

Rapid Gas-Chromatographic Assay of Lactulose and Mannitol for Estimating Intestinal Permeability

Mauro Celli,¹ Patrizia D'Eufemia,^{1,3} Roberto Dommarco,² Roberto Finocchiaro,¹ Daniela Aprigliano,¹ Francesco Martino,¹ Ettore Cardi,¹ and Omero Giardini¹

We developed a gas-chromatographic method to determine urinary mannitol and lactulose. The procedure for purification of urine by a resin was optimized for purification of analytes and high recovery; the aliquot of resin chosen (500 mg) was kept in contact with the urine for 1 min. The recoveries of mannitol and lactulose were >85% at concentrations that include both normal and pathological values. Sugars were converted to oximes before the silylation step to avoid multiple peaks for the anomeric forms. The calibration was linear over the range 0.1–1 µg of sugar injected. Analytical recovery of the sugars ranged from 90% to 95.3% for mannitol and from 90.4% to 95.8% for lactulose. The mean within-day imprecision (CV) was 6.2% for mannitol and 4.7% for lactulose; the between-day CV was 6.7% for mannitol and 5.1% for lactulose. A lactulose/mannitol ratio of 0.035 completely differentiated 28 normal children and 28 children with active gluten-sensitive enteropathy, whose mean ratios were 0.022 (SD 0.007) and 0.084 (SD 0.054), respectively.

Indexing Terms: celiac disease/urine/pediatric chemistry/sugars/enteropathy

The sugar intestinal permeability test is regarded as the most valuable screening test for mucosal damage of the small intestine in children (1, 2). This procedure is performed by orally administering two sugars of different molecular size and with different absorption routes and then measuring the urinary excretion. In disorders of the small intestine, transcellular permeability tends to decrease, reflecting a diminished number of mucosal cells, whereas paracellular permeability tends to increase, reflecting damaged tight junctions (3, 4). The change of the two types of intestinal permeability in opposite directions in intestinal diseases has been the rationale for using the ratio between lactulose and mannitol (L/M) urinary excretion for investigational purposes (5). This approach is based on the assumption that the two sugars are handled identically in all other physiological respects and thus intraindividual differences in gastric emptying, small intestinal transit, urinary excretion, and so forth can be eliminated (3, 6–11).

Since the first report of gas chromatography of carbohydrates (12, 13), numerous reports have appeared describing applications to the field of carbohydrate

chemistry (14–21). To date, however, no reports have used the desalting of urine with different aliquots of resin for a good purification of the sample with an adequate recovery of sugars. Therefore, we developed a gas-chromatographic method for the simultaneous measurement of lactulose and mannitol in urine after desalting the samples. We have used this method to determine the sugar intestinal permeability in normal children and in children with active gluten-sensitive enteropathy (GSE).

Materials and Methods

Materials

Pyridine, hydroxylamine chlorhydrate, and sodium azide were obtained from Merck (Darmstadt, Germany). Trimethylchlorosilane and bis(trimethylsilyl)trifluoroacetamide were obtained from Supelco (Bellefonte, PA). Inositol (1,2,3,5/4,6-hexahydroxycyclohexane), fructose (D-levulose), mannitol (mannite), and turanose (3-O- α -D-glucopyranosyl-D-fructose) were obtained from Sigma-Aldrich (Milan, Italy). D-Glucose (dextrose) and D-galactose were from Merck. Lactulose (Dia-Colon) was obtained from Vecchi e C. Piam (Genoa, Italy). Duolite MB 5113 (a mixture of Duolite C225 in the H⁺ form and A101 D in OH⁻ form) was supplied by BDH (Poole, UK).

Working solutions. Oxime solution: hydroxylamine chlorhydrate in pyridine (15 g/L); silylating solution: equal volumes of trimethylchlorosilane and bis(trimethylsilyl)trifluoroacetamide; solution A: aqueous mannitol, inositol, lactulose, and turanose, 1 g/L each; solution B: aqueous mannitol and lactulose, 1 g/L each; solution C: aqueous inositol and turanose, 1 g/L each. The internal standard solution consisted of inositol (1 g/L) and turanose (0.2 g/L) in H₂O (concentrations reflecting the mean content of each sugar in pathological urine).

