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Journal of Chromatography B, 836 (2006) 63–68
JOURNAL OF CHROMATOGRAPHY B
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Resolution of common dietary sugars from probe sugars for test of intestinal permeability using capillary column gas chromatography

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Received 10 January 2006; accepted 15 March 2006
Available online 18 April 2006

Abstract

Background: The most widely accepted method for the evaluation of intestinal barrier integrity is the measurement of the permeation of sugar probes following an oral test dose of sugars. The most-widely used sugar probes are sucrose, lactulose, mannitol and sucralose. Measuring these sugars using a sensitive gas chromatographic (GC) method, we noticed interference on the area of the lactulose and mannitol peaks.
Methods: We tested different sugars to detect the possible makeup of these interferences and finally detected that the lactose interferes with lactulose peak and fructose interferes with mannitol peak. On further developing of our method, we were able to reasonably separate these peaks using different columns and condition for our assay. Sample preparation was rapid and simple and included adding internal standard sugars, derivitization and silylation. We used two chromatographic methods. In the first method we used Megabore column and had a run time of 34 min. This resulted in partial separation of the peaks. In the second method we used thin capillary column and was able to reasonably separate the lactose and lactulose peaks and the mannitol and fructose peaks with run time of 22 min.
Results: The sugar probes including mannitol, sucrose, lactulose, sucralose, fructose and lactose were detected precisely, without interference. The assay was linear between lactulose concentrations of 0.5 and 40 g/L ($r^2 = 1.000$, $P < 0.0001$) and mannitol concentrations of 0.01 and 40 g/L ($r^2 = 1.000$). The sensitivity of this method remained high using new column and assay condition. The minimum detectable concentration calculated for both methods was 0.5 mg/L for lactulose and 1 mg/L for mannitol.
Conclusion: This is the first report of interference of commonly used sugars with test of intestinal permeability. These sugars are found in most of fruits and dairy products and could easily interfere with the result of permeability tests. Our new GC assay of urine sugar probes permits the simultaneous quantitation of sucralose, sucrose, mannitol and lactulose, without interference with lactose and fructose. This assay is a rapid, simple, sensitive and reproducible method to accurately measure intestinal permeability.

Keywords: Capillary column gas chromatography; Lactulose; Lactose; Mannitol; Fructose; Intestinal permeability

1. Introduction

The most widely accepted method for the evaluation of intestinal barrier integrity in vivo is the measurement of the permeation of sugar probes following an oral test dose of sugars [1–3]. The most-widely used sugar probes are sucrose, lactulose, mannitol and sucralose. In this setting, the urinary level of sucrose selectively reflects gastroduodenal permeability [2]. Urinary levels of mannitol (M) and lactulose (L) and particularly the L/M ratio reflect gastric and small intestinal permeability [4–9]. The urinary sucralose level reflects whole gut permeability [10]. These sugars are passively absorbed across the intestinal mucosa via the transcellular and/or paracellular pathways, escape metabolism, and are excreted unchanged in the urine in proportion to their permeation. Despite the availability of several analytical techniques for urinary sugar measurement, the separation and quantitation of closely related mono- and di-saccharides has often been challenging.

There are several methods for the quantitation of urinary sugars including paper and thin layer chromatography [11,12], enzymatic assays [13–16], gas chromatography [5,6,17–19], gas chromatography with mass specteroscopy and HPLC [1,20–25]. We recently reported [26] the use of capillary gas chromatography, for the measurement of silyl derivatives of urinary

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doi:10.1016/j.jchromb.2006.03.046

sucralose, sucrose, L and M, in our in vivo studies of intestinal permeability in man. We also realized that commonly used sugars interfered with the assay of these sugars. This observation suggested that the presence of lactose (milk sugar) and Fructose partially distorted the sugar probe peaks for lactulose and mannitol, respectively (Fig. 1a). Through spiking of urine samples with lactose and fructose we found that even the small amount of these sugars that were present in early morning urine samples,

