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Short Communication

Gas chromatography applied to the lactulose–mannitol intestinal permeability test

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Abstract

Intestinal permeability can be modified by various illnesses, trauma and sepsis. Alterations of the intestinal wall can facilitate the diffusion of potentially harmful substances such as endotoxins, as well as bacterial translocation. We describe the validation of a capillary gas chromatographic method for the determination of mannitol and lactulose, used as intestinal permeability probes. The method is linear up to 3 g/l for mannitol and 300 mg/l for lactulose; recovery from overload samples is between 92 to 110%. Intra-assay coefficients of variation (C.V.s) were 2.7 and 6.8% for mannitol and lactulose, respectively, and inter-assay C.V.s were 8.9 and 9.3%. Normal values for 25 healthy subjects (mean ± S.D.) were 14.5 ± 3.1% and 0.27 ± 0.15% for mannitol and lactulose, respectively. The GC method presented is rapid and precise.

1. Introduction

The epithelium of the small and large intestine separates the intestinal lumen from the internal environment of the body. It can be crossed by some molecules and macromolecules which are not transported actively but are thought to diffuse passively. Intestinal permeability is mainly determined by the size of the molecules and mucosal status. The intestinal epithelium is a selective barrier to the absorption of potentially harmful substances. In the differential sugar absorption test, two sugars are given simultaneously by mouth and the urinary recovery of each is determined; mannitol reflects the degree of absorption of small molecules (0.65 nm), while lactulose reflects the permeability of large molecules (0.93 nm). The simultaneous administration of two probe molecules and the expression of the urinary recoveries as a ratio reduces the influence of factors other than intestinal absorption. The lactulose–mannitol intestinal permeability test is applied widely to patients with Crohn's disease. Increased intestinal permeability is believed to result in bacterial translocation which, in part, could be a predisposition factor to systemic infections [1].

Several methods have been reported for the detection and quantification of mannitol and lactulose, including thin-layer chromatography [2], enzymatic methods [3,4], a colorimetric method for mannitol after oxidation [5], a liquid chromatographic method [6], gas chromatography (GC) for mannitol [7], and labelled mannitol or lactulose in animals [8]. GC methods using derivatives such as methyl, acyl deriva-

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tives, trimethylsilyl alditols or ethers are particularly suitable for assaying neutral sugars and sugar alcohols in biological fluids (reviewed in ref. 9). Two of the principal advantages of GC are its sensitivity and its precision, particularly when internal standardization is used.

We describe a GC technique based on the formation of trimethylsilyl (TMS) ethers of mannitol and lactulose, which permits the rapid separation and quantification of these two sugars in biological fluids on the same chromatogram. The method is validated and the normal range calculated.

2. Experimental

2.1. Reagents and chemicals

Amberlite MB8 was from Rohm and Haas (Lauterbourg, France); pyridine, mannitol, turanose and myoinositol were from Sigma (St. Louis, MO, USA). Lactulose (Duphalac®) was from Duphar et Cie (Villeurbanne, France); Tri-sil/TBT [TMS-imidazole, bis TMS-acetamide and trimethyl chlorosilane (3:3:2, v/v)] was from Pierce (Rockford, IL, USA).

2.2. Sample preparation

Urine samples and standard solutions: The first step is desalination of urine specimens. Amberlite MB8 resin is washed three times with distilled water and 0.5-ml aliquots are distributed in Poly-prep tubes. A 1-ml volume of urine or standard (mannitol 1 g/l, lactulose 100 mg/l) is added to the resin, and 500 μl of desalinated urine or standard, 30 μl of myoinositol (18 g/l) (internal standard for mannitol) and 30 μl of turanose (3 g/l) (internal standard for lactulose) are gently deposited in a silylation tube, then evaporated in a Speed Vac SVC 100. Standards and internal standard are prepared in distilled water. A 20-μl volume of anhydrous pyridine and 25 μl of Tri-Sil TBT are added to the evaporated residues, and the tubes are held at 60°C for 30 min. Aliquots (0.2 μl) are then injected onto the chromatograph.

2.3. Gas chromatography

The gas chromatographic studies were carried out with a Chrompack Packard Model 436 equipped with a flame-ionization detector (Middelburg, Netherlands). The SP 4290 integrator was obtained from Spectra Physics (Trimont, CA, USA). The capillary column [15 m $\times$ 0.53 mm I.D.; film thickness 1.5 μm, phase DB1 (100% dimethylpolysiloxane)] was obtained from J and W Scientifics (Folsom, CA, USA). The gas chromatograph is operated under the following conditions: carrier gas, nitrogen; flow-rate, 30 ml/min; injector temperature, 300°C; detector flame ionization, 320°C; hydrogen flow-rate, 26 ml/min; air flow-rate, 300 ml/min; temperature increase, 8°C/min from 250°C to 300°C; attenuation 2^{14} for 4 min and 2^{8} thereafter. Aliquots of 0.2 μl were injected directly onto the column.

