Abstract

Background: Evaluation of small intestinal permeability (SIP) is based on the estimation of the urinary excretion ratio of a large and a small molecule (lactulose and mannitol, L/M) after oral administration. We evaluated SIP using $^1$H-NMR spectroscopy.

Methods: In-vitro experiments on known concentration of mannitol and lactulose solutions were performed to measure accuracy and precision of quantification using $^1$H-NMR spectroscopy. Eighteen patients with malabsorption syndrome (MAS) and 28 healthy subjects (HS) underwent SIP evaluation using L/M excretion ratio over 6-h after oral administration of 15 mL (10g) lactulose and 5 g mannitol using $^1$H-NMR spectroscopy and trimethyl silyl propionic acid as external reference and for quantification.

Results: Median errors of estimation of mannitol and lactulose were 5% (range 1.2 to 5) and 1.3% (range 0.2 to 1.3), respectively in-vitro. Patients with MAS excreted higher quantity of lactulose in urine than HS (median 0.33 mmol vs 0.12, 0 to .676 mmol, p<0.008). There was a trend towards lower urinary excretion of mannitol in patients with MAS than HS (median 3.58, range 0.61 to 15.77 mmol vs. 3.82, 1.34 to 16.42 mmol, p = ns). L/M ratio was higher among patients with MAS as compared to HS (median 0.1172 vs 0.045, p< 0.002). A cut-off value of L/M excretion ratio by receiver-operating characteristic (ROC) curve of 0.049 had a sensitivity and specificity of 72% and 61%, respectively; a cut-off value of 0.078 had a specificity of 90% but low sensitivity (67%). Area under ROC curve was 0.77.

Conclusion: $^1$H-NMR spectroscopy is an analytical tool for assessment of SIP with reasonable sensitivity and specificity.

Key words

Malabsorption syndrome - intestinal function - inflammatory bowel disease - lactulose mannitol ratio.

Introduction

The small intestine is a major organ performing digestion and absorption of nutrients. It allows passage of digested nutrients into the bloodstream. It also prevents entry of toxic substances and undigested food macromolecules from getting into the blood stream. These functions of the intestinal mucosa are called small intestinal permeability (SIP) [1]. The rate limiting factor in SIP is the “tight junction” between the small intestinal epithelial cells [2]. Normal SIP is required for physiological functions of the small intestine. Several diseases of the small intestine are associated with abnormally increased permeability [3-6]. Assessment of SIP is an accurate, non-invasive method of evaluation of small intestinal function and correlates with jejunal biopsy [7] and is useful in screening for small intestinal diseases, in assessing response to treatment, and in predicting the prognosis [8]. The ratio of urinary excretion of lactulose and mannitol is a useful test for assessment of SIP [9-11]. The test is based on the coupled oral administration of two sugar probes of different molecular weight and on the determination of their excretion rate in urine [12]. Lactulose (disaccharide) and mannitol (a sugar alcohol) have various properties that make these suitable for measuring SIP: both are hydrophilic and lipophobic, also having a negligible affinity for the monosaccharide transport system and are passively absorbed [13]. Neither mannitol nor lactulose is metabolized in the body [12]. The sugar alcohol mannitol permeates the intestinal mucosa via a transcellular pathway through the water-filled pores on the cell membrane, whereas the disaccharide lactulose uses a paracellular route through the intercellular junctional complexes between adjacent enterocytes and extrusion zones at the villous tip [14]. The loss of mucosal integrity is likely to permit increased absorption of lactulose due to leaky intercellular tight junctions, whereas loss of absorptive
surface caused by associated villous shortening would be expected to reduce total absorption of mannitol [15]. Abnormal SIP means altered ratio of lactulose to mannitol excretion in urine as compared to healthy subjects [16]. Conventionally, lactulose and mannitol in urine are estimated by chromatographic methods and enzymatic assay [13, 14]. Among chromatographic methods, high-performance liquid chromatography (HPLC) is a quite sensitive method. HPLC must be coupled to refractive index, pulsed amperometric, fluorescence or evaporative light scattering detector [17, 18], while gas chromatography or gas chromatography-mass spectrometry need a derivatization step [19, 20]. NMR method is a simple and rapid technique, which is developing as an important tool in detecting a large number of metabolites in various bio-fluids simultaneously in a single experiment [21-24] which is not obtainable by existing techniques. NMR is based on the application of a radiofrequency pulse to the nuclear ensemble placed in a magnetic field and observing the response after the duration of the pulse. The resulting free indication decay is Fourier transformed to obtained characteristic frequency domain spectra with signature of the molecular structure. The present study therefore focuses on evaluation of SIP using urinary excretion of lactulose and mannitol by 1H-NMR spectroscopy.

