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Direct analysis of mannitol, lactulose and glucose in urine samples by high-performance anion-exchange chromatography with pulse amperometric detection

Clinical evaluation of intestinal permeability in human immunodeficiency virus infection

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Abstract

Clinically, the ratio of lactulose/mannitol excretion in urine after administration of these non-metabolized sugars has been used to evaluate the extent of malabsorption and intestinal permeability disruption in several infections and nutritional diseases, including human immunodeficiency virus (HIV) infection. A range of methodologies have been reported to determine the lactulose/mannitol ratio, including enzymatic assay, gas-liquid chromatography (GC), thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Most published methods involve tedious sample preparations, rendering them unsuitable for routine or automated clinical laboratory testing. We describe in this paper a method in which weak anion-exchange high-performance liquid chromatography in conjunction with a pulsed amperometric detector was used. It requires very simple sample preparation and avoids interference by other components present in the urine. The linear range of determination for mannitol, lactulose and glucose are up to 10 nmol, in a single injection. The limits of detection are 8, 12, 47 and 52 pmol, respectively, for mannitol, glucose, lactose and lactulose. The separation and quantification using this method are highly reproducible, yielding standard errors of less than 2.5% for retention times and less than 3.5% for quantitation. The ratios of lactulose/mannitol recovery in controls and in HIV-infected subjects with and without diarrhea showed striking differences, which are in close agreement with the published results derived with similar HPLC methods.

Keywords: Mannitol; Lactulose; Glucose

1. Introduction

Measurement of the urinary excretion of certain

orally administered nonmetabolized sugar probes provides an excellent noninvasive means of assessing small intestinal surface area, paracellular and trans-cellular permeability and integrity of mucosal barrier

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function, which may be altered by a number of diseases and trauma conditions [1–3]. Recent studies have also linked the deterioration of mucosal barrier function to the progression of human immunodeficiency virus (HIV) infection [4,5]. A useful and widely used indicator of mucosal damage and surface area is provided by the ratio of urinary recovery of the disaccharide lactulose over that of the monosaccharide mannitol following ingestion of standardized amounts of each. It has been theorized that mannitol diffuses through small water-filled pores in the enterocyte cell membrane, reflecting total transcellular transport of basal surface area while lactulose traverses the mucosa by way of intercellular channels between tight junctions, reflecting any disruption in paracellular permeability [6]. Patterns of malabsorption associated with HIV-infected individuals have been demonstrated using the lactulose/mannitol ratio as an index [7].

Chromatographic determination of mono- and polysaccharides has been greatly enhanced with the advent of the methodology of high-performance anion-exchange chromatography in conjunction with electrochemical detection [8–10]. By directly analyzing the sugar molecules without pre- or post column derivatizations, this methodology affords rapid analysis of multisugars with high sensitivity and minimal sample preparation. Some versions of this methodology have been reported successfully applied to evaluate the lactulose/mannitol index in clinical studies [5,7].

Herein we report an improved method to simultaneously separate and evaluate the urinary excretion of multiple sugars including lactulose and mannitol, which requires minimum sample preparation and offers rapid and reproducible assessment of these sugar probes of intestinal barrier functions.

2. Experimental

2.1. Sugars and chemicals

Myo-inositol, D-(+)-glucosamine hydrochloride, sorbitol, mannitol, D-(+)-cellobiose, D-(+)-glucose, melibiose, β -lactose and lactulose were purchased from Sigma as standards for the analysis. Low-

carbonate 50% (v/v) sodium hydroxide from Fisher Scientific was used as eluent for HPLC.

2.2. Specimen collection and handling

All patients were fasted overnight and had a pre-test urine sample collected for the purpose of comparison. A solution containing 5 g of lactulose and 1 g of mannitol in a volume of 20 ml was administered. Urine samples from control and patient subjects were collected up to 5 h after administration. A 1-ml volume of 20% (w/v) chlorohexidine was added to each collection as a preservative regardless of the final total volumes. The total urine volumes from individuals were measured and recorded. After thoroughly mixing, a portion of 5 ml was taken and stored at -20°C until analyzed.

