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The electroanalysis of mannitol, xylose and lactulose at copper electrodes: voltammetric studies and bioanalysis in human urine by means of HPLC with electrochemical detection[1]

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Abstract

The electrochemical behavior of mannitol, xylose and lactulose has been investigated at a copper working electrode. A sensitive, accurate and precise method employing HPLC with electrochemical detection in the d.c. amperometric mode, has been developed and validated for the determination of mannitol and lactulose in human urine. The ratio of these probe carbohydrates is altered in conditions that cause damage to the intestinal mucosal barrier. Systematic studies employing cyclic voltammetry indicate that the electrode reaction involves an electrocatalytic oxidation of each carbohydrate in a process yielding a single irreversible anodic wave that is dependent on the ionic strength of the sodium hydroxide supporting electrolyte solution. High performance liquid chromatography with electrochemical detection was performed using a thin-layer cell housing a custom manufactured copper working electrode. The optimized HPLC method can detect 72, 57 and 419 pg of mannitol, xylose and lactulose injected on column, respectively. The corresponding linear calibration ranges are 359 pg–2.24 μg, 57.4 pg–896 ng and 419 pg–262 ng, respectively. Solid-phase extraction of human urine on polar sorbents, and direct injection after simple 1 + 99 dilution in 0.025 M NaOH were compared for bioanalysis. Direct injection was selected for further method development as the technique proved robust and simple. The optimized method was validated for the determination of mannitol and lactulose in human urine over the concentration ranges predicted when assessing intestinal permeability (0.25–2.5 mg ml^{-1} mannitol and 0.05–1.0 mg ml^{-1} lactulose). Over these ranges intra- and inter-assay bias is $< \pm 6.5\%$, and imprecision (coefficient of variation) is $< 9\%$ for each carbohydrate. The validated method provides a useful alternative to HPLC with pulsed-amperometric detection at gold electrodes.

Keywords: Mannitol; Xylose; Lactulose; Copper electrode; Cyclic voltammetry; HPLC; Electrochemical detection; Human urine; Intestinal permeability

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1. Introduction

Determining the ratio of mannitol and lactulose excreted in fasting urine following an oral dose provides a valuable non-invasive way of assessing the permeability of the intestinal mucosal barrier. This adsorption model has proved useful for assessing intestinal permeability in patients with HIV infection [1,2], pediatric intestinal diseases [3], Coeliac's disease [4,5] and Crohn's disease [3,6] and for assessing intestinal damage following treatment with non-steroidal anti-inflammatory drugs [6]. The model has also proved useful in adsorption studies using laboratory rodents [7,8].

The bioanalysis of mannitol and lactulose in urine has proved challenging and numerous authors report that the lack of reliable methods has limited application of the model [3–5]. Several analytical techniques have been applied to the determination of mannitol and lactulose; including, HPLC with either pulsed amperometric (PAD)[9] or refractive index (RI) [5,8,10] detection, or gas-liquid chromatography [11–13]. Methods employing HPLC with pulsed-amperometric detection at gold electrodes have been successfully employed for clinical studies; [1,14–16] however, the pulsed-waveform generator is not available on all electrochemical detectors. We report the development of an alternative method to PAD, using HPLC-EC with conventional d.c. amperometry that can be performed with a standard electrochemical detector. The method employs a copper working electrode, and its performance has been determined for the bioanalysis of mannitol and lactulose in human urine.

Baldwin's research group have demonstrated that copper electrodes are useful for the determination of carbohydrates [17–19] amino acids [20] and peptides [19] using capillary electrophoresis. They also used HPLC with copper electrodes for the determination of sugars in food products [21] and underivatized polypeptides and proteins [22,23]. These reports were complemented by mechanistic studies that are referred to elsewhere in this article.

Our investigations were performed to examine the utility and robustness of copper electrodes for the bioanalysis of carbohydrates in human urine. We present results from systematic studies employing cyclic and hydrodynamic voltammetry to characterize the electrochemical behavior of mannitol, xylose and lactulose at a copper working electrode, and to optimize solution, instrumental and chromatographic conditions for their determination by anion-exchange HPLC-EC. Sample preparation of human urine was performed by either simple dilution followed by direct injection, or following purification by means of solid phase extraction on polar sorbents. Xylose was studied as a possible internal standard for the solid-phase extraction procedure. The accuracy and precision of the optimized bioanalytical method was determined to assess robustness for the bioanalysis of urine.

2. Experimental

2.1. Materials

All chemicals were of analytical-reagent grade. D-Mannitol, D(+) xylose and lactulose were purchased from Sigma. Solutions of supporting electrolyte were prepared immediately before use by adding an appropriate volume of stock 50% (m/v) sodium hydroxide (J.T. Baker) to deionized water. Mobile phase solutions were sparged continually with helium during HPLC; supporting electrolyte solutions were not de-aerated for cyclic voltammetry. Deionized water (>18 MΩ cm) was obtained from a Millipore Milli-Q system. Bond-Elute solid-phase extraction cartridges were obtained from Varian.

