The RRF values for each sugar were as follows:

D-Mannitol/myo-inositol	RRF= 1.18 ± 0.07	R ² =0.99
Sucralose/myo-inositol	RRF= 0.59 ± 0.3	R ² =0.99
Lactulose/myo-inositol	RRF= 1.04 ± 0.15	R ² =0.97
Sucrose/myo-inositol	RRF= 1.07 ± 0.2	R ² =0.97

Note that a representative gas chromatogram of control urine sample after sugar ingestion shown in Fig. 2 reveals that the peaks are clearly separated and also showed a single peak for each alditol acetate sugar probe derivative. The precision of the method was analyzed by measuring the same urine sample multiple times (7 times) within the same day (i.e., within-day variability) and between days (i.e., between-day variability) measured once a day for seven days. This is illustrated in Table 2 demonstrating a high degree of reproducibility (low variability; largest percent coefficient of variation (CV%) only 2.9%) both within and between days. (CV% is determined as the $SD/mean \times 100$).

3.3 Intestinal permeability: percent urinary excretion of sugar probes oral dose

The 4 sugar probes administered in the oral cocktail to patients were mannitol, sucralose, lactulose, and sucrose. The intestinal permeability profile of each sugar probe was calculated as the percent excretion of the oral dose in a 2-h period (Table 3). We should point out that because sucrose was administered in the oral solution but was not restricted in the diet, we present sucrose data to be complete but did not consider the sucrose data for further analysis. To determine if renal function had any impact on intestinal permeability, percent excretion was normalized to (divided by) the creatinine clearance of each subject (Table 4). In Table 3 we sought to look at the changes in the % excretion across time to see at what timepoint each sugar was predominantly excreted. We then compared these values to the same time periods for each probe normalized for creatinine clearance (CrCl) (non-normalized vs. normalized, respectively below; Note that after normalization for CrCl the probe data do not always equal exactly 100%). We observed that mannitol (61.5% vs. 52.9%) is mostly excreted in the first 5 hours while for sucralose (35.9% vs. 33.3%) and lactulose (29.3% vs. 30%) lower excretion is observed during this first 5 hour period. For the second urine collection period from 5 -12 h, mannitol excretion was (20% vs. 22.6%), for sucralose (29.7% vs. 38%) and for lactulose (26.8% vs. 32.4%). Cumulatively, for the first 12 h period mannitol excretion is the greatest (81.5% vs. 75.5%) with sucralose (65.6% vs. 71.3%) being the second highest followed by lactulose (56.1% vs. 60%). Interestingly, the amounts for the second 12 hours are more similar between %excreted dose vs. %excreted dose corrected for CrCl and are respectively: mannitol (18.5% vs. 23.5%), sucralose (34.4% vs. 28.2%) and lactulose (43.9% vs. 40%). Overall, the percent excretion numbers change only slightly when adjusted for renal function by dividing by creatinine clearance (CrCl) but were not statistically significantly different.

The percent excretion of the total urinary amount collected for each probe is shown for each collection time segment in Fig. 3. Note the clearly different rate of mannitol excretion in the first 5 h that was statistically different than both sucralose ($p<.001$) and lactulose ($p<.001$). Sucralose and lactulose rates are not statistically different at 5 h. At the second collection

time point of 5-12 h, again both sucralose ($p<.001$) and lactulose ($p<.001$) rates were significantly different than mannitol. Notably, sucralose is also significantly different than lactulose ($p<.0018$) at 12 h. Finally at the 12 h-24 h collection point, mannitol and sucralose rates are significantly different ($p<.0001$) and sucralose and lactulose are also significantly different ($p<.0119$). From 5-24 h clearly sucralose excretion is the most consistent. The cumulative data for the percent oral dose excreted for each sugar probe is shown in Fig.4. Once again this graphic representation of the data emphasizes that sucralose and lactulose are significantly different ($p<.001$) than mannitol at both the 5 h and 12 h timepoints, and are not statistically different from each other at 5 h and only slightly at 12 h ($p<.0018$).