2.3. Equipment

The BioLC carbohydrate analyzer HPLC system, which was composed of a Module GPM-2 gradient pump, an EDM-II eluent degasing module and a PAD-II pulsed amperometric detector with gold working electrode, was from Dionex (Sunnyvale, CA, USA). Also from Dionex was a CarboPac MA-1 anion-exchange column (250 \times 4.0 mm I.D., particle size 8.5 μm , pellicular resin) with associated guard column. Automatic sample injection was performed by a Waters intelligent sample processor (WISP, Model 710B, Waters, Millipore, MA, USA).

2.4. Sample preparation

A 50- μl volume of stored urine sample was mixed with 3.0 ml of deionized water which contained 60 mmol/l melibiose as internal standard. From the diluted sample, a volume of 200 ml was filtered by centrifugation through a 0.22- μm cellulose acetate membrane (Spin-X Centrifuge Filter Unit, Costar, Cambridge, MA, USA).

2.5. Chromatographic conditions for HPLC analysis

High-performance anion-exchange chromatography of the sugar alcohol and disaccharides was carried out on the Dionex BioLC system. Elution of

the sugar alcohols, monosaccharides and disaccharides was achieved with isocratic eluant of 480 mM NaOH at a flow-rate of 0.4 ml/min. Column temperature was ambient. Detection was conducted with the pulsed amperometric detector with a waveform consisting of the following potential–duration profile: sampling=0.15 V, 720 ms; oxidation=0.70 V, 120 ms; reduction=-0.30 V, 360 ms. Output range of the detector was set at 1.0 mA with integration response time of 3 s. A 50- μ l volume of each sample was injected automatically using the WISP. Quantitation of the analytes was performed using the BioAutoIon 450 Data System (Dionex). The amounts of all components of interest in each injection were calibrated against the amount of internal standard, i.e., melibiose, to compensate for the possible variation in injected quantity between runs. Duplicate determinations were performed for each sample and the results were reported as the average.

3. Results and discussion

3.1. Separation and calibration of the saccharides

The fact that mannitol and lactulose belong to two

different categories of saccharide, namely, mono-sugar alcohol and disaccharide respectively, contributes to the difficulties encountered in the development of the separation methods. We tested several columns, such as strong and weak anion-exchange, and strong and weak cation-exchange HPLC columns, and a number of sets of conditions in an attempt to establish a method that is rapid, reproducible and tolerant of the multiple analytes present in a complex matrix such as urine. Here we present the method using a CarboPac MA-1 column. This particular column, with resin packed in a non-metallic column body, is designed for the separation of weakly ionizable analytes in high concentration of sodium hydroxide. A typical separation profile of the sugars tested is shown in Fig. 1. As seen in the chromatogram, the first component of interest, i.e. mannitol, elutes after at least four column volumes, making it possible for the strongly charged electrolytes commonly found in human urine to elute well beforehand and therefore minimizing the possibility of their interference. This is in contrast to many previously published methods in which the major analytes were eluted close to the void volume and hence susceptible to interferences, making pre-cleaning of the samples necessary [5,7].