In the case of samples from patients with glycosuria, the chromatographic conditions are modified as follows: initial temperature: 220°C for 10 min, increase 8°C/min from 220°C to 300°C.

2.4. Quantification of trimethylsilylated sugars

Trimethylsilylated sugars were quantified by measuring the area under the peak obtained on the integrator, or by measurement of peak height. The molar response factor for each sugar was determined relative to that of the internal standards (myoinositol for mannitol and turanose for lactulose). Using the response factor obtained with standard solutions, the concentration of the sugars in urine was derived from the ratio of the test samples to the internal standards. We used two internal standards with retention times very close to those of the measured sugars. The amounts of sugars injected were 0.2 μg for mannitol and 20 pg for lactulose.

3. Results and discussion

The mannitol and lactulose peaks were easily identified, since the retention times were highly

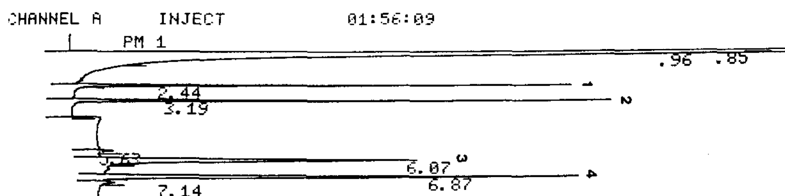

Fig. 1. Gas chromatogram of a standard mixture of mannitol and lactulose. Peaks: 1 = mannitol (1 g/l), 2 = myoinositol, 3 = lactulose (100 mg/l), 4 = turanose.

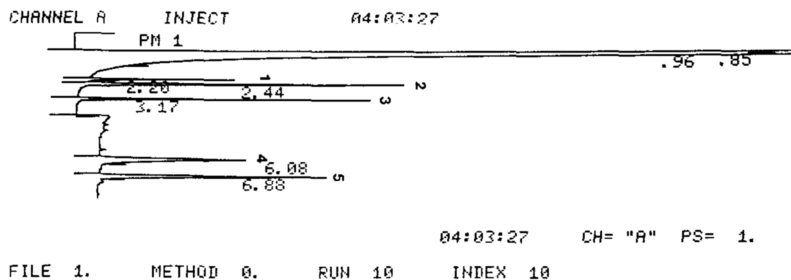

Fig. 2. Gas chromatogram of urine containing glucose after intestinal permeability test. Peaks: 1 = β-glucose, 2 = mannitol, 3 = myoinositol, 4 = lactulose, 5 = turanose.

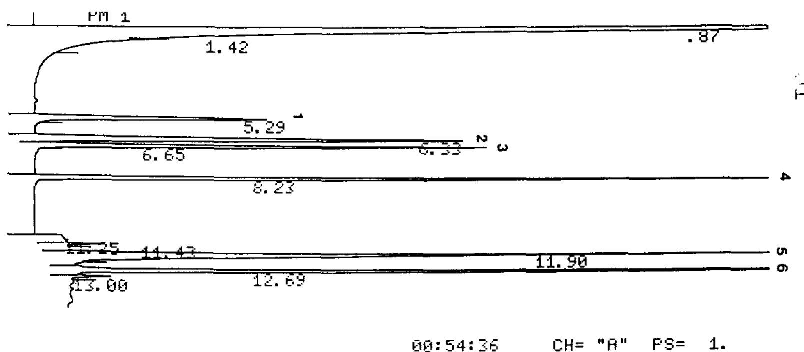

Fig. 3. Gas chromatogram of glucose-containing urine after intestinal permeability test. Chromatographic conditions: initial temperature: 220°C for 10 min, temperature increase 8°C/min from 220°C to 300°C. Peaks: 1 = β-glucose, 2 = mannitol (1.08 g/l), 3 = α-glucose, 4 = myoinositol, 5 = lactulose (118 mg/l), 6 = turanose.

reproducible. Ten repeated analyses of the samples gave coefficients of variation in the retention time of 2.4% for mannitol and 3.0% for lactulose. The retentions times for mannitol, lactulose, myoinositol and turanose were respectively 2.43 ± 0.058; 6.05 ± 0.12; 3.21 ± 0.06; and 6.85 ± 0.28 min. Fig. 1 shows the gas chromatogram of a standard mixture of mannitol, lactulose and internal standard.

