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Analysis of multiple sugar probes in urine and plasma by high-performance anion-exchange chromatography with pulsed electrochemical detection

Application in the assessment of intestinal permeability in human immunodeficiency virus infection

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ABSTRACT

Mannitol, 3-O-methylglucose and lactulose administered orally are used to investigate small intestinal absorption pathways and mucosal integrity. Current methods of analysis include thin-layer chromatography, gas chromatography (GC) and enzymatic analysis, which require separate estimation of mono- and disaccharides and for GC, prior derivatization. We describe a high-pressure anion-exchange chromatographic method coupled with pulsed electrochemical detection allowing simultaneous measurement of all three sugars and its clinical application in monitoring intestinal damage in human immunodeficiency virus (HIV) infection. Sample preparation is simple and fast. All sugars are resolved within 10 min. Mean recovery is 93.3% for all sugars and the overall relative standard deviation is 4.2%. Intestinal permeability (lactulose/mannitol ratio) rises with disease progression to AIDS, indicating mucosal damage. The greatest increase in permeability is associated with chronic diarrhoea. The method is an ideal non-invasive test to assess gut mucosal damage in HIV infection.

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INTRODUCTION

The measurement of the urinary excretion of orally administered non-metabolized sugar probe molecules has been used to assess the permeability of small bowel mucosa in a variety of intestinal diseases [1–3]. Several probe molecules, including mannitol, 3-O-methylglucose and lactulose, have been used to assess active and passive transport in the small intestine.

Mannitol, a low-molecular-weight sugar alcohol, is thought to diffuse through water-filled pores in the enterocyte membrane. Lactulose, a synthetic disaccharide, crosses the mucosa via the intercellular tight junctions. 3-O-Methylglucose is transported in a similar manner to glucose and, therefore, acts as a marker of active intestinal transport [4]. Expressing the ratio of these sugars in a timed urine sample provides an index of intestinal permeability [1].

The difficulty in quantifying urinary and plasma sugars has limited the widespread use of these tests. The introduction of high-performance liquid chromatography (HPLC) with pulsed amperometric detection had enabled the rapid and simultaneous determination of lactulose and mannitol in urine [5]. We have further developed this method to allow simultaneous measurement of mannitol, 3-O-methylglucose and lactulose both in plasma and urine, for use in intestinal permeability studies.

We also report the clinical application of such permeability testing in subjects with human immunodeficiency virus (HIV) infection.

EXPERIMENTAL

Sugars

Lactulose (4-O-D-galactopyranosyl-D-fructofuranose) and 3-O-methylglucose (3-O-methyl-α-D-glucopyranose) were supplied by Sigma (Poole, UK). Mannitol and melibiose (6-O-α-D-galactopyranosyl-D-glycopyranose) were supplied by BDH (Poole, UK).

Chemicals

Sodium hydroxide (50%, w/v), zinc acetate (anhydrous), 5-sulphosalicylic acid, Amberlite resins IR-120 H^+ and IRA-400 Cl^- were obtained from BDH. Deionized water (18 MΩ/cm) was produced with an in-house deionizer (Elga, High Wycombe, UK).

Equipment

A quarternary gradient HPLC system with pulsed electrochemical detector was supplied by Dionex UK (Camberley, UK). A high-performance anion-exchange column (Carbopac PA-1; 250 mm × 40 mm I.D.) with associated guard column was also supplied by Dionex UK.

Eluent preparation

Deionized water (989.4 ml) was degassed with helium for 20 min. A 1-ml volume of 0.5 mol/l zinc acetate solution was added and mixed. A 9.6 ml volume of 50% (w/v) sodium hydroxide was added with degassing for a further 10 min.

Sample preparation

Urine. Depending on collection volume, samples were diluted between 1:10 and 1:40 with deionized water to a volume of 1 ml. This dilution was performed on an aliquot of the neat frozen urine. A 1-ml volume of internal standard (melibiose, 250 mg/l) was added and the mixture de-salted with hydrogen ion-exchange resin (Amberlite IR-120 H^+) occupying up to a third of the total volume.