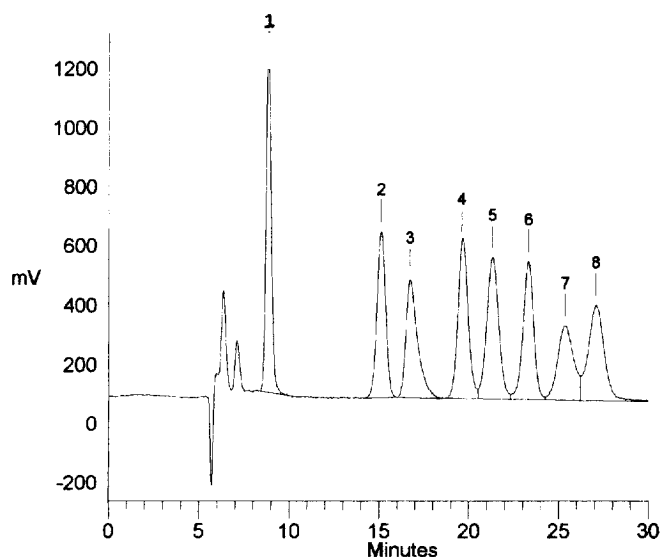


Fig. 1. Chromatogram of the standards. Each numbered peak represented an amount of 3 nmole and their identities are as follows: (1) myo-inositol, (2) glucosamine, (3) sorbitol, (4) mannitol, (5) glucose, (6) melibiose, (7) lactulose, (8) lactose. The chromatographic conditions used are described in the text in Section 2.

Reproducibility of the separation was assessed by making 6 identical injections of 3 nmole of each standard in 50 μ l and then comparing the corresponding retention times and the measured quantities. The results are shown in Table 1. The standard errors for retention times are less than 2.5% while that of quantitation less than 3.5%, although they vary to some extent for the different components.

3.2. Range of linearity and limits of detection

In this method the detection range remained constant throughout the course of separation and in doing so provided a good signal-to-noise ratio. The range of linearity varied among the analytes. However, linearity was maintained up to 10 nmole per injection of all the test standards. A working calibration curve is shown in Fig. 2. The results were the average of duplicate determinations. For myo-inositol, $y=7.069x$ ($r^2=0.9999$, S.D.=0.0706); for glucosamine, $y=6.042x$ ($r^2=0.9999$, S.D.=0.0472); for sorbitol, $y=6.232x$ ($r^2=0.9999$, S.D.=0.0304); for mannitol, $y=6.803x$ ($r^2=0.9998$, S.D.=0.114); for glucose, $y=5.934x$ ($r^2=0.9981$, S.D.=0.319); for

lactulose, $y=4.731x$ ($r^2=0.9985$, S.D.=0.209); for lactose, $y=5.691x$ ($r^2=0.9998$, S.D.=0.105).

The limits of detection for those analytes with this method, defined arbitrarily as a signal-to-noise level greater than 2.0, are shown in Table 2. A build-up of hard-to-remove deposit on the gold surface of the electrode is common with amperometric detectors, especially during the run of a large batch of samples. This phenomenon may gradually reduce the sensitivity of the detection between the electrode cleaning procedures. To compensate for that, a standard solution at an amount of 3 nmole for each analyte was inserted among every 5 samples in the sample batch and calibration was done accordingly.

3.3. Interference

Pre-treatment of the urine samples is required in basically all of the published methods of similar applications, as shown in the literature. The purpose of pre-treating the samples is first of all to remove the components that may co-elute or overlap with the analytes of interest and by doing so to ensure the accuracy of the assay. Secondly, some components,

Table 1
Retention times and integrated amounts of six replicates of standards along with their means and standard deviations

Analytes	Rep1	Rep2	Rep3	Rep4	Rep5	Rep6	Mean	S.D.	R.S.D. (%)
<i>A. Retention times (min)</i>									
Myo-inositol	9.15	8.95	9.11	9.25	9.22	9.00	9.11	0.119	1.3
3-O-MeGlc	14.30	14.10	14.22	14.39	14.30	14.25	14.26	0.097	0.7
Glucosamine	16.00	15.75	16.05	16.12	16.10	16.05	16.01	0.135	0.8
Sorbitol	18.15	18.00	18.22	18.30	18.45	18.16	18.21	0.152	0.8
Mannitol	21.53	21.35	21.58	21.75	21.90	21.80	21.65	0.202	0.9
Melibiose	23.15	23.08	23.15	23.26	23.22	23.06	23.15	0.077	0.3
Glucose	25.40	25.13	25.36	25.45	25.60	25.48	25.40	0.157	0.6
Lactulose	27.99	27.69	27.55	28.09	28.05	28.12	27.92	0.216	0.8
Lactose	30.33	30.21	31.00	30.42	30.50	30.25	30.45	0.289	0.9
<i>B. Integrated amounts (pmol)</i>									
Myo-inositol	3000	3018	3056	3033	2998	3155	3043	58.8	1.9
3-O-MeGlc	3000	3153	2897	3123	3086	3123	3064	97.3	3.2
Glucosamine	3000	3056	2866	3187	3048	3056	3036	103.9	3.4
Sorbitol	3000	2985	2989	3096	3102	3058	3038	53.9	1.8
Mannitol	3000	2987	2879	3048	3052	3008	2996	62.9	2.1
Melibiose	3000	2899	2981	3107	2987	3018	2999	67.1	2.2
Glucose	3000	2969	3054	3025	3016	3089	3026	41.9	1.4
Lactulose	3000	3089	3102	3182	3113	3042	3088	62.5	2.0
Lactose	3000	3012	3058	3108	3123	3057	3060	49.4	1.6