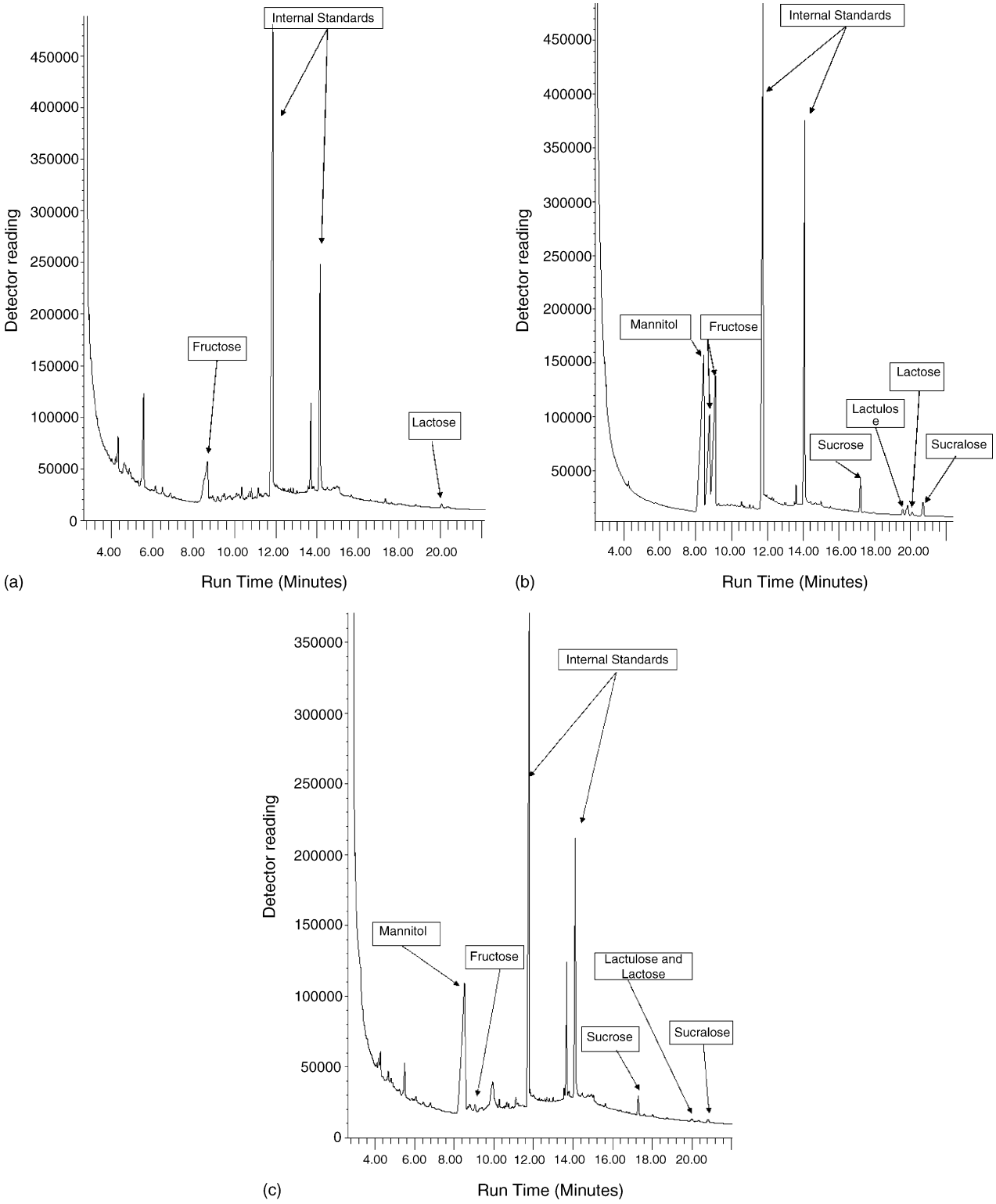

Fig. 1. (a) Chromatogram of a subject baseline urine samples containing fructose and minimal lactose. Different part of the chromatogram needs different magnification (lactose peaks). (b) Whole chromatogram of a blank urine sample spiked with mannitol, fructose, internal standards, sucrose, lactulose, lactose and sucralose. (c) Chromatogram of a subject 12 h-collected urine after ingestion of sugar for test of intestinal permeability. Sample containing mannitol, fructose, internal standards, sucrose, lactulose, lactose and sucralose. Different part of the chromatogram needs different magnification (lactose and lactulose peaks).

after 8 h of fasting, could confound our probe sugar measurements. In this study we modified our chromatographic technique to reasonably separate these sugar peaks, in order to accurately measure lactulose and mannitol in urinary samples.