Plasma. A 200-μl volume of plasma was added to 200 μl of internal standard (melibiose, 250 mg/l) and mixed. To this, 200 μl of ice-cold 5-sulphosalicylic (35 g/l) was added to precipitate plasma proteins, and the samples were left on ice for 20 min. Samples were centrifuged at 9000 *g* for 5 min and the supernatant removed. Supernatant was mixed with ion-exchange resin (IR-120 H^+ and IRA-400 Cl^- in mass ratio 1:1.5) and centrifuged again.

HPLC analysis

Supernatant (25 μl) was injected onto the column. For urine analysis, samples were eluted with 120 mmol/l sodium hydroxide, 0.5 mmol/l zinc acetate at a flow-rate of 1 ml/min at 20°C. For plasma, analysis samples were eluted with 160 mmol/l sodium hydroxide, 0.675 mmol/l zinc acetate. At the end of each plasma sample run a 4-min wash with 1 *M* sodium hydroxide was employed to maintain stability of retention times.

Detection was with a pulsed electrochemical detector, with a detection potential of −0.01 V (0–0.5

s), an oxidation potential of +0.75 V (0.51–0.64 s) and a reduction potential of −0.75 V (0.65–0.75 s) with an integration period of 0.05–0.5 s. Peak heights or peak areas were measured with internal standardization.

Subjects

Controls. Nineteen healthy caucasians (twelve males and seven females), age range 21–39 years, with no history of gastrointestinal disease acted as control subjects.

HIV group. Forty HIV antibody-positive males living in London were recruited prospectively to the study, after giving informed consent. The HIV clinical status was: AIDS, 26; AIDS-related complex (ARC), 7; asymptomatic, 7. Of the 33 subjects with AIDS or ARC 15 had chronic diarrhoea.

Protocol

All subjects were fasted overnight, being allowed only water to drink. On the morning of the test, subjects provided a pre-test urine sample. They then drank a solution containing 10 g of lactulose and 5 g of mannitol dissolved in 50 ml of water (osmolality, 1350 mmol/kg). Urine was collected over 6 h into a container with 1 ml of 20% (w/v) chlorhexidine as preservative.

The total urine volume was recorded on completion of the test, and a 40-ml aliquot was stored at −20°C until analysis. Subjects were encouraged to drink fluids freely after the first hour of the test to maintain an adequate urine output. All subjects avoided ingesting alcohol or non-steroidal anti-inflammatory drugs for at least 12 h prior to the test.

RESULTS

Linearity

The linearity of the method was assessed by adding known amounts of analytes to urine and plasma against a fixed concentration of internal standard. The method was linear for each sugar to the following concentrations: mannitol, 125 mg/l; 3-O-methylglucose, 300 mg/l; and lactulose, 40 mg/l. Standard curves are represented by the following equations: mannitol, $y = 0.0062x + 0.0185$ (standard error of the estimate, S.E.E., 0.035); 3-O-methylglucose, $y = 0.0021x + 0.0001$ (S.E.E., 0.005); and lactulose, $y = 0.0014x + 0.002$ (S.E.E. 0.051).

Detection limit

Lactulose was detectable to levels of 0.4 mg/l in urine and plasma.

Recovery

The analytical recovery was determined by adding known amounts of sugars to urine and plasma and comparing the concentration measured with the amount added. The results are shown in Table I. Recovery ranged between 89 and 105% for all analytes, at various concentrations.

Precision

The imprecision of the method was determined by the repeated measurement of sugars in test urine and plasma samples and the results are summarised in Table II. Overall relative standard deviations ranged from 1.8 to 8.5%.

Interference

No other peaks were found in urine with the same retention times as the probe markers, and all sugars were well resolved within 10 min. In plasma, the eluent conditions had to be changed slightly to avoid interference from an unidentified peak that sometimes co-eluted with lactulose. An increase in eluent concentration from 120 to 160 mmol/l sodium hydroxide resolved the two peaks as shown in Fig. 1.