2.2. Apparatus

Cyclic voltammetry was performed with either a BAS CV27 and BAS x-y flat bed recorder or with an Eco-Chemie PGSTAT10 potentiostat interfaced to either a 4DX33 or 4DX66 personal computer (PC). A three-electrode cell was employed incorporating a planar copper electrode (BAS, 3.1 mm diameter), an Ag/AgCl reference electrode (BAS, model RE-5) and a platinum wire counter electrode (BAS). Analyses were performed in a polystyrene cell held in a BAS C-1 stand.

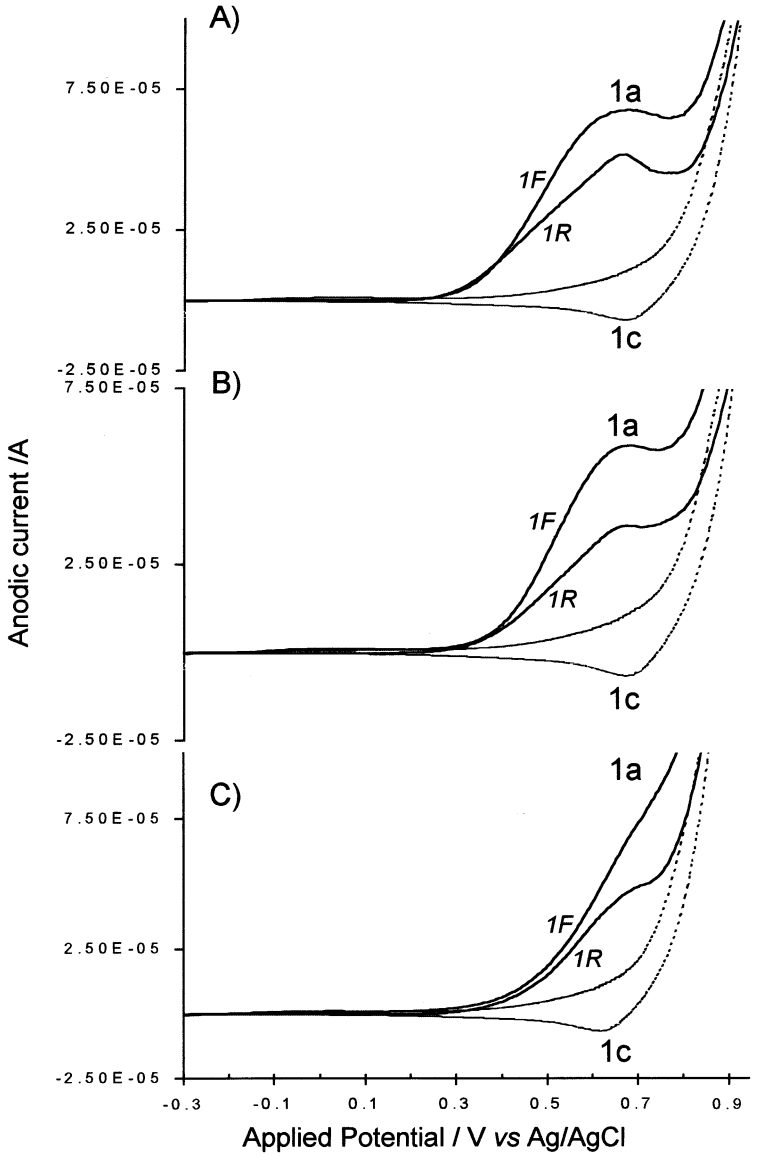

Fig. 1. Cyclic voltammograms for: (A) 0.22 mg ml^{-1} (1.2 mM) mannitol, (B) 0.16 mg ml^{-1} (1.1 mM) xylose, (C) 0.42 mg ml^{-1} (1.2 mM) lactulose at a copper working electrode, and (D) blank 0.1 M NaOH at a freshly polished copper electrode. The solid lines represent voltammograms for the carbohydrates; the broken line (A–C) for blank 0.1 M NaOH supporting electrolyte solution. Scan rate, 50mV s^{-1}. 1F and 1R refer to the first forward and first reverse scans, respectively.