2. Experimental

2.1. Materials

Most of the materials and chemicals were previously described [26]. Lactose (α-Lactose monohydrate, purity >98%), Fructose (D-Fructose, purity >99%) were obtained from Sigma (St. Louis, MO). Two forms of active lactase were used. Lactaid (obtained from McNeil-PPC Philadelphia, PA) and purified Lactase from *Escherichia coli* (Sigma St. Louis, MO).

2.2. Equipment

Gas chromatography was performed using a Hewlett Packard instrument (HP5890A Palo Alto, CA) equipped with a Flame Ionization Detector (FID). We used DB-1701 capillary column (J&W, Folsom, CA), which was 30 m × 250 μm I.D. column, with a 0.25 μm film thickness.

2.3. Sample preparation

Urine samples were obtained from healthy volunteers before and after sugar ingestion for test of intestinal permeability. Known amount of sugar standards were added to blank urine before sugar ingestion. Samples were prepared as previously reported by us [26].

2.4. Enzymatic degradation of lactose

Known amounts of lactulose and lactose were added to control urine samples. Lactase (either Lactaid or *E. coli* derived lactase) was added to the samples and the mixtures were incubated at 37 °C for 1, 5, 15 or 30 min. Following enzymatic degradation of lactose, chromatography was carried out.

2.5. Chromatography

The chromatographic condition was different from our previous report. The detector temperature was 280 °C and the injector temperature was 250 °C. The initial column temperature of 180 °C was held for 9 min and then increased at a rate of 20 °C/min to 250 °C which was maintained for 9.5 min. The total run time was 22 min. The rest of the conditions were similar to our initial method [26].

3. Results

3.1. Enzymatic separation

Both enzyme preparations effectively degraded lactose. However, to our surprise, the enzymes also degraded Lactulose. Therefore, neither enzyme preparation could be used to differentiate the lactose from lactulose.

3.2. Chromatographic separation

We could not clearly resolve the lactose and lactulose peaks or the mannitol and fructose peaks using our established method previously described [26].

In our newer method, we used a thin column and detect all of the sugars that we use as probes for the test of intestinal permeability such as mannitol, sucrose, lactulose and sucralose in spiked urine (Fig. 1b) and real patient's sample (Fig. 1c). Some sugars eluted as multiple adjacent peaks, including mannitol, lactulose, lactose, fructose and sucrose. Although some peaks were overlapped, we were still able to successfully separate the peaks that are not overlapped and quantify the amount of lactulose, lactose, mannitol and fructose without significant interference. There were no significant changes in our assay of sucrose or sucralose in our new method.

In this method, both lactose and lactulose eluted as two adjacent peaks (Fig. 2a). The retention times were 19.4 and 19.6 min for the lactulose peaks and 19.7 and 19.9 min for the lactose peaks. As evident in Fig. 2a, the second peak of lactulose still overlapped with the first peak of lactose and a sample that contained both lactose and lactulose produce three adjacent peaks which correspond to pure lactulose, combination of lactulose and lactose, and pure lactose, respectively (Fig. 2b). We used samples containing different concentrations of lactulose and lactose to see how effective this method was for measuring the lactulose concentration independently of the lactose concentration in the mixture. Plotting the concentration of lactulose standards against the area under the first peak of lactulose and analyzing the correlation using linear regression, we showed that the area under the first lactulose peak closely correlated with the lactulose concentration of a spiked sample ($P < 0.0001$, $r^2 = 1.000$) (Table 1). In addition, the concentration of lactulose calculated by measurement of the area under the first lactulose peak was independent of the lactose concentration in the sample (Fig. 3). The area under the second peak however, correlated with the concentration of both sugars in the sample (figure not shown). The area under the third peak correlated to the concentration of lactose. There was minimal run to run variability in the ratio of the first to the second peak of lactulose in one day assay for lactulose. The average of this ratio in 6 consecutive days was 55.36 ± 2.44%.