Subjects infected with HIV

The lactulose/mannitol ratio (mean ± 2 S.D.) was higher in subjects with AIDS (0.064 ± 0.014, $p = 0.03$) and in subjects with ARC (0.042 ± 0.013, $p = 0.03$) than in asymptomatic HIV antibody subjects (0.027 ± 0.004) and control subjects 0.024 ± 0.002). In addition HIV-infected subjects with diarrhoea had a higher lactulose/mannitol ratio (0.072 ± 0.017, $p = 0.001$) than HIV-infected individuals without diarrhoea (0.034 ± 0.006). There was no significant difference between asymptomatic HIV antibody-positive subjects and control subjects. Thus, changes in intestinal permeability were only detected in patients with AIDS and ARC particularly in the presence of diarrhoea. Mannitol absorption in subjects with AIDS (11.33 ± 1.02%) and subjects with ARC (8.84 ± 2.6%) and asymptomatic HIV-positive subjects (9.71 ± 2.34%) was not different from that of control subjects (11.4 ±

TABLE I

ANALYTICAL RECOVERIES OF MANNITOL, 3-O-METHYLGLUCOSE AND LACTULOSE FROM URINE AND PLASMA SAMPLES

Matrix	Amount added (mg/l)	Measured concentration[a] (mg/l)	Recovery[a] (%)
Mannitol			
Urine	31.25	28.5 ± 0.2	91.3 ± 0.7
Plasma	31.25	28.9 ± 0.3	92.3 ± 0.8
Urine	125.0	111.4 ± 1.4	89.1 ± 1.1
Plasma	125.0	118.6 ± 1.5	94.9 ± 1.2
3-O-Methylglucose			
Urine	75.0	66.8 ± 0.7	89.1 ± 0.9
Plasma	75.0	77.6 ± 1.2	103.4 ± 1.5
Urine	150.0	149.21 ± 4.6	99.5 ± 3.1
Plasma	150.0	145.6 ± 5.2	97.1 ± 3.6
Lactulose			
Urine	6.25	6.1 ± 0.4	97.6 ± 6.8
Plasma	6.25	6.6 ± 0.6	105.4 ± 9.1
Urine	12.5	12.1 ± 0.7	97.1 ± 6.0
Plasma	12.5	12.0 ± 0.9	95.7 ± 7.5
Melibiose			
Urine	250.0	244.9 ± 2.7	98.0 ± 1.1
Plasma	250.0	248.6 ± 3.3	99.4 ± 1.3

[a] Mean ± 2 S.D.

0.98%). Similarly, there was no significant difference in lactulose permeation between all the groups. Only the lactulose/mannitol ratio showed significant differences between the groups, and this clearly demonstrates the discriminative value of expressing intestinal permeability as a ratio of two markers.

DISCUSSION

High-performance anion-exchange chromatography coupled with pulsed electrochemical detection overcomes many of the problems previously encountered in the analysis of carbohydrates in low

TABLE II

IMPRECISION OF ESTIMATION OF MANNITOL, 3-O-METHYLGLUCOSE AND LACTULOSE IN URINE AND PLASMA FROM A CONTROL SUBJECT

Analyte	Matrix	Within batch (mean ± S.D., n = 10)		Overall (mean ± S.D., n = 20)	
		Concentration (mg/ml)	R.S.D. (%)	Concentration (mg/ml)	R.S.D. (%)
Mannitol	Urine	104.3 ± 0.42	0.4	107.8 ± 2.7	2.5
	Plasma	28.07 ± 0.193	0.69	29.7 ± 0.95	3.2
3-O-Methylglucose	Urine	226.0 ± 0.90	0.4	222.6 ± 3.9	1.8
	Plasma	29.6 ± 0.13	0.4	30.2 ± 0.8	2.7
Lactulose	Urine	3.08 ± 0.15	4.9	3.23 ± 0.18	5.6
	Plasma	5.35 ± 0.45	8.5	5.25 ± 0.29	6.8

[a] Mean ± S.D. (CV %).