HPLC with electrochemical detection was performed using a Hewlett Packard HP1090 chromatograph controlled using the HP ChemStation data acquisition and reduction software (Rev. A.03.03) installed on an HP Vectra 4DX66 PC. Chromatography was performed on either a Dionex Carbopac PA-100 or PA-10 analytical column (250 × 4 mm) protected by a Dionex Carbopac PA-100 or amino trap guard column, respectively. Mobile phase, unless indicated otherwise, was 0.1 M NaOH; flow rate 1.0 ml min^{-1}; sample volume 20μl. Amperometric detec-

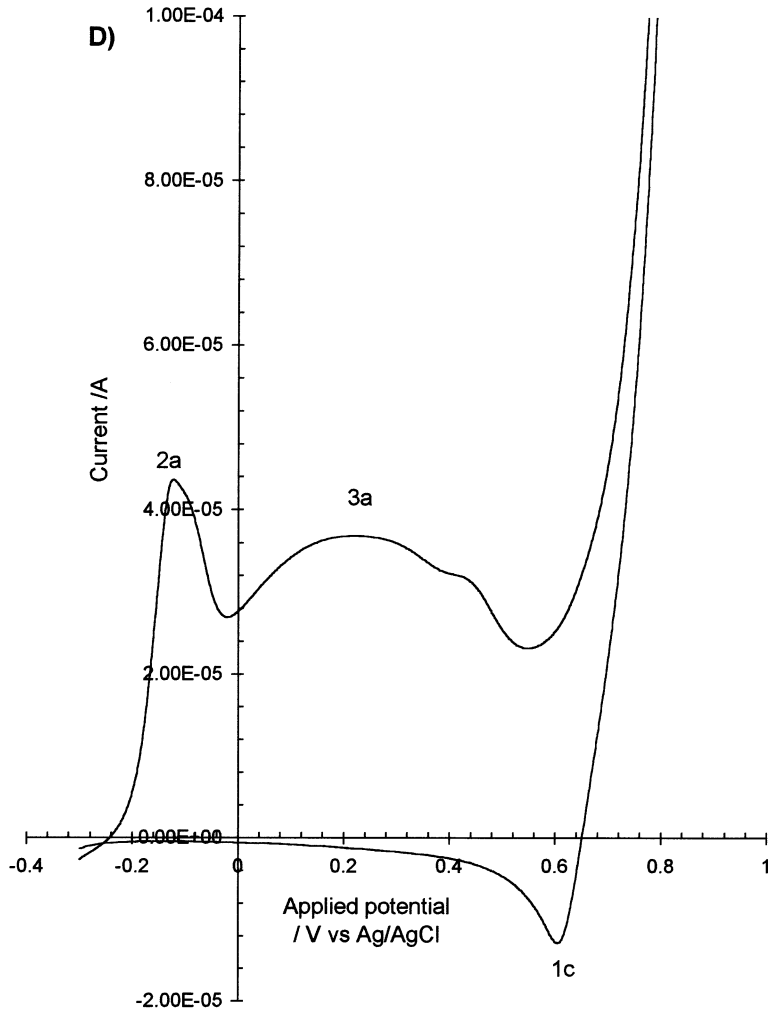

Fig. 1. (*Continued*)

tion was performed with an ESA Coulochem 2 detector equipped with a 5040 thin-layer cell housing a custom manufactured copper working electrode kindly donated by ESA. The copper electrode was polished weekly as part of a routine maintenance schedule.

2.3. Voltammetric procedures

2.3.1. Cyclic voltammetry

Cyclic voltammetry (CV) was performed on solutions containing either NaOH supporting electrolyte or the same solutions containing the indicated concentrations of test carbohydrate. The effects of NaOH concentration were investigated over the concentration range 0.024–0.19 M. The instrumental conditions for cyclic voltammetry were typically: initial potential -0.3 V; scan rate 50 mV s^{-1} and final potential $+0.9$ V. Between successive runs the working electrode was cleaned by washing with methanol, followed by deionized water, and finally dried with tissue paper.

The nature of the electrochemical process was investigated by varying the scan rate used to record cyclic voltammograms for 1.2 mM solutions of each carbohydrate in 0.1 M NaOH.