Mannitol produced two separate peaks with retention times of 8.08 and 9.65 min (Fig. 4a). The area under the first and the second peaks of mannitol closely correlated with mannitol concentration in the spiked sample (peak 1 $r = 1.000$, $P < 0.0001$, peak 2 $r = 0.952$, $P < 0.0001$). Either mannitol peaks could be used for measurement of mannitol in the samples. However, the second peak of mannitol is smaller in height and interfered with the third fructose peak. There was minimal run to run variability in the ratio of the first to the second peak of mannitol in one day assay for lactulose. The average of this ratio in 3 consecutive days was 88.08 ± 1.17%. Fructose produced three peaks with retention times of 8.37, 8.68 and 9.45 min (Fig. 4a). The first and

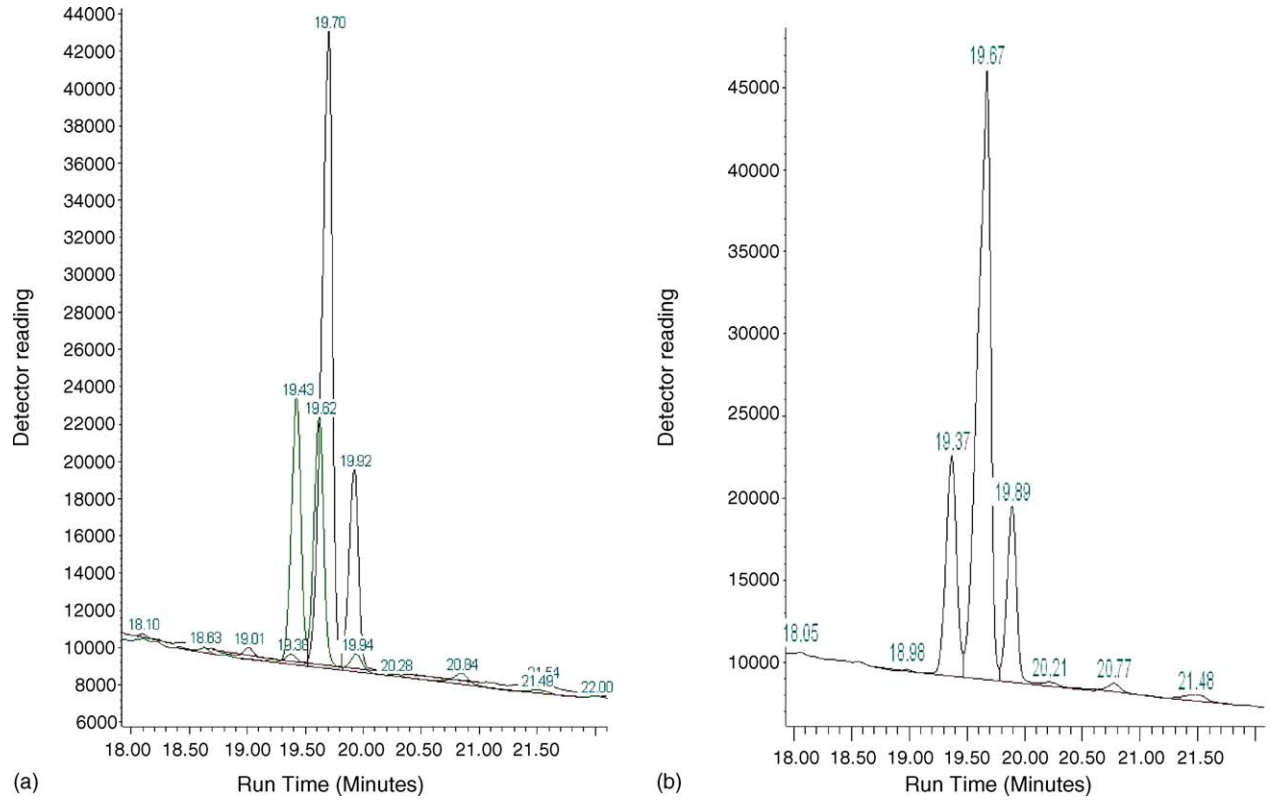

Fig. 2. (a) Composite of two chromatograms showing how the second peak of lactulose curves (with retention time of 19.62 min) overlap the first peak of lactose curves (with retention time of 19.70). (b) GC chromatogram of a sample containing both lactulose and lactose shows three peaks corresponds to pure lactulose, mixed lactose and lactulose and pure lactose in the sample, respectively.

Table 1
Detection of lactulose, lactose, mannitol and fructose using capillary column gas chromatography

	Lactulose (first peak)	Lactose (second peak)	Mannitol (first peak)	Fructose (second peak)
Calibration range (g/L)	0.0005–2	0.0005–2	0.001–40	0.001–40
Linearity[a] (r^2)	1.000	0.979	1.000	1.000
Slope of regression line	0.00027 (±0.0001)	0.0011 (±0.0001)	0.016 (±0.000)	0.010 (±0.0001)
Regression line intercept	0.00012 (±0.0001)	0.0038 (±0.001)	0.004 (±0.004)	0.005 (±0.004)
Limit of detection[b]	0.5 ng	0.5 ng	1 ng	1 ng
Detection limit (mg/L)[c]	0.5	0.5	1	1
Coefficient of variation[d] (%)	2.06	1.89	3.4	3.1

[a] Regression line was based on 8 point spiked standard line.
[b] Amount per injection.