Methods

In-vitro experiments

Detection and estimation of mannitol and lactulose

The 1H-NMR spectrum of D-mannitol (Hexane-1,2,3,4,5,6-hexol, S.D., Fine chemicals, Gujarat, India, Fig.1A) and lactulose (4-O-b-D-galactopyranosyl-D-fructofuranose, Sigma Aldrich, Fig 1B) in D2O solution were recorded separately. To estimate the T1 relaxation times of characteristic signals of mannitol and lactulose, inversion recovery experiments were performed at 400 MHz NMR spectrometer on standard chemicals (mannitol 3.86 [dd, 2H]; lactulose b-anomeric peak-CH). Parameters used were as follows: spectral width: 4,800 Hz; time domain points: 32K; relaxation delay: 15 s; number of scans: 8; inversion recovery delay: varied between 10 ms and 10 s in 10 to 13 steps; spectrum size: 32 K and line broadening: 0.3 Hz. T1 relaxation time was calculated using the intensities of the recovered signals with respect to the recovery delays using Bruker Xwinmr software version 3.1.

Analytical recovery of mannitol and lactulose

NMR experiments were performed to test the accuracy and precision of quantitative estimation of mannitol and lactulose in urine samples. Urine from three patients having different concentrations of mannitol and lactulose was used in the recovery experiments. Each urine sample was divided into three parts of 400 ml each. 100 ml of D2O was added to one part of urine in a 5 mm NMR tube, which served as a control. To the remaining two parts a known quantity of mannitol was added using the standard mannitol stock solution. 1H-NMR spectra were recorded for all the solutions under identical conditions using a single pulse sequence by suppressing the residual water signal by presaturation. Integral of mannitol was determined relative to trimethyl silyl propionic acid (TSP) reference. Using these integrals, the quantities of mannitol were calculated individually from all the control spectra and those obtained after the addition of standard mannitol solution. The accuracy and the reproducibility of quantitative estimation of the mannitol were compared by integrating each signal five times, independent of one another. Same procedure was followed for the lactulose recovery test.

In-vivo experiments

Study subjects

From December 2006 to December 2007, 18 patients with MAS due to various causes (8 with tropical sprue, 5 with Crohn’s disease, 4 with small intestinal bacterial overgrowth, one with intestinal tuberculosis) and 28 healthy volunteers were subjected to evaluation of SIP using estimation of lactulose mannitol urinary excretion by 1H-NMR spectroscopy after obtaining informed consent and clearance from the Ethics Committee of the Institute. The diagnosis of patients with MAS was done according to the standard method [25].

Sample collection

The subjects were not allowed to take milk or milk products within 24-h before the test. Next morning, a urine specimen was obtained after overnight fast. Subsequently, 5 g D-mannitol and 10 g (15mL) lactulose (Duphalac™, Solvay Pharm., Brussels, Belgium) were orally administered with 50 mL distilled water and the patient was allowed to drink water ad libitum 1 hour later. Patients were allowed to take a sugar free diet after two hours. Urine was collected in preservative (sodium azide) till 6-hours after ingestion of lactulose and mannitol and total volume of urine collected in next 6-h was measured and noted. 20 mL urine was stored in -80° refrigerator till further experiments.