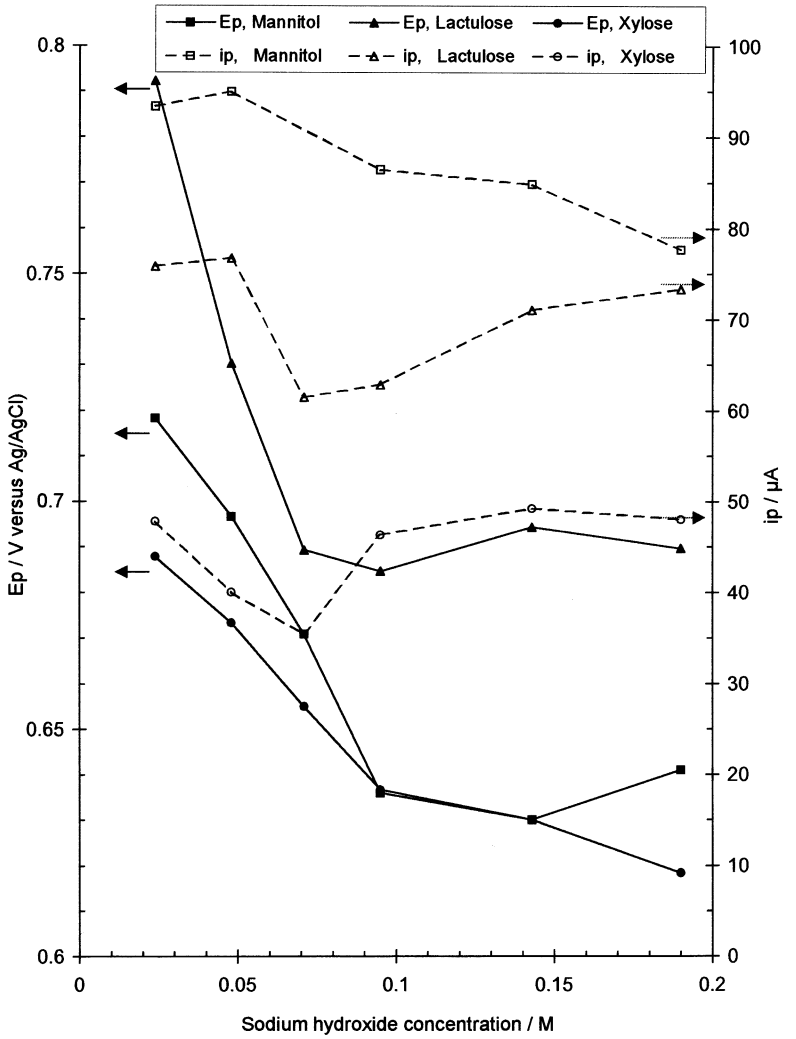

Fig. 2. Effect of sodium hydroxide concentration on peak potential (solid lines) and peak current (broken lines) for 0.22 mg ml^{-1} (1.2 mM) mannitol, 0.16 mg ml^{-1} (1.1 mM) xylose and 0.42 mg ml^{-1} (1.2 mM) lactulose at the copper electrode. Each data point represents the mean of triplicate measurements. The E_p data for lactulose correspond to the minima in plots of Δi_p vs. applied potential and should be considered approximate. Construction of these plots was necessary because the values could not be read directly from the original cyclic voltammograms. Peak current values were measured at the selected E_p potential.

2.3.2. Hydrodynamic voltammetry

Hydrodynamic voltammetry was performed by injecting 20 μl volumes of a mixture containing 2.77 μg ml^{-1} (15.2 μM) mannitol, 2.10 μg ml^{-1} (14.0 μM) xylose and 5.37 μg ml^{-1} (15.7 μM) lactulose onto the HP1090 chromatograph. The mobile phase was 0.1 M NaOH, and the applied potential was increased in 50 mV steps from 0.00 to +0.65 V. Hydrodynamic voltammograms were constructed by plotting the recorded current against applied potential.

2.4. Effects of mobile phase composition and calibration of the HPLC-EC method

The effects of mobile phase ionic strength were investigated over the range 0.005–0.12 M NaOH.

Calibration graphs of peak current versus mass of carbohydrate were constructed over the range 0.574 pg–3.067 μg mannitol, 0.458 pg–2.45 μg xylose and 3.35 pg–17.9 μg lactulose loaded on column; the mobile phase was 0.1 M NaOH.

2.5. Determination of mannitol, xylose and lactulose in human urine by HPLC-EC

Urine samples collected from healthy male and female volunteers after an overnight fast were either injected directly on to the column (after 1 + 99 dilution in 0.025 M NaOH), or subjected to solid-phase extraction (SPE). The general procedure for SPE was as follows: condition with 1 ml of acetonitrile, then add urine sample (10 μl) diluted in 2 ml of acetonitrile; wash with 1 ml of acetonitrile, dry cartridge by drawing air through packing material; then elute with 1 ml of deionized water. Finally, 20 μl of the carbohydrate containing extract were injected on to the column. Aminopropyl, silica, diol, cyanopropyl and C2 sorbents were evaluated for SPE; 100 mg, 1 ml volume cartridges were employed in each instance.