Instrumentation

The gas chromatograph (Model 5890 A), integrator (Model 3390 A), and column [HP1; 25 m $\times$ 0.32 mm (i.d.) $\times$ 0.17 µm film thickness] were all from Hewlett-Packard, Palo Alto, CA. The injector was a split-splitless used in a split mode of 1:80. The detector operated by flame ionization detection. Temperatures were: column, 150–270°C (8°C/min); injector, 270°C; detector, 270°C. The carrier gas was nitrogen (5 mL/min); the make-up gas was nitrogen (30 mL/min).

¹ Istituto di Clinica Pediatrica, Università di Roma "La Sapienza," Viale Regina Elena 324, 00161 Rome, Italy.

² Istituto Superiore di Sanità, Rome, Italy.

³ Author for correspondence. Fax 396/4462767.

Received September 23, 1994; accepted February 13, 1995.

Procedures

Derivatization. Known amounts (100–200 μL) of sugar solutions A, B, or C were dried in a screw-top vial at 60°C under a gentle stream of nitrogen; 100 μL of oxime solution was then added, the vial was capped, and the sample was incubated at 60°C for 20 min. After cooling the sample to room temperature, 100 μL of silylating solution was added and the incubation period was repeated.

Internal standard calibration. Inositol and turanose were used as internal standards for the quantitative determination of mannitol and lactulose, respectively. Investigation of the response factors (RF) over a wide range of concentrations (0.05–1.5 g/L) gave the following results:

$$\text{Inositol/mannitol RF} = 1.03 \pm 0.025$$

$$\text{Turanose/lactulose RF} = 1.05 \pm 0.041$$

Urine purification. Many methods described do not mention urine purification before derivatization and gas-chromatographic analysis. However, our preliminary tests without urine purification yielded gas-chromatographic patterns showing peaks with retention times close to those of investigated sugars. We therefore used Duolite MB 5113 as supplied by BDH to desalt and to purify urine samples. We evaluated the use of different amounts of resin to achieve both the best purification and the greatest recoveries of sugars. To 1-mL aliquots of sugar-free urine we added 125, 250, 500, 750, or 1000 mg of resin, shaking these for 1 min in a capped vial. We then transferred 200 μL of the supernates to screw-top vials and dried the samples at 60°C under a gentle stream of nitrogen. After derivatization, the samples were analyzed by gas chromatography.

To assess recovery, we mixed 1-mL aliquots of sugar-free urine with 1 mL of solution B and treated the mixtures with the scalar quantities of resin previously described. We then added 100 μL of solution C to 100 μL of the supernates, and dried and processed the aqueous samples as described above.

Statistical analysis. Statistical analysis was performed with the Mann-Whitney *U*-test for nonparametric data.

Clinical Studies

Patients. To evaluate the procedure described, we studied 56 children of both sexes: 28 control children (12 boys and 16 girls), ages 2–15 years (mean 9, SD 5.3 years), without evidence of gastrointestinal or systemic disease; and 28 patients (10 boys and 18 girls) with active GSE, ages 3–16 years (mean 10, SD 4.7 years). In each case the diagnosis was based on the European Society of Pediatric Gastroenterology criteria (22).

L/M test. The subjects followed a diet free of mannitol, lactulose, mannose, and fructose for 24 h before the test (17, 23–25). After an overnight fast, the subjects voided a pretest urine sample and then ingested (0.55 mL/kg body weight) a solution containing 18.2 g

of mannitol and 18.2 g of lactulose in 100 mL of deionized water. We used a hypertonic solution (1500 mosmol/L) because hypertonicity increases the absorption of intact disaccharides (26–28) and enhances the sensitivity of the test (26).

After 2 h from the beginning of test, the subjects were encouraged to drink ~100 mL of water to ensure adequate urine production. All urines voided in the subsequent 5 h were collected with sodium azide as a preservative. The volume was recorded and an aliquot was kept at –20°C for subsequent analysis.

After the frozen urine aliquots had reached room temperature, 1 mL of urine was shaken with 500 mg of resin; 100 μL of this supernate was then added to 100 μL of internal standard solution, dried, and processed as described above.