Overall then, these data support a model in which both sucralose and lactulose exhibit similar excretion kinetics but differ significantly in their kinetics of secretion compared to mannitol. As shown in Table 3, this is reflected in the lactulose/mannitol (L/M) ratio varying more than 8-fold (800%) with a peak in the second 12 hours over 24 h with 5 h: .065, 5-12 h: 0.25, 12-24 h: 0.534, and 0-24 h: 0.124. However the sucralose/lactulose ratio remains almost constant for each time segment over 24 h at 5 h:0.53, 5-12 h: 0.54, 12-24 h: 0.36, and 0-24 h: 0.49 with no dramatic peak and the largest variability only 25% from the mean of 0.48. Finally, we should point out that when the 24 h percent excretion of oral dose data is divided into 3 tertile groups ranging from lowest excretion to highest, the ranges of the tertile means were: mannitol (9.7%-22.3%), sucralose (0.4%-2.4%), lactulose (0.5%-1.7%) and sucrose (0.3%-0.6%).

3.4 Application of the sucralose/lactulose ratio to a disease case group of patients

We tested our hypothesis that the use of the sucralose/lactulose ratio might be a valuable measure of intestinal permeability and is more discriminatory than sucralose excretion to detect gut leakiness (increased intestinal permeability) in disease states. To this end, we compared 24 h urinary sucralose and the sucralose/lactulose ratio in patients with Parkinson's disease that we have previously shown to have significantly increased intestinal permeability using 24 h urinary sucralose as a marker of gut leakiness [18]. Parkinson's disease (PD) is the second most common neurodegenerative disorder of aging, and is projected to affect nearly 10 million citizens of the world's most populous countries by 2030 [28,29]. We have previously published intestinal permeability data for newly diagnosed Parkinson's disease (PD) patients and BMI/age-matched healthy controls using the same orally administered four sugar cocktail and analysis of 12 h urine samples for lactulose and mannitol and 24 h urinary sucralose as described above in Methods [18]. The demographic data for these healthy controls and PD patients are summarized in Table 5 and were reported previously [18]. The 24 h mean urinary excretion sucralose value for controls (0.58 ± 0.1) compared to PD patients (1.12 ± 0.1) was almost double for PD patients and significantly different ($p = 0.015$) (Fig. 5A). Most important to our hypothesis, the mean urinary excretion sucralose/lactulose ratios for controls (0.21 ± 0.1) were significantly different than PD patients (0.48 ± 0.1 ; $p = 0.001$) (Fig. 5B). As depicted in Figs. 5A and 5B, although both mean urinary sucralose and the sucralose/lactulose ratio were significantly different between control and PD patients, the sucralose/lactulose ratio was more discriminatory with less overlap than urinary sucralose. Thus, at least in the case of these PD patients, our data support the use of the sucralose/lactulose ratio as an additional valuable measure of whole

gut permeability in the context of a disease state. The advantage of a ratio over simply the excretion value of a single sugar is that it will correct for incomplete urine collection and minimize the potential impact of non-permeability factors on urinary sugar values like differences in intestinal transit and volume of distribution between groups as has been previously reported [9,21]

3.5 The effects of age, gender, smoking and BMI on intestinal permeability

There were no statistically significant effects of age, gender, smoking or BMI on markers of intestinal permeability in our subjects (Fig. 6 and Table 6). Thus, in these healthy controls these factors have little impact on intestinal permeability to the sugar probes that were tested. In addition, there were no significant correlations between the urinary sugar probes percent excretion of oral dose and serum endotoxin (a measure of gut leakiness) in healthy controls (Table 6).

4. Discussion

Normal intestinal barrier function is essential for maintaining optimal health and for preventing intestinal and even systemic inflammation and immune activation. It is the first line of defense against increasingly toxic, immunogenic and pro-inflammatory environmental factors. Thus, an easy to use and non-invasive tool to assess intestinal barrier function is critical to assess the impact of intestinal barrier function in healthy and disease states. In addition, an ideal method of analysis should also be able to assess intestinal barrier function in different parts of the gastrointestinal (GI) tract because functional characteristics of the different parts of the GI tract are not the same. The method outlined in this paper demonstrates that the alditol acetate method of measuring orally administered sugar probes in the urine is a relatively straightforward and non-invasive tool to measure intestinal permeability.