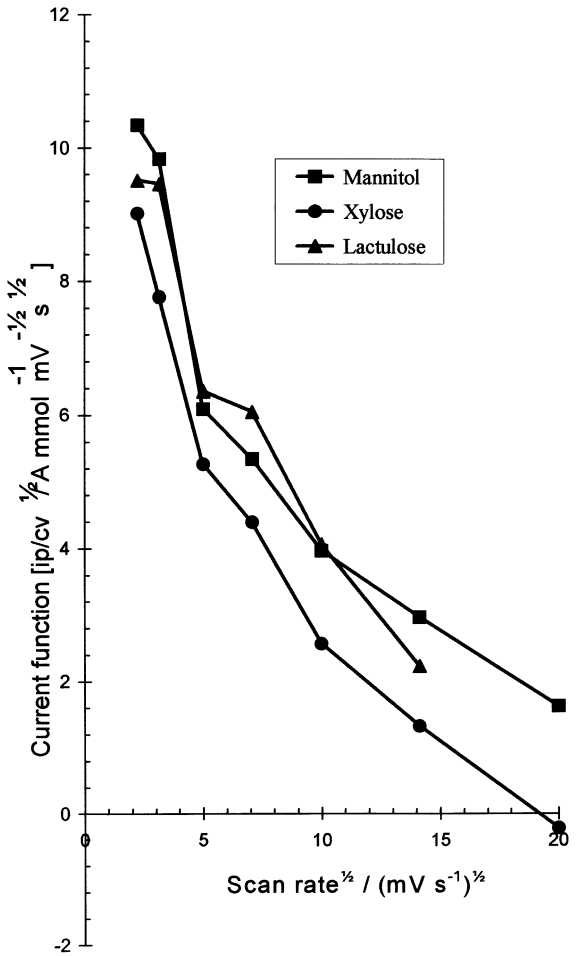

Fig. 3. Current function vs. $v^{1/2}$ for 0.22 mg ml^{-1} (1.2 mM) mannitol, 0.18 mg ml^{-1} (1.2 mM) xylose and 0.43 mg ml^{-1} (1.3 mM) lactulose in 0.1 M NaOH. Each data point is the mean of at least triplicate measurements.

2.6. Accuracy and precision of the HPLC-EC method for mannitol and lactulose in human urine

Method accuracy and precision were determined according to published guidelines [24–26] that establish the performance of a method with regard to its working limits of quantification. Intra- and inter-accuracy and precision of the direct injection method were determined, over six independent assay runs, by repeat analysis of a pool of fasted human urine spiked with mannitol and lactulose. Intra-assay data were obtained by assaying the spiked urine samples as discrete series so that an individual sample at a given concentration was separated by the other concentrations before the next sample at that level was assayed. This way samples at each concentration were spaced throughout the entire run. The concentration ranges studied reflect levels predicted in urines collected during intestinal permeability modeling. Calibration standards (mannitol, 0, 0.25, 0.5, 1.0, 2.0 and 3.0 mg ml^{-1}, lactulose, 0, 0.05, 0.1, 0.25, 0.5 and 1.0 mg ml^{-1}) were assayed before and after each sequence of spiked urine samples; standards were also included after every 10 samples. Over two approximately 11 h runs for mannitol the mean drift in detector response was −16% and +17%, and over a single lactulose run of 14 h the mean drift was −27%. The drift did not appear to be caused by fouling as the original response