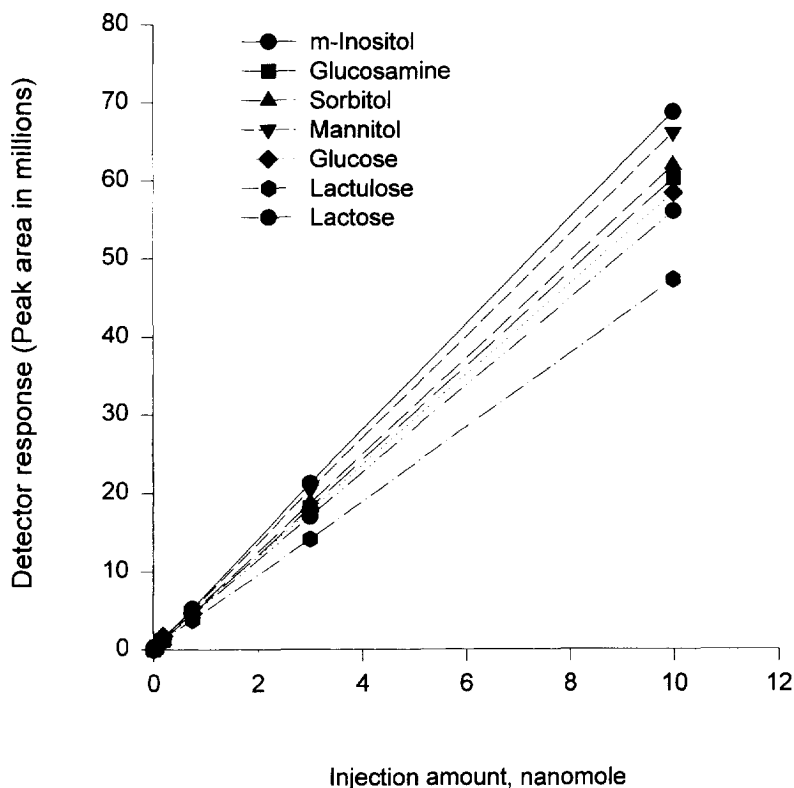


Fig. 2. Calibration curve of the analytes showing the linear range and the order of response factors. The statistical data is presented in the text.

although not interfering with the separation per se, may present problems to the lifespan of the columns and guard columns. In other cases, some components

Table 2
Detection limits of the analytes and minimum concentration of the analytes in the injection solution to be quantified

	Detection limits (pmol) ^a	Minimum concentration (mol/l)
Myo-inositol	5	0.10
3-O-MeGlc	12	0.24
Glucosamine	12	0.24
Sorbitol	12	0.24
Mannitol	8	0.16
Melibiose	12	0.24
Glucose	12	0.24
Lactulose	52	1.04
Lactose	47	0.94

^a Defined as having a signal-to-noise ratio equal to or greater than 2.

may poison the detector if present in prevailing amounts. With this method, it was found that the pre-administration urine samples from the three categories of subjects, i.e. healthy control, HIV-positive without diarrhea, and HIV-positive with diarrhea, produced no significant peaks co-eluting with the analytes of interest, i.e. mannitol and lactulose. A chromatogram containing the urine profiles from the three types is shown in Fig. 3. Thus we eliminated the pretreatment procedures, which is counter-productive and presents a potential obstacle for automation of the assay. In addition, it was observed in a related control study that the preservative used in the urine samples, chlorohexidine, did not interfere with the separation or quantitation even at a two-fold higher concentration than the most concentrated urine sample in the lot.