A mixture of poorly absorbed sugars appears to represent the ideal approach to assess intestinal permeability that permits the determination of regional specificity. Different sugar probes with specific physicochemical properties can be chosen to match different structural characteristics of the GI tract in order to be able to measure regional differences in intestinal barrier function [9,30]. Sucrose is degraded so rapidly in the small intestine that its measurement in urine is only used as a measure of gastric permeability [9,30]. In the small intestine, the enterocyte is the most abundant cell type with a large surface area, due to surface-amplification through villi and microvilli [31,32]. In the large intestine, the colonocyte is the most abundant cell type without villi and thus there is far less surface area than in the small intestine. In addition, the large intestine has tighter cell-to-cell junctions than those found in the small intestine [33]. This prevents molecules the size of disaccharides (e.g., lactulose or sucralose) from permeating across the intestinal barrier; whereas, monosaccharides such as mannitol can cross with relative freedom [9]. Thus, mannitol is an appropriate probe to assess small intestinal permeability and its rate of permeation across the intestine is not only impacted by leakiness; but also by the surface area of the intestine (i.e., less surface area equates to lower rate of permeation). In contrast, the rate of permeation of larger sugar probes like lactulose and sucralose is not impacted by

surface area and is increased by the level of leakiness of the tight junctions (i.e., paracellular path) in the small intestine and colon [9].

Another factor that impacts the measurement of intestinal barrier function is the availability of the sugar probe in the intestinal segment to be tested. For example, sucrose is an appropriate probe to test gastroduodenal permeability. This is true because sucrose while available in the lumen of the stomach and duodenum is no longer available in the more distal small intestine or colon because it is metabolized by the small intestinal epithelial enzyme sucrase [30]. Similarly, a portion of mannitol and lactulose that are not absorbed in the small intestine will be fermented by bacteria in the colon and thus mannitol and lactulose are not ideal probes to assess colonic permeability. Thus, mannitol and lactulose given orally are virtually absent from the large intestine due to absorption and degradation in the small intestine[30]. In contrast, sucralose, which has a molecular mass comparable to lactulose and is probably passively absorbed through the same paracellular pathway as lactulose, is an appropriate probe to assess colonic permeability because it is not metabolized and fermented by the colonic bacteria [34-38]. Sucralose is excreted unchanged in the urine and is a good marker to access whole gut permeability [39]. This conjecture is supported by our data showing relatively consistent absorption throughout the GI tract (Fig.3; Table 3) as well as data shown in previously published literature[9, 40].

In order for a sugar mixture to gain widespread acceptability by the clinician and clinician scientists and investigators as a practical tool to assess intestinal permeability, an easy and cost effective technique to simultaneously measure all sugar probes in the urine is the key. There are several prior studies that reported successful use of HPLC to measure all sugars simultaneously [30, 40] and we have already published a GC-based technique to measure all four sugar probes in the urine [24]. In order to further increase the sensitivity of our method, we modified our method based on the use of alditol acetate derivatives and found the new method is far more sensitive than our previous method (Figs. 1 and 2). Our finding is supported by a published study that reported a highly sensitive and reproducible measurement of urinary rhamnose, lactulose and sucrose using GC based on the use of alditol acetate derivatives[26]. That report and ours now provide strong evidence to support this GC technique and the use of alditol acetate derivatives as a reproducible and easy to use method to measure all widely used permeability sugar probes.

Traditionally, it has been advocated and proposed that urinary sugar data should be presented as the ratio of two sugars: the differential urinary excretion principle [21]. The rationale for this proposed analysis is that the ratio will eliminate non-intestinal barrier function related factors that could affect urinary sugar values [21]. Usefulness of the urinary sugar ratios has been shown in celiac disease where urinary values of a monosaccharide (i.e., mannitol, rhamnose) are decreased and lactulose increased resulting in an increased L/M ratio [9]. However, it should be noted that celiac disease is associated with a reduction in surface area (i.e., villous atrophy) and thus a marked decrease in small bowel surface area that impacts permeation of monosaccharides like mannitol. In contrast to celiac disease, there is no villous atrophy or change in intestinal surface area in several diseases with paracellular gut leakiness and the reliability of this ratio in these pathological conditions has not been validated [23]. Use of a sugar probe ratio is valid to eliminate the potential impact of non-