NMR experiments

NMR analysis

From the total volume of urine of each patient, 3 mL was lyophilized using HetoLyolab lyophilizer (HETO Lyolab Freeze Dryer, UK), residue was dissolved in 500ml D2O and was directly taken in a 5 mm cleaned NMR tube. A reusable sealed capillary tube containing 30 ml of 0.375% of sodium salt of TSP in deuterium oxide was inserted into the NMR tube before recording the spectra. TSP served as a chemical shift reference as well as an internal standard for quantitative estimation, whereas deuterium oxide served as the ‘field-frequency lock’.

One dimensional NMR experiments using single pulse sequence were performed with water suppression by presaturation. Spectral width used was 4800 Hz, with time domain data points 32 K. The flip angle of the radio frequency pulse was 45° with a total delay of 8.4s to ensure maximum recovery of magnetization equilibrium between the scans. Typically, 512 scans were obtained for each sample and the resulting data were Fourier transformed after multiplying by an exponential window function using a line broadening function of 0.3 Hz and an FT size of 32 K.
Small intestinal permeability using 1H-NMR spectroscopy

points. The NMR spectra of all the pre test samples were also recorded at similar conditions in order to see the difference in the pre and post lactulose and mannitol urine spectra.

Statistical analysis

Continuous data were expressed as median and range. Differences between continuous and categorical variables were analyzed using Mann-Whitney U-test and Chi-square test with Yates’ correction as applicable, respectively. Receiver operating characteristic (ROC) curves were constructed to evaluate the cut-off value of lactulose mannitol urinary excretion ratio that best differentiated healthy subjects from those with MAS. The sensitivity and specificity of SIP determined using 1H-NMR spectroscopy were calculated

Results

1H-NMR spectrum of mannitol

1H-NMR spectrum of mannitol contained strongly-coupled multiplets covering a chemical shift range from 3.88-3.62 ppm (Fig. 1A). T1 relaxation time for the resonance at 3.86 (dd 2H) ppm, which was used for the quantification of mannitol in the urine was found to be 819 ms. The number of protons contribution to the signal at 3.86 ppm was determined by recording known concentration of D-mannitol spectrum in D2O in presence of external reference TSP.

1H-NMR spectrum of lactulose

1H-NMR spectrum of lactulose in D2O is shown in Fig. 1B. 1H-NMR studies of lactulose in D2O showed the presence of three isomeric β-anomeric galactosyl protons 4-O-(β-D-galactopyranosyl)-β-D-fructopyranose, 4-O-(β-D-galactopyranosyl)-β-D-fructofuranose and 4-O-(β-D-galactopyranosyl)-α-D-fructofuranose at 4.48, 4.39 and 4.37 in a ratio of 66:25:9 with reference to HOD signal in D2O solution was shown in a previous study [26]. The β-anomeric galactosyl protons of 4-O- β-D-galactopyranosyl moiety of lactulose resonating at 4.57 ppm with respect to TSP calibrated at 0.0 ppm and representing 66% of the major isomeric form was used for the quantification of lactulose excreted in the urine. The number of protons contribution to the signal at 4.57 ppm was determined by recording the known concentration of lactulose spectrum in D2O in presence of external reference TSP. The T1 relaxation time for resonance at 4.57 ppm was found to be 489ms.

Recovery test for mannitol and lactulose in urine

Table I shows percentage recovery of mannitol and lactulose from urine specimens. Median errors of estimation of mannitol and lactulose were 5% (range 1.2 to 5) and 1.3 % (range 0.2 to 1.3), respectively.