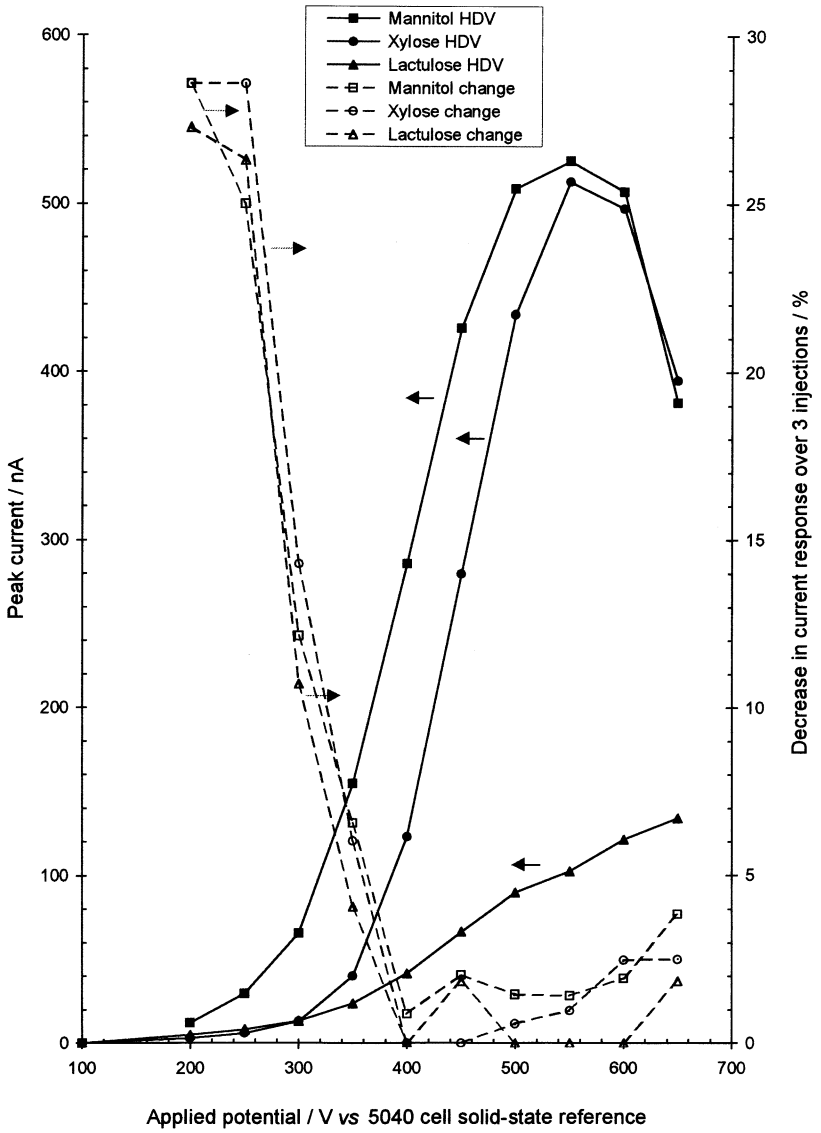

Fig. 4. Hydrodynamic voltammograms and decreases in detector responses for 2.8 μg ml^{-1} (15.2 μM) mannitol, 2.1 μg ml^{-1} (14.0 μM) xylose and 5.37 μg ml^{-1} (15.7 μM) lactulose in 0.1 M NaOH. Data points on the HDVs represent the mean of triplicate measurements.

was restored shortly after completing a batch of analyses. Sample concentrations were interpolated from the response data for adjacent sets of calibration standards. Future method development will focus on the use of a suitable internal standard to simplify calibration.

3. Results and discussion

3.1. Voltammetric behavior of mannitol, xylose and lactulose at a copper electrode

Cyclic voltammograms were recorded at the copper working electrode, for mannitol, xylose

and lactulose solutions prepared in 0.024–0.2 M sodium hydroxide. In all instances the first anodic scan gave one peak (Fig. 1 (A–C), 1a) with no cathodic peaks appearing on the reverse scan; this indicated that the overall process was irreversible. In contrast, the voltammograms for the copper electrode in plain sodium hydroxide solutions revealed a small cathodic wave on the reverse scan (Fig. 1(A–D), 1c) but did not reveal any discernable anodic waves; although several additional anodic peaks were observed for a freshly polished electrode (Fig. 1(D)). These latter waves have been described previously [27–29] and attributed to the formation of Cu(I) (Fig. 1(D), 2a), Cu(II) (Fig. 1(D), 3a) and Cu(III) surface species during the anodic scan, and reduction to Cu(II) (Fig. 1(A–D), 1c) on the cathodic scan. Luo [27] and Marioli [29] have described additional cathodic peaks attributed to the formation of Cu(I) and Cu(0) species at -0.5 and -0.8 V vs. Ag/AgCl respectively, when the scan is extended to -1 V. The CuII/CuIII anodic wave is reported to occur at the same potential as the onset of solvent oxidation and was not observed in our studies [27,29]. We have not elucidated the origin of the small anodic wave at approximately 0.4 V.

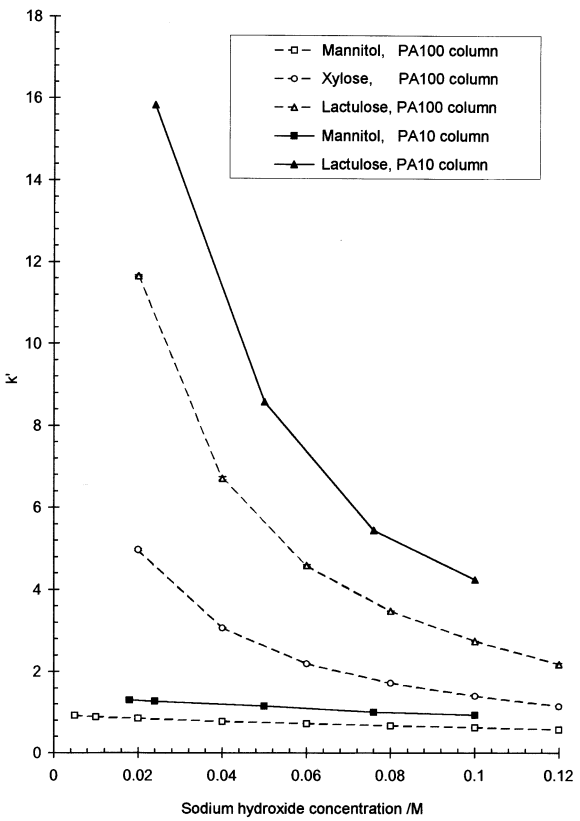

Fig. 5. Effect of mobile phase NaOH concentration on the retention of mannitol, xylose and lactulose on Dionex PA-10 and PA-100 anion exchange columns. Analyte concentrations employed: for the PA-1-0 column, 544 ng ml^{-1} (3.0 μM) mannitol, 432 ng ml^{-1} (2.9 μM) xylose, 3.16 μg ml^{-1} (9.2 μM) lactulose; and for the PA-100 column, 1.39 μg ml^{-1} (7.6 μM) mannitol, 1.06 μg ml^{-1} (7.1 μM) xylose, 2.72 μg ml^{-1} (7.9 μM) lactulose.