intestinal permeability factors on the urinary sugar values but only if the intestine handles the two sugars the same, otherwise the ratio can be misleading as it was elegantly shown by Rao et al. in the case of the L/M ratio [23]. There is no doubt that non-intestinal factors like volume of distribution of the sugar probe, renal function and integrity of urine collection can impact outcomes and potentially cause misleading conclusions. So the question becomes, how can we take advantage of a ratio of probes without its potential deficiencies? Our data demonstrates that the kinetics of mannitol excretion is significantly different from those observed for lactulose or sucralose and thus in healthy subjects the use of the L/M ratio would not be ideal. In contrast, the kinetic profiles of sucralose and lactulose are nearly identical and thus the use of a sucralose/lactulose ratio would fulfill the essential criteria for ratio calculation (i.e., 2 sugars that are handled similarly by the intestine). Our findings are compatible with the findings reported by Rao et al. [23] that mannitol and lactulose will continue to be absorbed in the colon. However, we showed that the majority of mannitol is absorbed early with over 800% variability in the L/M ratio over 24 h, and thus the most likely site of absorption is proximal and mid small intestine where the surface area is high. We also showed that sucralose and lactulose are absorbed from the entire intestine. Thus our findings are not compatible with a recent study showing decreased intestinal permeability in IBS patients using the same 4 sugar probes as our study but only a 5 h urine sample[41]. Since sucralose is not fermented by colonic bacteria, it is a more reliable probe to assess colonic permeability than lactulose especially in pathological conditions where colonic microbiota could be abnormal. One example of this is in Parkinson's disease. Our data in Fig. 5 now show that at least in this specific disease, calculation of the sucralose/lactulose ratio reveals a significant increased intestinal permeability similar to the sucralose excretion data but slightly more discriminatory.

4.1 Conclusions

Based on our findings we recommend using: (1) The sucralose/lactulose ratio in the first 5 h urine collection to represent primarily small intestinal permeability, (2) The sucralose/lactulose ratio in the first 12 hour urine collection to represent small and large intestinal permeability, (3) The sucralose/lactulose ratio in the second 12 h urine collection to represent primarily large intestinal permeability, and (4) The sucralose/lactulose ratio in the 24 h urine collection to represent total gut permeability. We found that kinetic absorption of lactulose and sucralose is similar in healthy subjects, however, similar kinetic analysis should be done for each pathological condition before considering the appropriateness of using the ratio to represent changes in intestinal permeability. Thus, we propose that data for the original report of intestinal permeability in a given pathology should be reported for individual urinary sugar value changes over the period of the urine collection. We proposed that urinary sugar ratio should only be calculated if the two sugars have a similar kinetic as we found for sucralose and lactulose from healthy subjects and PD. Use of a ratio of sugars with different kinetics could potentially lead to incorrect conclusions and misinterpretation of intestinal permeability data. In healthy controls, renal function (i.e., creatinine clearance), age, gender, race, and BMI did not affect urinary sugar values; however, it has yet to be determined if these factors may be important considerations impacting intestinal permeability in various disease states.

In summary, our study provides an important new insight into the value of using specific orally administered sugar probes and urinary excretion to measure intestinal permeability. Our data using alditol acetate derivatives of sugars is sensitive and highly reproducible with little variability. Our data also do not support the use of the widely used L/M ratio as an accurate estimate of intestinal permeability, but instead argue in favor of the use of the sucralose/lactulose (S/L) ratio and specific urine collection times to assess either segmental or whole gut intestinal permeability. We also show that this sucralose/lactulose ratio principle applies not only to healthy subjects but also to a disease state, such as Parkinson's disease.

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Glossary

L/M	ratio lactulose to mannitol ratio
PD	Parkinson's disease
PTSD	post-traumatic stress disorder
RF	response factor
S/L	ratio sucralose to lactulose ratio
TFA	trifluoroacetic acid

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Highlights

- Healthy volunteers were used to measure intestinal permeability.
- Urinary excretion of oral sucrose, mannitol, lactulose and sucralose was measured.
- Lactulose and sucralose kinetics were similar and less variable than mannitol.
- The sucralose/lactulose ratio is as an excellent measure of total gut permeability.
- Sucralose/lactulose ratio in Parkinson's disease patients was also a valuable measure.