A considerable decrease of resolution of the CarboPac MA-1 column was observed after 100–200 hundred injections of urine samples. However, the

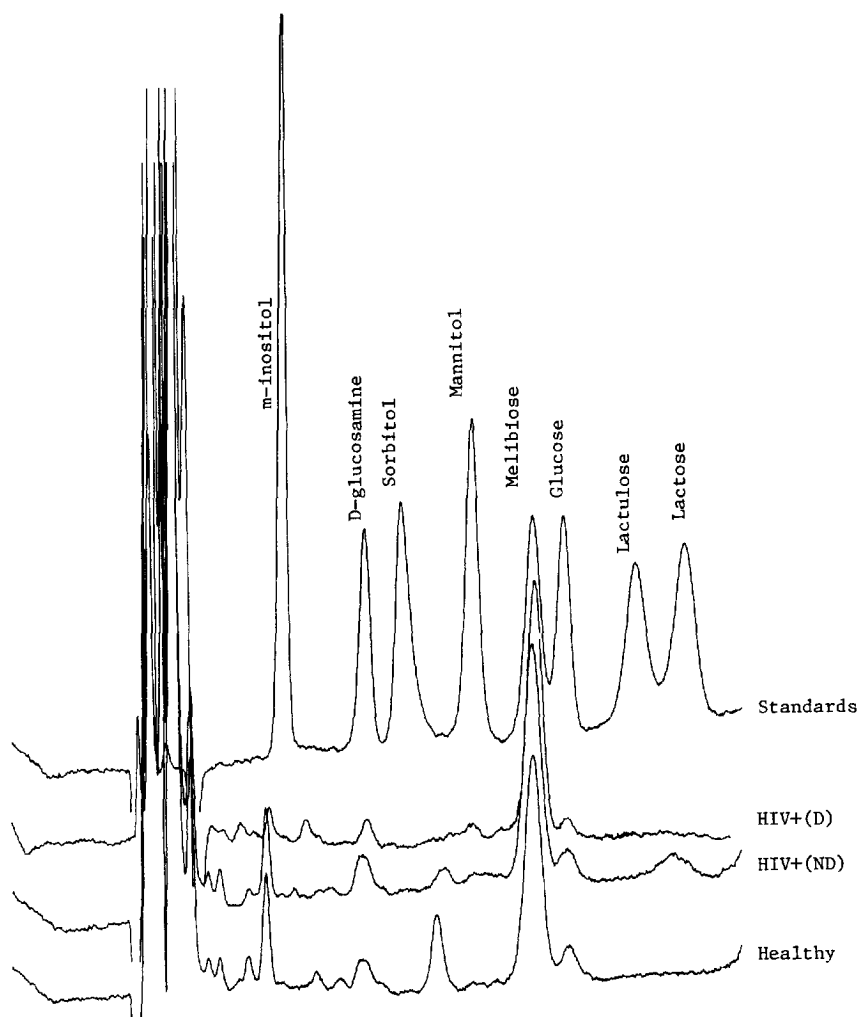


Fig. 3. Chromatographic profiles of pre-administration urine samples from three different subjects: (a) a healthy subject, (b) an HIV-positive subject without diarrhea, and (c) an HIV-positive subject with diarrhea. The volume of each urine sample injected was $0.33 \mu\text{l}$. The upper trace shows the standards, as indicated, at an amount of 3 nmole.

resolving power was restored by cleaning the column with $0.6 M$ sodium hydroxide– $1.2 M$ sodium acetate at 0.3 ml/min for 16 h and then equilibrating with the separation eluant.