The variation in the anodic peak current and peak potential with NaOH concentration is presented in Fig. 2. The magnitude of the current response appeared independent of NaOH concentration. In contrast, a break appears in the E_p vs. concentration graph, with E_p becoming independent of concentration above 0.1 M NaOH. It is unlikely that this break results from deprotonation of carbohydrate hydroxyl groups because the break is observed at the same point for all three carbohydrates while pK_a values for xylose and mannitol are 12.14 and 13.50, respectively. Consequently, the break is more likely to occur owing to mechanisms involving surface copper species.

Increasing sodium hydroxide concentration above 0.1 M, in the absence of carbohydrate, yields marked increases in the magnitude of the wave arising from the formation of Cu(I) species (Fig. 1(D), 2a). This suggests that the break in the E_p vs. NaOH graph corresponds to the hydroxide concentration required for facile surface modification. The anodic Cu(I) wave diminishes in the presence of carbohydrate.

The above observations support the mechanisms proposed previously [27–29] where carbohydrate is considered to absorb onto the surface of the electrode bearing either Cu(I) or (II) oxide/hydroxide species and affects the complete formation of the Cu(I) and Cu(II) layers. Subsequently, absorbed carbohydrate undergoes an electrocatalytic oxidation by a mechanism possibly involving the reduction of a catalytically active Cu(III) species that is generated electrochemically at high hydroxide ion concentrations. The existence of the catalytically active Cu(III) species is supported by the anodic response for each carbohydrate on the

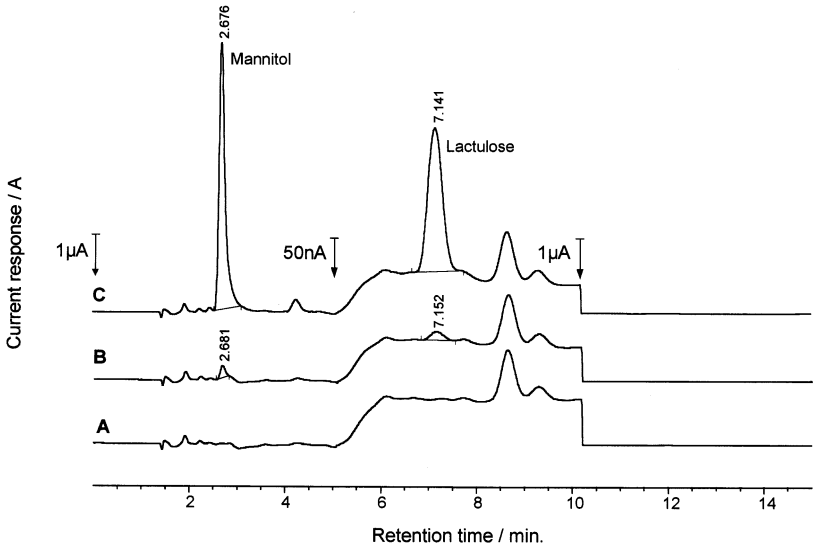

Fig. 6. Chromatograms of (a) control human urine pool, (b) control human urine pool containing 125 μg ml^{-1} mannitol (2.5 μg on col.) and 50 μg ml^{-1} lactulose (1.0 μg on col.) and (c) control human urine pool containing 2.5 mg ml^{-1} mannitol (50 μg on col.) and 1.0 mg ml^{-1} lactulose (20 μg on col.). Urine samples were diluted 1 + 99 in 0.025 M NaOH before analysis. Detector sensitivity, (0–5 min) 10 μA FSD, (5–10 min) 500 nA FSD and (10–15 min) 10 μA FSD.

reverse CV scans. Furthermore, the graph of current function vs. scan $rate^{1/2}$ (Fig. 3) revealed negative slopes for each carbohydrate confirming the electrocatalytic process.

3.2. Hydrodynamic voltammetry and calibration

Hydrodynamic voltammograms (HDVs) were constructed for each carbohydrate (Fig. 4) to identify the optimum potential for electrochemical detection following HPLC. The maximum current responses for mannitol and xylose were achieved at a potential of + 0.55 V, which was used for all subsequent analyses. At higher potentials the current response decreased which is characteristic of an electrocatalytic process [30]. A defined plateau was not observed for the oxidation of lactulose.

Optimizing applied potential also proved important for precision. At low applied potentials (< 400 mV) there was a marked decrease in the magnitude of the peak response over triplicate injections (Fig. 4). The poorest stability was observed at + 0.2 V where peak current decreased by approximately 30% between the first and third injections. Between + 0.4 and + 0.55 V the decrease was less than 2% which is within the imprecision of the method (see below).

The calibration graphs of peak area vs. mass of each carbohydrate were linear over the ranges 359 pg–2.24 μg, 57.4 pg–896 ng and 419 pg–262 ng of mannitol, xylose and lactulose loaded on column, respectively. The corresponding limits of detection were 71.8, 57.4 and 419.1 pg of carbohydrate; (based on a signal to noise ratio of 3:1 and a full scale deflection (FSD) of 20 nA), and the calibration responses were 2.138, 1.467 and 0.301 mA μmol^{-1}.