Figure 1. Calibration curves for each sugar probe

Calibration curves for mannitol, sucralose, lactulose and sucrose were determined for alditol acetate derivatives using gas chromatography (GC) as described in Methods. Each curve shows the molar ratio of sugar/internal standard versus peak area of sugar/peak area of internal standard ranging from **A**: 1.1 to 1.3 for mannitol, **B**: 0.2 to 1.1 for sucralose, **C**: 0.8 to 1.2 for lactulose and **D**: 0.7 to 1.35 for sucrose.

Figure 2. Representative gas chromatogram

A representative gas chromatogram is shown of a urine sample analyzed as alditol acetates derivatives from a healthy subject after ingestion of the 4 sugar probe cocktail containing mannitol(1), sucralose(2), lactulose(3) and sucrose(4) and internal standard myo-inositol(5) as described in Methods.

Figure 3. Percent of total urinary excretion for each sugar probe expressed as urinary recovery for each collection time segment

Urine was collected for the designated time segments from healthy subjects over 24 h after administration of an oral cocktail containing the sugar probes mannitol, sucralose, lactulose and sucrose as described in Methods. Alditol acetate derivatives of sugar probes were analyzed by GC as described in Methods. * $p < .001$ at time 5 h for both sucralose and lactulose compared to mannitol. ** $p < .001$ at time 5-12 h for sucralose and lactulose compared to mannitol; ++ $p < .0018$ at time 5-12 h for sucralose compared to lactulose. For the 12-24 h collection point, mannitol and sucralose are significantly different (** $p < .0001$) and sucralose and lactulose are also significantly different (+++ $p < .0119$).

Figure 4. Cumulative percent excretion of total sugar probe urinary excretion for each collection time segment over 24 h

Urine was collected from healthy subjects at the designated time segments over 24 h after administration of an oral cocktail containing the sugar probes mannitol, sucralose, lactulose and sucrose as described in Methods. Alditol acetate derivatives of sugar probes were analyzed by GC as described in Methods. * $p < .001$ at time 5 h for both sucralose and lactulose compared to mannitol. ** $p < .001$ at time 5-12 h for sucralose and lactulose compared to mannitol; ++ $p < .018$ at time 5-12 h for sucralose compared to lactulose.

Figure 5. The sucralose/lactulose ratio in Parkinson's disease patients is more discriminatory than sucralose excretion alone

Urine was collected for 24 h from Parkinson's disease (PD) patients as well as age and BMI matched healthy Control subjects after administration of an oral cocktail containing the sugar probes mannitol, sucralose, lactulose and sucrose as described in Methods. Data were analyzed as described in Fig. 4. 5A. Comparison of 24 hour sucralose urinary excretion in healthy controls vs. PD patients, * $p < .015$. 5B. Comparison of 24 h sucralose/lactulose ratio for healthy controls vs. PD patients, * $p < .001$.

Figure 6. The effects of gender, BMI, age and smoking on the 5 h percent urinary excretion of the oral dose of sugar probes

Four sugar probes (mannitol, sucralose, lactulose and sucrose) administered to healthy subjects by oral cocktail were analyzed in 5 h urine samples for total % excretion of oral dose over 5 h by GC as described in Methods. After statistical analysis described in Methods, no significant differences were found for any of the 4 sugar probes.

Table 1

Patient Demographics

Variable	Controls
Gender- n(%)	(N=40)
Male	16(40)
Female	24(60)
Race – n(%)	
Caucasian	16(40)
African American	24(60)
Age (mean $\pm$ SD)	42.35 $\pm$ 13.84
Range (years)	(20-72)
BMI (mean $\pm$ SD)	29.37 $\pm$ 7.55
Range (kg/m ²)	(19.6-45.4)
BMI 30	N=10
CrCl (mean $\pm$ SD)	91.7 $\pm$ 21.9
Range (ml/min)	(50.6-135.7)
Endotoxin (mean $\pm$ SD)	0.86 $\pm$ 0.59
Range (EU/ml)	(0.04-2.5)
Smokers	13.56%

Table 2

Precision of the GC measurement method in urine:

	<u>Within-day (7)</u>			<u>Between-day (7)</u>		
	Mean	± S.D	CV, %	Mean	± S.D	CV, %
Mannitol	1.126	± 0.008	0.71	1.135	± 0.004	0.36
Sucralose	0.182	± 0.005	2.90	0.180	± 0.003	1.90
Lactulose	0.521	± 0.003	0.61	0.524	± 0.002	0.5
Sucrose	0.489	± 0.002	0.35	0.491	± 0.002	0.35

Table 3

Percent urinary excretion of each oral sugar probe for each collection period over 24h.