Results

Analytical Performance

Linearity. The linearity of the method was tested for mannitol, inositol, lactulose, and turanose, inositol and turanose being tested for their suitability as internal standards. After derivatizing 10, 20, 50, and 100 μL of solution A, we injected 2 μL of each sample twice directly into the gas chromatograph. This procedure was repeated three times. A linear response (Fig. 1) was obtained in the range 0.1–1 μg of sugars injected, as shown: mannitol, $y = 173924x - 750714$ ($r = 0.999$, $S_{x|y} = 0.016$); inositol, $y = 173541x - 603267$ ($r = 0.999$, $S_{x|y} = 0.012$); lactulose, $y = 114810x - 154469$ (r

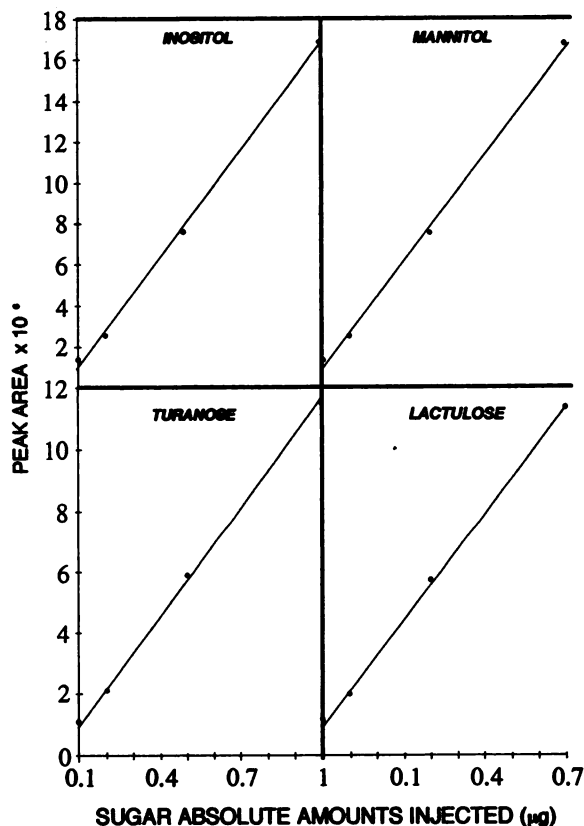


Fig. 1. Linearity of detector response.

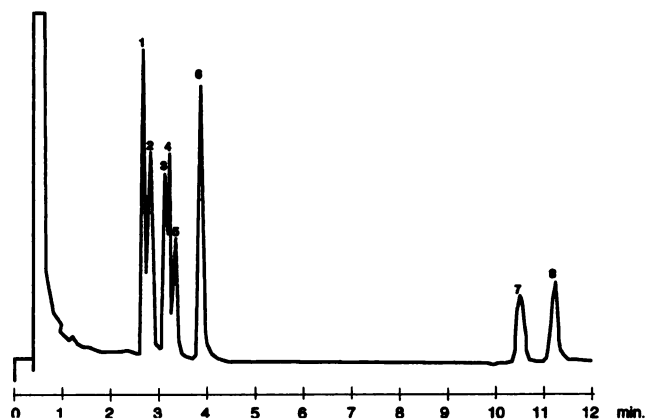


Fig. 2. Chromatogram of a standard solution containing 1 g/L each of mannitol (1), fructose (2), galactose (3,4), glucose (5), inositol (6), lactulose (7), and turanose (8).

= 0.999, $S_{x|y}$ = 0.011); and turanose, $y = 116719x - 26358$ ($r = 0.999$, $S_{x|y}$ = 0.019).

Interferences. No overlapping peaks were found in a chromatogram from a solution containing glucose, fructose, galactose, mannitol, inositol, lactulose, and turanose (Fig. 2).

Urine purification and recovery. Table 1 shows the mean recoveries at different resin amounts. The best results were achieved with 500 mg of resin, which yielded chromatograms free of interferences (Fig. 3) and gave average recoveries of 91.5% (SD 4.3%) and 92.5% (SD 5.2%) for mannitol and lactulose, respectively ($n = 4$ trials).

We tried also to optimize the contact time between

Table 1. Mannitol and lactulose recoveries from 1 mL of sugar-supplemented urine samples treated with scalar quantities of Duolite MB 5113 resin.

Resin, mg	Recovery %*	
	Mannitol	Lactulose
125	94.6 ± 4.7	96.0 ± 5.0
250	94.0 ± 5.1	93.2 ± 6.1
500	91.5 ± 4.3	92.5 ± 5.2
750	86.2 ± 5.8	85.1 ± 6.7
1000	82.5 ± 5.4	83.3 ± 6.4

* Recoveries are the arithmetic mean ± SD of four trials.