[c] The quantitation limit (the lowest amount of an analyte in a sample which can be quantitatively determined with suitable precision and accuracy is usually three times of DL).
[d] Based on the concentration in mid range and using 6 consecutive day experiments for lactulose and lactose and 3 consecutive day experiment for mannitol and fructose.

second peaks were adjacent and the third peak was smaller and could only be detected in higher concentration (detection limit was 8 times of the other peaks). All three fructose peaks were well correlated with the concentration of fructose in the solution (peak 1 $r = 1.000$, $P < 0.0001$; peak 2 $r = 1.000$, $P < 0.0001$; peak 3 $r = 0.955$, $P < 0.0001$). Either the first or the second peak of fructose could be used for quantitation measures. The third peak of fructose, however, interfered with the second peak of mannitol (Fig. 4a, b). Using different concentration of mannitol and fructose we showed that the area under the first peak of mannitol is independent of fructose concentration in the sample (Fig. 5).

The sensitivity and specificity of this method remained high using new column and assay condition (Table 1).

4. Discussion

The assessment of small intestinal permeability using L/M ratios has been an important tool for the in vivo investigation of GI barrier function in many research and clinical settings [27,28]. Since, the use of minimally absorbed, non-metabolized sugars as probes for the assessment of barrier function is convenient and non-hazardous [7,29], measurement of the silyl

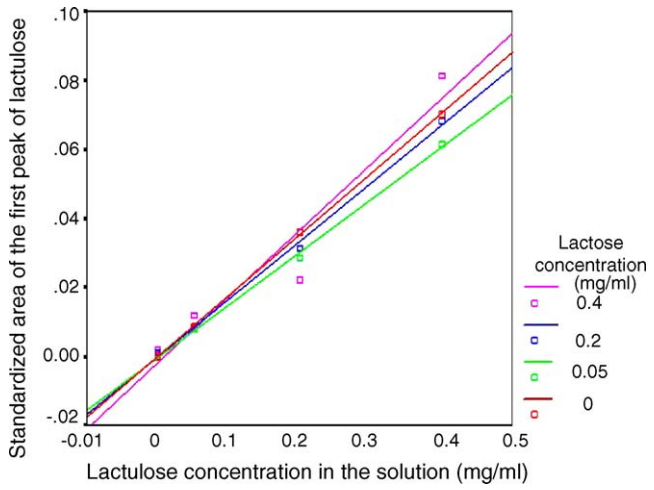

Fig. 3. Scatter plot of the instrument reading of the standardized first lactulose peak at various concentrations of lactulose in the sample. Each line represents a different concentration of lactose. The proximity of the lines together shows that the reading of the first peak of lactulose is independent of the concentration of lactose in the solution.