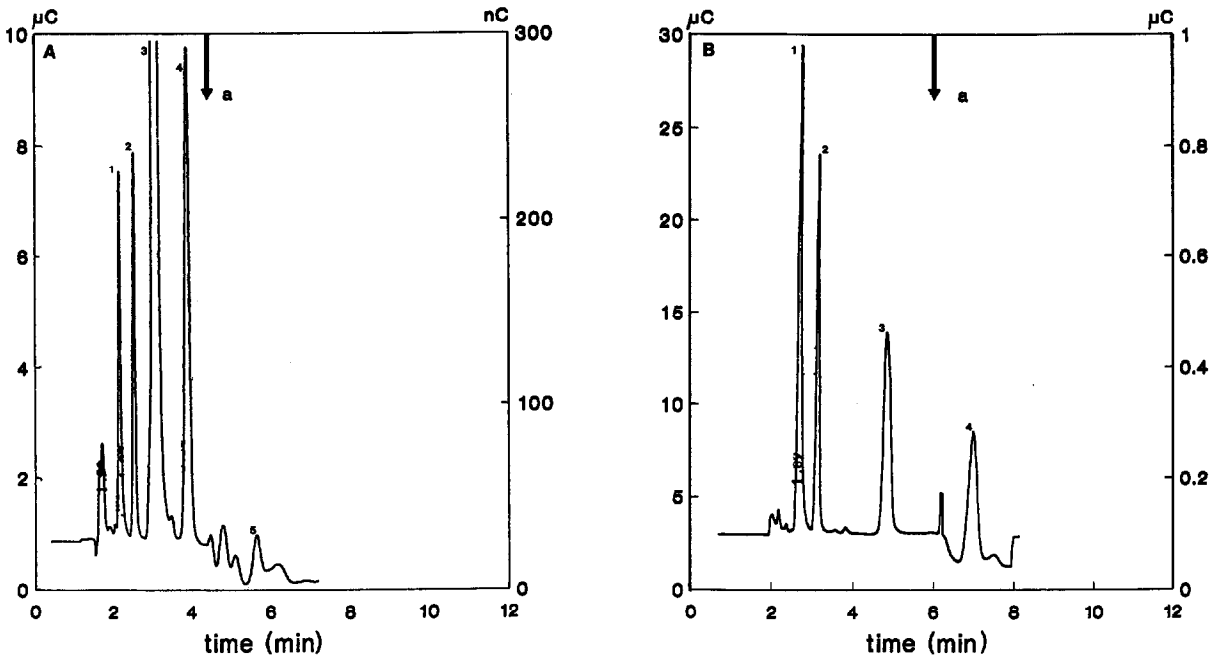

Fig. 1. (A) Chromatographic analysis of a plasma sample 90 min post sugar ingestion in a healthy subject. Eluent: 160 mmol/l sodium hydroxide, 0.675 mmol/l zinc acetate at 1 ml/min. Note sensitivity scale change at a: monosaccharides at 10 μC = disaccharides at 300 nC. Peaks (retention times of sugars in parentheses): 1 = mannitol (2.1 min); 2 = 3-O-methylglucose (2.5 min); 3 = glucose (3.2 min); 4 = melibiose (4.0 min); 5 = lactulose (5.9 min). (B) Chromatographic analysis of a 6-h urine sample post sugar ingestion in a healthy subject. Eluent: 120 mmol/l sodium hydroxide, 0.5 mmol/l zinc acetate at 1 ml/min. Note sensitivity scale change at a: monosaccharides at 30 μC = disaccharides at 1 μC. Peaks (retention times of sugars in parentheses): 1 = mannitol (2.2 min); 2 = 3-O-methylglucose (2.7 min); 3 = melibiose (4.6 min); 4 = lactulose (6.9 min).

concentrations in biological fluids. The method described here is precise, reproducible and highly sensitive. The inclusion of zinc acetate in the eluent effects separation of lactulose and lactose, a potential problem in the paediatric age group. Sample preparation is straightforward and fast for both urine and plasma samples. Analysis time is short with all the sugar probes being resolved within 10 min, enabling a large number of samples to be analyzed within one day.

The use of non-metabolized sugar probes in intestinal permeability testing is well recognized [6]. Such a test is a convenient, non-hazardous method of assessing small intestinal absorption pathways and mucosal integrity in HIV-infected subjects. The results presented here demonstrate altered small intestinal permeability in male subjects infected with HIV. Diarrhoea appears to be the major clinical correlate with intestinal mucosal damage, although an increase in permeability is seen with disease progression to AIDS.

The lactulose/mannitol test has potential application in HIV disease, and its longitudinal use may be helpful in the monitoring of progression of HIV-related intestinal damage.

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