Clinical and laboratory parameters of study subjects

Patients with MAS were comparable to HS in age (median 37-y, range 18-68 vs. 29-y, range 23-48, p > 0.05) and gender (14/18 vs. 22/28 male, p>0.05). Causes of MAS included tropical sprue (8, 44%), Crohn’s disease (5, 28%), small intestinal bacterial overgrowth (4, 22%) and intestinal tuberculosis (1, 6%). Median Hb of patients with MAS was 10 g/dL (range 5.6 to 15), number of fat droplets in stool by Sudan III stain 18 (range 8 to 30) per high power field (normal ≤ 10), quantitative fecal fat by Van de Kamer’s method 8 g/24-h (range 2 to 15 g, normal ≤ 7 g/24-h) and urinary excretion of D-xylose 0.59 g/5 g/5-h (range 0.22 to 1.0 g, normal ≤ 1g/5 g/5-h).

Urinary excretion of lactulose, mannitol and its ratio in patients with MAS and controls

Table II and Fig. 2 show urinary excretion of lactulose and mannitol and its ratio in patients with MAS and healthy subjects. Patients with MAS excreted a higher quantity of lactulose in urine than healthy subjects (median 0.33, range 0 to1.09 mmol Vs median 0.12, range 0 to 0.676 mmol, p=0.008). There was also a trend towards lower urinary
Table II. Urinary excretion of lactulose and mannitol (mmol) in patients with malabsorption (MAS) and in healthy subjects.

<table>
<thead>
<tr>
<th>Urinary excretion</th>
<th>Patient with MAS (N=18)</th>
<th>Healthy subjects (N=28)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactulose</td>
<td>0.33 (1.09)</td>
<td>0.12 (0.67)</td>
<td>≤ 0.008</td>
</tr>
<tr>
<td>Mannitol</td>
<td>3.58 (0.61-15.77)</td>
<td>3.82 (1.34-16.42)</td>
<td>NS</td>
</tr>
<tr>
<td>Lactulose/Mannitol</td>
<td>0.12 (0-0.40)</td>
<td>0.05 (0-0.25)</td>
<td>≤ 0.002</td>
</tr>
</tbody>
</table>

*Mann-Whitney U test

Discussion

This study showed that it is feasible to evaluate SIP using an estimation of urinary excretion ratio of lactulose/mannitol by $^1$H-NMR spectroscopy and to present cut-off values that differentiate MAS from healthy subjects with reasonable sensitivity and specificity.

Evaluation of SIP is useful in screening for small intestinal diseases, in assessing response to treatment, and in predicting prognosis [8]. Use of minimally absorbed, non-metabolized sugars as probes for the assessment of barrier functions is convenient and non-hazardous. Conventionally, lactulose and mannitol excreted in urine are estimated by HPLC, which is quite sensitive method. However, HPLC must be coupled to refractive index, pulsed amperometric, fluorescence or evaporative light scattering detector [17, 18], not always available in all analytical laboratories, while gas chromatography or gas chromatography-mass spectrometry needs a derivatization step [19, 20]. Thus the establishment of urinary excretion ratio by ROC curve of 0.049 had a sensitivity and specificity of 72% and 61%, respectively; a cut-off value of 0.078 had a specificity of 90% but sensitivity was low (67%). Area under ROC curve was 0.77 (Fig. 4).

Fig 3. The cut-off values of lactulose mannitol urinary excretion ratio in patients with malabsorption syndrome (MAS) and healthy subjects (HS) and the sensitivity and specificity of these cut-off.