Mannitol	0-5 hour	5-12 hour	12-24 hour	0-24 hour
Mean $\pm$ SD	15.6 $\pm$ 0.8	6.03 $\pm$ 0.96	6.4 $\pm$ 1.3	28.9 $\pm$ 1.3
% Total (cumulative)	61.5%	20% (81.5%)	18.5%	100
Sucralose				
Mean $\pm$ SE	0.5 $\pm$ 0.8	0.51 $\pm$ 0.13	0.3 $\pm$ 0.04	1.23 $\pm$ 0.14
% Total (cumulative)	35.9%	29.7% (65.6%)	34.4%	100%
Lactulose				
Mean $\pm$ SE	0.95 $\pm$ 0.6	1.1 $\pm$ 0.19	1.4 $\pm$ 0.1	3.11 $\pm$ 0.2
% Total (cumulative)	29.3%	26.8% (56.1%)	43.9%	100%
Sucrose				
Mean $\pm$ SE	0.5 $\pm$ 0.15	0.50 $\pm$ 0.08	0.8 $\pm$ 0.7	1.82 $\pm$ 0.17
% Total	29.6%	27.4% (57%)	43%	100%
Lactulose /Mannitol Ratio				
Mean $\pm$ SE	0.065 $\pm$ 0.05	0.25 $\pm$ 0.03	0.534 $\pm$ 0.11	0.124 $\pm$ 0.010
Sucralose/Lactulose Ratio				
Mean $\pm$ SE	0.53 $\pm$ 0.10	0.54 $\pm$ 0.13	0.36 $\pm$ 0.06	0.49 $\pm$ 0.08

Table 4

Percent urinary excretion of each oral sugar probe for each collection period over 24h corrected for CrCl.

Mannitol	0-5 hour	5-12 hour	12-24 hour	0-24 hour
Mean $\pm$ SE	0.18 $\pm$ 0.01	0.077 $\pm$ 0.01	0.08 $\pm$ 0.016	0.34 $\pm$ 0.029
% Total (cum)	52.9%	22.6% (75.5%)	23.5%	99%
Sucralose				
Mean $\pm$ SE	0.005 $\pm$ 0.001	0.0057 $\pm$ 0.001	0.004 $\pm$ 0.0006	0.015 $\pm$ 0.002
% Total (cum)	33.3%	38% (71.3%)	28.2%	99.5%
Lactulose				
Mean $\pm$ SE	0.012 $\pm$ 0.001	0.012 $\pm$ 0.002	0.016 $\pm$ 0.001	0.04 $\pm$ 0.002
% Total (cum)	30%	30.0% (60%)	40%	100%
Sucrose				
Mean $\pm$ SE	0.006 $\pm$ 0.0007	0.006 $\pm$ 0.001	0.009 $\pm$ 0.0009	0.0223 $\pm$ 0.002
% Total (cum)	26.9%	26.9% (53.8%)	40.8%	94.6%

Table 5

Parkinson's Disease Study Patient Demographics

Variable	Controls	Parkinson's Disease
Gender-n(%)	(N=10)	(N=9)
Male	7(70)	7(77.8)
Female	3(30)	2(22.2)
Race-n(%)		
Caucasian	7(70)	9(100)
African American	2(20)	0
others	1(10)	
Age (median)	49	57
BMI (mean $\pm$ SD)	25.9 $\pm$ 1.7	26.0 $\pm$ 2.7

Table 6

Spearman correlations of the sugar probes % excretion of oral dose (5 hour urine collection) with BMI, Age, Sex and Serum Endotoxin

	BMI	Age	Sex	Endotoxin
Mannitol	−0.02045	0.13806	−0.202	−0.1268
Sucralose	−0.06651	0.07295	−0.066	−0.0627
Lactulose	0.0682	0.0895	0.065	−0.02241
Sucrose	0.1116	0.13899	0.024	−0.01695
BMI	1.000	0.12347	0.279	−0.16365
Age	0.12347	1.000	−0.157	−0.23913
Sex	0.279	−0.157	1.000	−0.062
Endotoxin	−0.16365	−0.23913	−0.062	1.000