The method gives very good peak separation. If urine contains glucose, another peak (β-glucose) appears between the solvent front and the mannitol peak (Fig. 2); chromatographic conditions are then modified as follows: initial temperature, 220°C for 10 min; temperature increase, 8°C/min from 220°C to 300°C to separate the second glucose peak (α-glucose) which overlaps with the mannitol peak. Under these conditions it is possible to quantify mannitol, which is clearly separated from the two glucose peaks, as shown in Fig. 3, which represents the chromatogram for a urine sample after a per-

Table 1
Recovery of sugars added to urine samples

Sugars	Amount added (mg/l)	Amount measured (mg/l)	Recovery (%)
Mannitol	0	415	
	100	483	94
	250	653	98
	500	970	106
	750	1126	97
	1000	1293	92
Lactulose	0	42	
	11	57	107
	27.5	71	102
	55	106	110
	82.5	120.5	97
	110	148	97

meability test in a patient with glycosuria. In addition, the separation of lactulose and mannitol isomers is not necessary, since patients receive only one isomeric form of each sugar.

To test the linearity of the method, a set of calibration curves was made by derivatizing aqueous samples of mannitol and lactulose at five different concentrations (0.5, 1, 1.5, 2, 3 g/l and 25, 50, 100, 200, 300 mg/l, respectively). The method was linear up to 3 g/l for mannitol and 300 mg/l for lactulose. The correlation coefficients were 0.997 ($y = 1.05 \times -3.49$) and 0.998 ($y = 1.04 \times -0.002$) for lactulose and mannitol respectively. Linearity is adequate, which is particularly important for lactulose, since its urinary concentration can be high when intestinal permeability is modified.

The detection limits of the method were 5 mg/l and 3 mg/l for mannitol and lactulose, respectively.

Accuracy was determined by overloading urine. Increasing amounts of sugars were added to pooled urine from a normal subject who had undergone an intestinal permeability test. Mean ($n = 20$) urinary concentrations of mannitol and lactulose were 415 and 42 mg/l before overloading. Table 1 shows the recoveries of mannitol and lactulose. Recovery was very satisfactory for a chromatographic method, with values between 92 and 106% for mannitol and 97 and 110% for lactulose. This indicated the good accuracy of the method, comparable to that reported by Shippee *et al.* [10] using oxime-trimethylsilyl derivatives.

The precision of the measurements is reported in Table 2. Intra-assay variation was determined from ten successive measurements of sugars in pooled frozen urine. The coefficients of variation for mannitol and lactulose were 2.7 and 6.8 respectively. These results are within the range of those reported for urinary mannitol by Laker and Mount [7]. Inter-assay run variations were measured using another aliquot of pooled urine in twenty successive assays. The C.V.s for mannitol and lactulose were 8.9% and 9.3%, respectively.

Reference values were obtained with samples from 25 fasting healthy subjects (12 females and 13 males) free of intestinal disorders. The 100-ml test solution contained 5 g of mannitol and 10 g of lactulose in distilled water. Urine was collected for six hours after ingestion. The urinary concentrations of mannitol and lactulose were multiplied by 6-h diuresis to obtain the amount excreted during this period. The results are

Table 2
Precision

	Intra-assay ($n = 10$)		Inter-assay ($n = 20$)	
	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)
Mannitol	516	2.7	604	8.9
Lactulose	48	6.8	54.5	9.3

Table 3
Normal values[a]

	Mannitol	Lactulose	Ratio
Concentration	14.5 ± 3.1	0.27 ± 0.15	0.0186 ± 0.01
Normal range	11–18	0.10–0.50	0.01–0.035

[a] Percentage of the ingested dose recovered in 6-h urine (mean ± S.D., $n = 25$).

expressed as percentage excretion of the administered doses.

Before the test, lactulose was undetectable (below 3 mg/l). Mannitol concentrations varied from 0 to 49 mg/day, with a mean of 22 ± 15 mg/day ($n = 25$); similar results have been reported by Haga and Nakajima [11]. These values of 24-h endogenous mannitol excretion were low relative to those found in 6-h urine samples after the test. As it was impossible to determine the amount of endogenous mannitol excreted during the 6-h urine collection after the test, and as it is no doubt smaller than the 24-h value, we did not take endogenous excretion into account in the results.

The normal values (mean ± S.D.) for the 25 healthy subjects were 14.5 ± 3.1% for mannitol, 0.27 ± 0.15% for lactulose and 0.0186 ± 0.01 for the lactulose/mannitol (L/M) ratio (Table 3). The range was as follows: 11–18% for mannitol, 0.10–0.50% for lactulose and 0.01–0.035 for the L/M ratio. These values are comparable to those reported by other authors [2,5,8,12,13] using a similar test protocol with regard to the amounts of mannitol and lactulose administered and the period of urine collection. Intestinal permeability is increased when the L/M ratio is above 0.035. It is essentially the L/M ratio which is used to interpret the results. Indeed, it compensates for technical errors such as incomplete urine collection and particular pathophysiological conditions such as renal impairment, accelerated gastric emptying or accelerated intestinal transit.