3.4. Validation of the test results in clinical disease

Numerous enteric infections may disrupt the intestinal mucosa and compromise the integrity of the cell membranes. The intestinal mucosa has been found to be highly susceptible in HIV-infected patients and diarrhea often occurs as the disease

progresses [4,5]. The lactulose/mannitol recovery ratio, a noninvasive assay conventionally used as an indicator for the assessment of the permeability of the gastrointestinal tract, may therefore reflect the progress of the HIV infection. Preliminary results from a clinical study of lactulose/mannitol recovery in HIV-infected subjects with and without diarrhea are illustrated in Fig. 4. As shown in the figure, the ratio of recovery with lactulose over mannitol (L/M) is greatly increased in the patient with diarrhea ($L/M=0.187 \pm 0.050$, $n=19$, $p<0.05$) compared to that found in the HIV-infected patient without diarrhea ($L/M=0.050 \pm 0.0063$, $n=19$, $p<0.001$). The

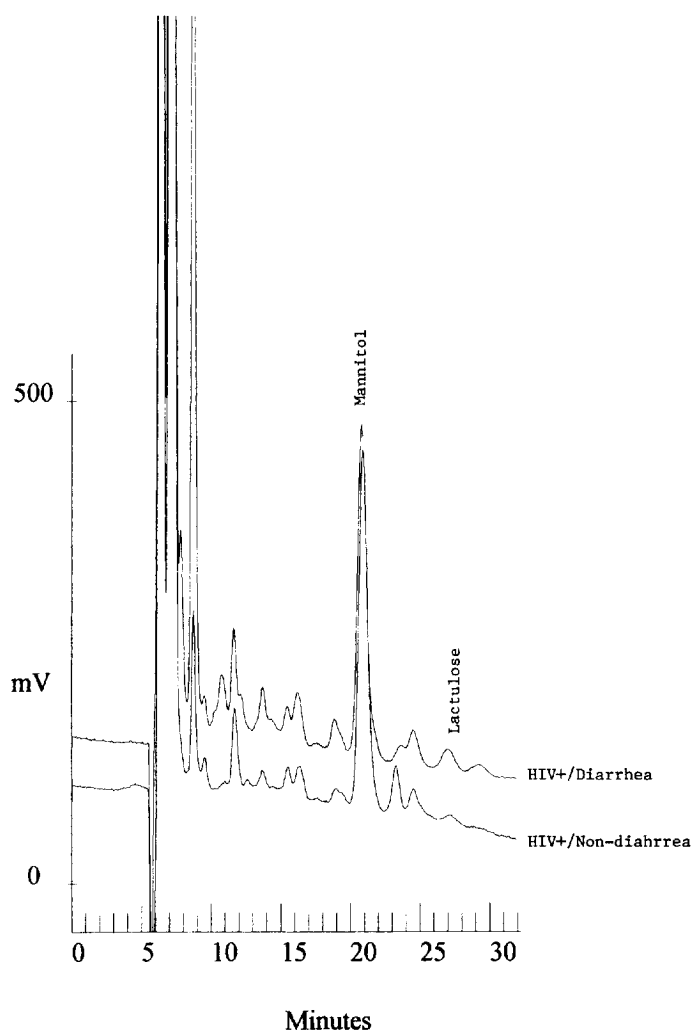


Fig. 4. Comparison of the sugar secretion profile between two typical HIV-positive subjects with and without symptomatic diarrhea. Note that the lactulose/mannitol recovery ratios are 0.179 and 0.0653 respectively for the diarrhea and the non-diarrhea.

study also included a group of healthy subjects for comparison. The L/M ratio for this group was $L/M = 0.0146 + 0.0036$ ($n=8$, $p < 0.05$). These findings are in close agreement with those published elsewhere [3–5,7].

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