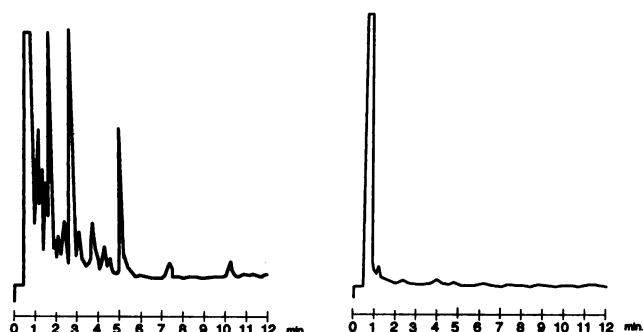


Fig. 3. Chromatogram of normal urine untreated (left) and treated with resin (right).

Table 2. Analytical recoveries of mannitol and lactulose added to urine.

Added amount, g/L	Mean ± SD recovery, %	
	Mannitol	Lactulose
0.02	90.0 ± 5.6	91.7 ± 6.1
0.10	92.0 ± 4.3	90.4 ± 4.8
0.50	91.6 ± 4.4	92.3 ± 5.0
1.00	93.6 ± 3.9	94.5 ± 4.4
2.50	95.3 ± 4.0	95.8 ± 5.3

urine and resin. We found that the purification step was not critically time dependent.

Precision. One urine sample of a patient with active GSE was analyzed four times per day for 5 days. The mean within-day imprecision (CV) for mannitol was 6.2%, for lactulose 4.7%. The between-day CV was 6.7% for mannitol and 5.1% for lactulose.

Accuracy. The accuracy of the method, calculated by testing recoveries in sugar-free urine samples mixed with known amounts of mannitol and lactulose (between 0.02 and 2.5 g/L urine), is summarized in Table 2.

Clinical Results

The subjects' results are expressed as the percentage of mannitol and lactulose recovered in the urine samples and as the L/M recovery ratio. The control subjects showed a mean mannitol recovery of 15.61% (SD 5.8%) and a mean lactulose recovery of 0.28% (SD 0.04%). In contrast, the GSE patients showed a mean recovery of 8.72% (SD 3.5%) and of 0.73% (SD 0.5%) for mannitol and lactulose, respectively. The mean L/M recovery ratio value was 0.022 (SD 0.007) in the control subjects and 0.084 (SD 0.054) in the patients (Table 3).

Chromatograms of urine samples from a GSE patient taken before and 5 h after receiving the mannitol-lactulose solution are shown in Fig. 4.

The differences between mean lactulose and mannitol urinary recovery and mean L/M in GSE children compared with controls were highly statistically significant ($P < 0.001$). Moreover, in all 28 GSE patients, L/M was >0.035 , whereas in the controls, all L/M values were <0.035 . In contrast, by receiver-operating characteristic curve analysis, neither urinary lactulose nor urinary mannitol alone provided sensitivities $>80\%$ at a specificity of 80% (data not shown).

Discussion

Ratios of L/M excretion are used to assess the intestinal permeability in various diseases and trauma

Table 3. Results of the lactulose/mannitol test.

Urinary recovery	Mean ± SD		P
	Controls	Active GSE	
Mannitol, %	15.61 ± 5.8	8.72 ± 3.5	<0.001
Lactulose, %	0.28 ± 0.04	0.73 ± 0.5	<0.001
L/M ratio	0.022 ± 0.007	0.084 ± 0.054	<0.001

$n = 28$ each.

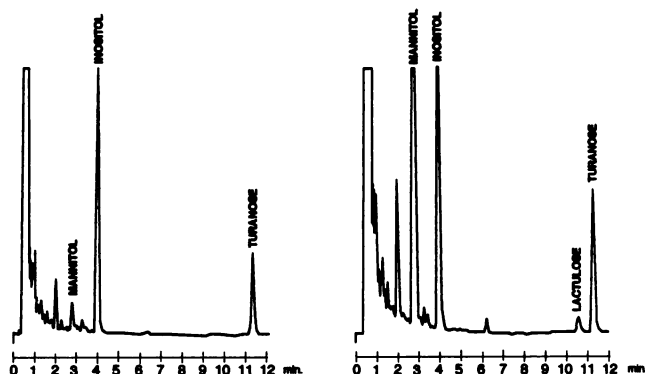


Fig. 4. Chromatogram of urine from a GSE patient before (left) and after (right) ingesting the mannitol-lactulose solution.

conditions. Several methods have been used for determining urinary sugars: thin-layer chromatography (29), colorimetric/enzymatic procedure (30), gas chromatography (2, 14, 16, 17), and HPLC (31–33).