Fig. 4 represents the chromatogram of urine from a patient with Crohn's disease, after a permeability test. The 6-h mannitol excretion was only slightly modified (13%) relative to normal values whereas that of lactulose was considerably increased (1.26%). The L/M ratio was also markedly increased, at 0.096. These

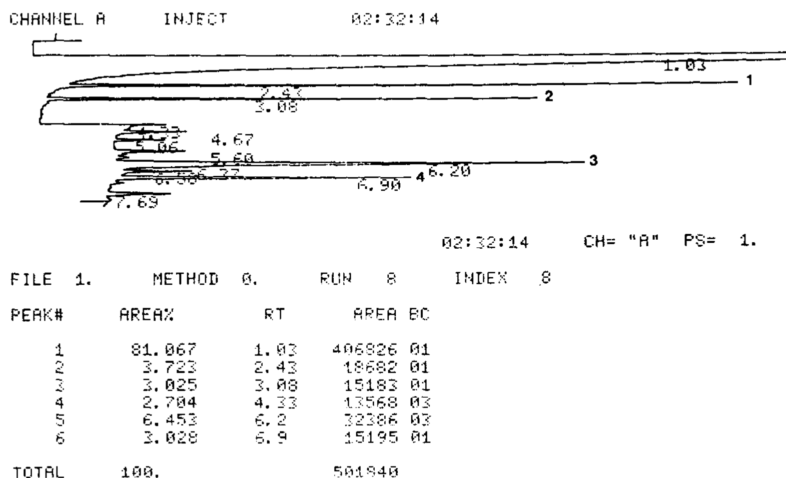

Fig. 4. Gas chromatogram of urine from a patient with Crohn's disease after intestinal permeability test. Peaks: 1 = mannitol (1.36 g/l), 2 = myoinositol, 3 = lactulose (263 mg/l), 4 = turanose.

values reflect the increase in intestinal permeability due to the degradation of the mucosa and poor intestinal barrier function. The test may also prove useful for assessing the effects of treatment and following the course of the disease.

We chose to use trimethylsilylated derivates, as their preparation is simple and rapid. Hydrolysis was reduced by the use of a powerful silyl donor. Despite the fact that the two sugars have very different concentrations, both can be quantified on the same chromatogram under the conditions used. This GC assay is more specific than a colorimetric method [5] and more precise than quantitative paper chromatography [2]. The enzymatic assay for the determination of mannitol and lactulose [3,4] is an excellent technique, but mannitol dehydrogenase is not available commercially.

Liquid chromatographic methods have the advantage of dispensing with sample derivatization, which leads to a gain in time. However, detection by use of the refractivity index often limits the spectrum of applications. The technique developed by Dela Hunty and Hollander [6] gave good intra-assay, but inter-assay variations were not measured. In addition, analytical recoveries from urine were poor and there was an overlap between the lactulose and glucose peaks.

As the indications of the intestinal permeability test widen, this rapid and precise GC method should be of value.

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5. References

[1] B.F. Rush, J.A. Redan and J.J. Flanagam, *Ann. Surg.*, 210 (1989) 342.
[2] I.S. Menzies, J.N. Mount and M.S. Wheeler, *Ann. Clin. Biochem.*, 15 (1978) 65.
[3] C.A. Northrop, P.G. Lunn and R.H. Behrzens, *Clin. Chim. Acta*, 187 (1990) 79.
[4] P.G. Lunn, C.A. Northrop and A.J. Northrop, *Clin. Chim. Acta*, 183 (1989) 163.
[5] R.H. Behrens, H. Docherty, M. Elia and G. Neale, *Clin. Chim. Acta*, 137 (1984) 361.
[6] T. Dela Hunty and D. Hollander, *Clin. Chem.*, 32 (1986) 1542.
[7] M.F. Laker and J.N. Mount, *Clin. Chem.*, 26 (1980) 441.
[8] J. Wyatt, H. Vogelsang, W. Hubl, T. Naldhoer, H. Lochs, *Lancet*, 341 (1993) 1437
[9] S.C. Churm, *J. Chromatogr.*, 500 (1990) 555.
[10] R.L. Shippee, A.A. Johnson, W.G. Cioffi, J. Lasko, T.E. Le Voyer and B.S. Jodran, *Clin. Chem.*, 38 (1992) 343.
[11] H. Haga and T. Nakajima, *Biomed. Chromatogr.*, 3 (1989) 68.
[12] P.G. Lunn, C.A. Northrop-Clewes and R.M. Downes, *Lancet*, 338 (1991) 907.
[13] F. Andre, C. Andre, Y. Emery, J. Forichon, L. Descos and Y. Minaire, *Gut*, 29 (1988) 511.