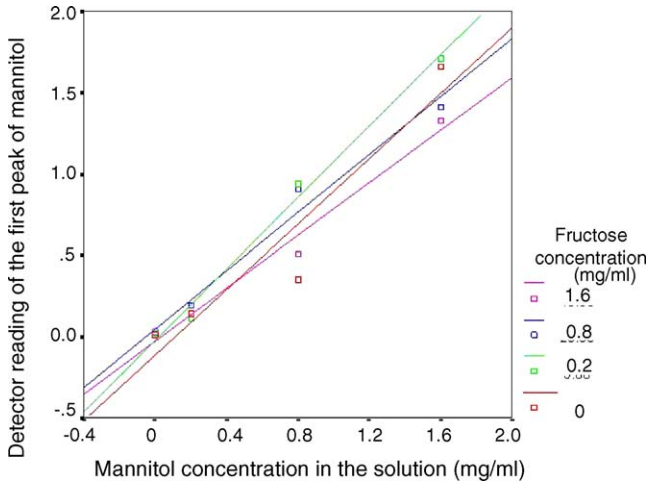

Fig. 5. Scatter plot of the instrument reading of the first peak of mannitol at various concentration of mannitol in the sample. Each line represent the different concentration of fructose. The proximity of the lines together shows that the reading of the first peak of mannitol is independent of the concentration of fructose in the solution.

derivatives of urinary sugars using GC has been a widely used method for analysis of these sugars [5,6]. We previously showed that the adaptation of this approach to capillary column-based separation provides high sensitivity for the detection of lactulose, mannitol, sucrose and sucralose [26]. Upon improvement of our method, the presence of peak distortion in the area of lactulose and mannitol and possibility of interference with commonly used sugars led us to try a different enzymatic and chromatographic techniques. In our hands, the enzymatic digestion of lactose using lactase was not a useful tool for separation of lactose and lactulose. Using a new chromatographic method, we were able to separate these sugars in our assay. Meanwhile, we