Our method represents a further development of the gas-chromatographic methods previously described, the main modification being an improved purification of urine. Using a Duolite MB 5113 resin yielded chromatograms free of interferences.

The best results for quantifying the sugars were obtained by using 500 mg of resin per milliliter of urine, relatively less than amounts previously described in the literature (2, 14, 16, 31, 32, 34). We found that lower amounts were insufficient for good purification, whereas greater amounts greatly reduced the quantitative recovery of sugars. The recovery efficiency of 500 mg of resin per milliliter of urine was tested for a wide range of sugar concentrations (0.02–2.5 g/L). Recoveries were nearly 90% for each sugar, as described in *Results*.

We also found that 1 min of contact time between resin and urine was sufficient even if a longer period did not affect the recovery of sugars. This seems to be in contrast with a previous report (2, 14).

As recommended by Shippee et al. (35), we converted sugars to the relative oximes before the silylating process. The oxime derivatives are essential to avoid the presence of multiple gas-chromatographic peaks due to the anomeric forms of sugars.

We conclude that the present technique is an improvement on the sensitivity and selectivity of some previous methods. Furthermore, the reliability and brevity of this procedure encourage its use in routine investigations of intestinal diseases.

References

1. Hodges S, Ashmore SP, Patel HR, Tanner MS. Cellobiose: mannitol differential permeability in small bowel disease. *Arch Dis Child* 1989;64:853–4.
2. Hamilton I, Hill A, Bose B, Bouchier IAD, Forsyth JS. Small intestinal permeability in pediatric clinical practice. *J Pediatr Gastroenterol Nutr* 1987;6:697–701.
3. Menzies IS. Transmucosal passage of inert molecules in health and disease. In: Skadhauge E, Heintze K, ed. *Intestinal absorption and secretion*. Falk Symposium 36. Lancaster, UK: MTP Press, 1984:527–43.