Fig 4. The receiver operating characteristic (ROC) curve showing the area under the curve.
of a simpler, rapid and convenient method of analysis for urinary sugars is important. 1H-NMR spectroscopy is emerging as a powerful technique for the measurement of small molecules in various bio-fluids [21, 22, 27]. Most body fluid or biological molecules possess nuclei such as 1H, 13C, 31P, 15N which provide NMR signals with frequencies and intensities characteristic of chemical/biological environments of the nuclei under study. From the frequency one gets information on the molecular environments (or identification of a particular metabolite) and from the intensity, its quantity. This permits to identify and quantify various metabolites in the sample in a single experiment without any additional sample preparation. This makes NMR an important tool for the study of metabolic profile in complex mixtures in a straightforward manner. In addition, with the current technological advances, the sensitivity of NMR has drastically increased and it has become one of the most powerful physical techniques to study the complex mixtures such as bio-fluids containing numerous structurally similar molecules in rather low concentrations. This makes it a valuable simple tool for the diagnosis of different diseases and monitoring the treatment. We have previously used this technique for estimating urinary D-xylene and for studying the mechanism of MAS resulting from small intestinal bacterial overgrowth [21, 22].

Our current study demonstrates that the 1H-NMR spectroscopy is a useful technique to measure SIP. Though this technique is costly for a new setup, it will be cheap for those centers that have NMR setup already established. A cut-off value of 0.049 had sensitivity and specificity of 72% and 61%, respectively, whereas a value of 0.078 had specificity of 90% but sensitivity was 67%. We believe that these two cut-offs can be judiciously used depending upon the purpose of the test. For example, if the test is being used as a screening test before undertaking other invasive investigations such as endoscopic intestinal biopsy, a low specificity but high sensitivity as given by a cut-off value of 0.049 is acceptable. On the other hand, if the test is being used as only an investigation to assess small intestinal permeability, a low specificity of 90% but sensitivity was 67%. We believe that these two cut-offs can be judiciously used depending upon the purpose of the test. For example, if the test is being used as a screening test before undertaking other invasive investigations such as endoscopic intestinal biopsy, a low specificity but high sensitivity as given by a cut-off value of 0.049 is acceptable. On the other hand, if the test is being used as only an investigation to assess small intestinal function such as in studies to know the effect of a therapeutic intervention on small bowel functions [11], then a cut-off with high specificity is better.

Small intestinal permeability has been used as a screening for various diseases such as celiac disease particularly among family members [28, 29] inflammatory bowel disease [30, 31] and hypolactasia [32], evaluating response to therapy [11], studying pathophysiology of various diseases such as small intestinal bacterial overgrowth syndrome [33], intestinal parasitic infestation [34, 35], functional bowel disease such as irritable bowel syndrome [36], primary biliary cirrhosis [37], cirrhosis of liver [18, 38], malnutrition [10], studying toxicity of drugs on the small intestine such as non-steroidal anti-inflammatory drugs [39], biphosphonates [40] and alcohol [41] and differentiation between organic and functional diarrhea [42]. Since an assessment of SIP is non-invasive, it can be used in screening for various small intestinal diseases before undertaking more invasive tests such as small bowel endoscopy and biopsy [8, 43]. Assessment of SIP is of particular importance in pediatric practice due to its non-invasive nature [44]. Considering the varied clinical and investigational utility of studying SIP, the novel technique of assessment of SIP using lactulose mannitol urinary excretion ratio has determined that using 1H-NMR spectroscopy would open a new avenue in gastroenterology practice and research. Though the sample size in the present study is somewhat low, we have standardized the technique of 1H-NMR by in-vitro experiments and then tested this in-vivo for the first time.

We conclude that 1H-NMR spectroscopy is a reliable and rapid technique to estimate lactulose and mannitol in the urine samples therefore, studying small intestinal permeability will differentiate healthy subjects from those with malabsorption syndrome with reasonable sensitivity and specificity.

Acknowledgement

The authors thank the Department of Science and Technology, Government of India for financial support in the Gastrointestinal Pathophysiology and Motility Laboratory at the Department of Gastroenterology, through grant no.SR/SO/HS-23/2003 and SR/SO/HS-15/2007 and to the Centre of Biomedical Magnetic Resonance at SGPGI, Lucknow.

Conflicts of interest

None to declare.

References

10. Lunn PG, Northrop-Clewes CA, Downes RM. Intestinal permeability, mucosal injury, and growth faltering in Gambian