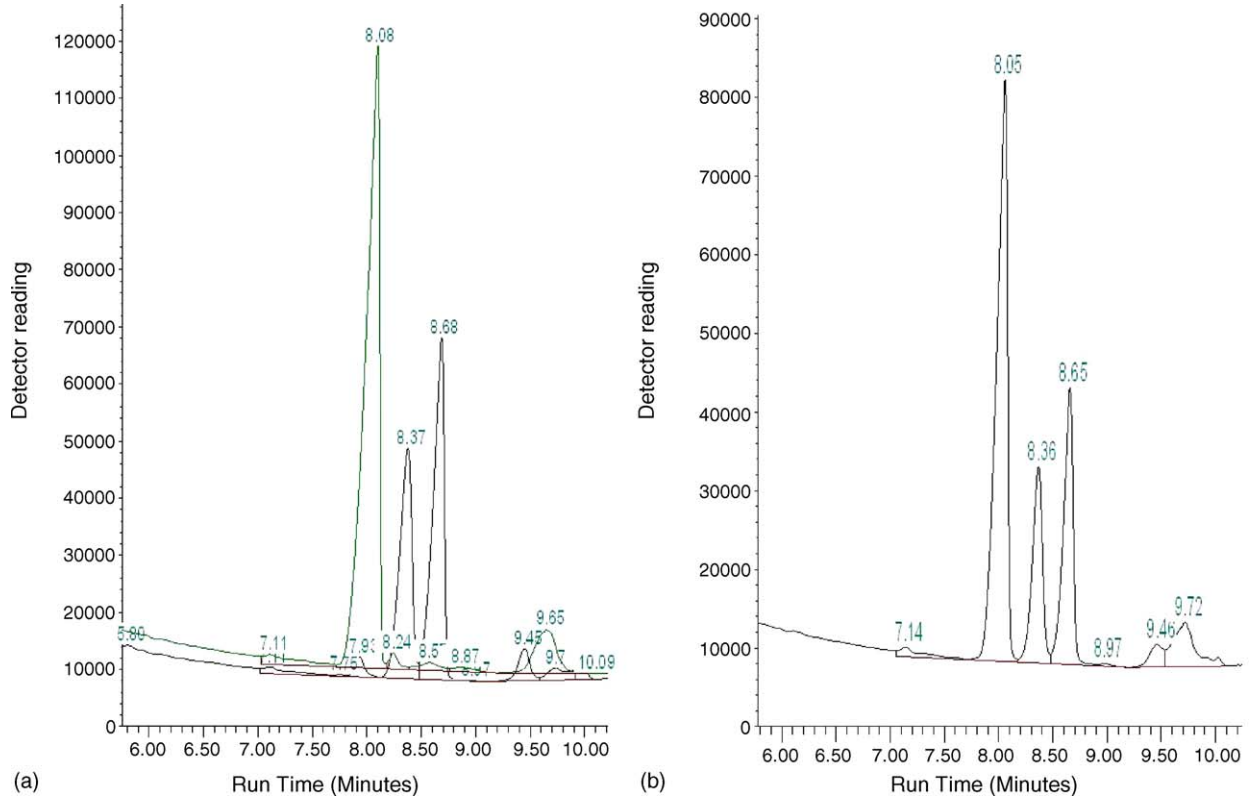

Fig. 4. (a) Composite of two chromatograms showing how the second peak of mannitol (with retention time of 9.65 min) overlap the third peak of fructose curves (with retention time of 9.45). (b) Chromatogram of a sample containing both mannitol and fructose showing the first peak of mannitol separated from fructose /mannitol mix peak and fructose peak.

noted that most of sugars produce multiple peaks. The reason for creation of the multiple peaks is not clear. However, it might be due to production of multiple silyl derivatives of these sugars. As adapted from Bartolozzi, two-step derivitization of organic acids and sugars with methoxamine and silylating reagent replaces the labile protons of hydroxy, carboxyl and phenolic functional groups by trimethylsilyl (TMS) group, while carbonyl groups are converted to methoxime functionalities. This method results in reducing the number of chromatographic peaks arising from different isomers to only two peaks corresponding to syn-oxime and anti-oxime [30,31]. For example, reducing sugars like fructose and glucose may potentially yield up to six peaks upon direct silylation. In our new technique the sensitivity (limit of detection) is still very high and other assay characteristics remained same as compared to our older method.

Prior studies have addressed the separation of lactulose and lactose using enzymatic assay [13] and HPLC [32–35], or mannitol and fructose using anion-exchange chromatography with pulsed amperometric detection [36–39], HPLC [40], gas chromatography [41–43], GC/mass spectrometry [40,44–45] and liquid chromatography [46]. There is no prior report in the literature dealing with the interference of lactulose/lactose and mannitol/fructose in the assessment of intestinal permeability. In fact, this article is the first to address this interference in the measurement of urine sugar for the assessment of intestinal permeability using gas chromatographic method. Using capillary column GC method has the several advantages such as being practical, reproducible, accurate and highly sensitive. Besides, there is a simple and rapid sample preparation method available, and the samples can be stored for later analysis. Using this newer GC method, it was possible for us to measure urinary sugars including lactulose and mannitol accurately, in the presence of urinary commonly used dietary sugars such as lactose and fructose. In addition, the run time with the new method is 22 min, which makes this technique even more practical. This approach should now make it possible for investigators to measure intestinal permeability without needing to be concerned about instituting dietary restrictions to minimize interference.

Acknowledgements

This research was funded in part by the Research Grant 5R01, AA013745-03 of National Institute of Health and National Institute on alcohol abuse and alcoholism.

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