4. Hollander D. The intestinal permeability barrier. A hypothesis as to its regulation and involvement in Crohn's disease [Review]. *Scand J Gastroenterol* 1992;27:721–6.
5. Travis S, Menzies I. Intestinal permeability: functional assessment and significance. *Clin Sci* 1992;82:471–88.
6. Pearson ADJ, Eastham EJ, Laker MF, Craft AW, Nelson R. Intestinal permeability in children with Crohn's disease and coeliac disease. *Br Med J* 1982;285:20–1.
7. Behrens RH, Szaz KF, Northrop C, Elia M, Neale G. Radio-nuclide tests for the assessment of intestinal permeability. *Eur J Clin Invest* 1987;17:100–5.
8. Juby LD, Rothwell J, Axon ATR. Lactulose/mannitol test: an ideal screen for celiac disease. *Gastroenterology* 1989;96:79–85.
9. Katz KD, Hollander D, Vadheim CM, McElree C, Delahunty T, Dadufalza VD, et al. Intestinal permeability in patients with Crohn's disease and their healthy relatives. *Gastroenterology* 1989;97:927–31.
10. Ukabam SO, Clamp JR, Cooper BT. Abdominal small intestine permeability to sugars in patients with Crohn's disease of the terminal ileum and colon. *Digestion* 1983;27:70–4.
11. Katz KD, Hollander D. Intestinal mucosal permeability and rheumatological disease. *Bailliere's Clin Rheumatol* 1989;3:271–84.
12. Catassi C, Pierani P, Natalini G, Gabrielli O, Coppa GV, Giorgi PL. Clinical application of a simple HPLC method for the sugar intestinal permeability test. *J Pediatr Gastroenterol Nutr* 1991;12:209–12.
13. Menzies IS. Quantitative estimation of sugars in blood and urine by paper chromatography using direct densitometry. *J Chromatogr* 1973;81:109–27.
14. Laker MF. Estimation of disaccharides in plasma and urine by gas-liquid chromatograph. *J Chromatogr* 1979;163:9–18.
15. Laker MF, Mount JM. Mannitol estimation in biological fluids by gas-liquid chromatography of trimethylsilyl derivatives. *Clin Chem* 1980;26:441–3.
16. Debord P, Postaire M, Pham-Huy C, Hamon M. Dosage par chromatographie gazeuse capillaire du mannitol et du lactulose dans les urines. *Ann Biol Clin* 1992;50:77–80.
17. Blomquist L, Bark T, Hedenborg G, Svenberg T, Norman A. Comparison between the lactulose/mannitol and ^{51}Cr -ethylenediaminetetraacetic acid/ ^{14}C -mannitol methods for intestinal permeability. Frequency distribution pattern and variability of markers and marker ratios in healthy subjects. *Scand J Gastroenterol* 1993;28:274–80.
18. Manius G, Mahn FP, Venturella VS, Senkowski BZ. GLC (gas-liquid chromatographic) determination of sorbitol and mannitol in aqueous solutions. *J Pharm Sci* 1972;61:1831–5.
19. Marunaka T, Matsushima E, Umeno Y, Minami Y. GLC mass fragmentographic determination of mannitol and sorbitol in plasma. *J Pharm Sci* 1983;72:87–9.
20. Sondack DL. Determination of sorbitol and mannitol in pharmaceuticals by GLC of tri-*n*-butyldiboronate esters. *J Pharm Sci* 1975;64:128–9.
21. Muller M, Walker-Smith J, Shmerling DH, Curtius HC, Prader A. Lactulose: a gas-liquid chromatography method of determination and evaluation of its use to assess of intestinal mucosal damage. *Clin Chim Acta* 1969;24:45–9.
22. Meeuwisse GW. Roundtable discussion. Diagnostic criteria in coeliac disease. *Acta Paediatr Scand* 1970;59:461–3.
23. Laker MF, Bull HJ, Menzies IS. Evaluation of mannitol for use as a probe marker of gastrointestinal permeability in man. *Eur J Clin Invest* 1982;12:485–91.
24. Ng WG, Donnel GN, Bergren WR. Mannitol excretion in galactosemia patients. *Clin Chim Acta* 1975;64:39–44.
25. Blau K. Increased mannitol excretion in controlled hereditary galactosemia. *Clin Chim Acta* 1972;38:441–5.
26. Cobden I, Hamilton I, Rathwell J, Axon ATR. The cellobiose/mannitol test: physiological properties of probe molecules and influence of extraneous factors. *Clin Chim Acta* 1985;148:53–62.
27. Laker MF, Menzies IS. Increase in human intestinal permeability following ingestion of hypertonic solutions. *J Physiol* 1977;265:881–94.
28. Menzies IS. Absorption of intact oligosaccharide in health and disease. *Biochem Soc Trans* 1974;2:1042–7.
29. Menzies IS, Mount JN, Wheeler MJ. Quantitative estimation

of clinically important monosaccharides in plasma by rapid thin-layer chromatography. *Ann Clin Biochem* 1978;15:65-76.

30. Strobel S, Brydon WG, Ferguson A. Cellobiose/mannitol sugar permeability test complements biopsy histopathology in clinical investigation of the jejunum. *Gut* 1984;25:1241-6.

31. Delahunty T, Hollander D. Liquid-chromatographic method for estimating urinary sugars: applicability to studies of intestinal permeability. *Clin Chem* 1986;32:1542-4.

32. Kynaston JA, Fleming SC, Laker MF, Pearson ADJ. Simultaneous quantification of mannitol, 3-O-methyl glucose, and lactulose in urine by HPLC with pulsed electrochemical detection, for use in studies of intestinal permeability. *Clin Chem* 1993;39:453-6.

33. Willems D, Cadranel S, Jacobs W. Measurement of urinary sugars by HPLC in the estimation of intestinal permeability: evaluation in pediatric clinical practice. *Clin Chem* 1993;39:888-90.

34. Leke L, Barau E, Barbet JP, Epelbaum S, Vierin Y, Dupont C. Intérêt du test de perméabilité intestinale par lactulose-mannitol pour le dépistage et la surveillance de la maladie coeliaque chez l'enfant. *Arch Fr Pediatr* 1992;49:33-7.

35. Shippee RL, Johnson AA, Cioffi WG, Lasko J, LeVoyer TE, Jordan BS. Simultaneous determination of lactulose and mannitol in urine of burn patients by gas-liquid chromatography. *Clin Chem* 1992;38:343-5.