Kano et al [28] predicted that the electro-oxidation of mannitol and xylose at copper should produce single-carbon products in a process yielding 14 and 10 electrons, respectively. This ratio is supported by our calibration data as the mannitol to xylose current response is 1.46.

3.3. Optimization of chromatographic conditions for the determination of mannitol, xylose and lactulose in human urine

The effect of sodium hydroxide concentration on the capacity factor (k') was studied using the Dionex CarboPac PA-100 and PA-10 columns (Fig. 5). The comparison was performed because

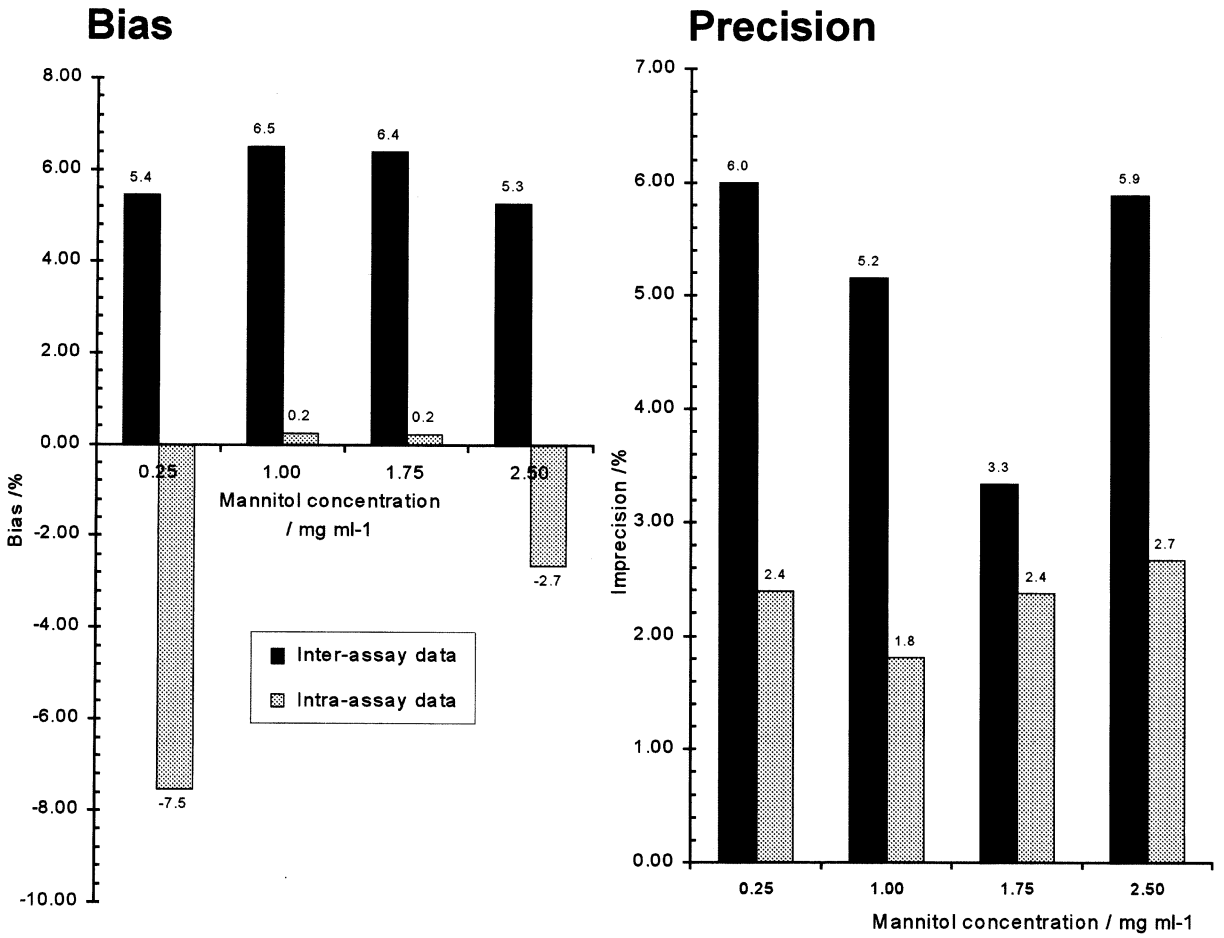

Fig. 7. Summary inter- and intra-assay bias and precision data for the determination of mannitol in human urine; $n = 6$ for each concentration level for inter- and intra-assay studies.

the PA-10 column was a recent development that the manufacturer claimed would afford improved performance for the separation of simple carbohydrates.

As expected, capacity factor was inversely related to OH^- concentration, with the largest effects observed for lactulose and xylose; mannitol retention was not markedly affected. The PA-10 column was selected for further method development because it afforded slightly superior retention of mannitol.

Solid-phase extraction on polar sorbents and direct injection of urine were compared as sample pretreatment techniques for the analysis of human urine. Mannitol and lactulose showed promising recovery from urine on NH_2, CN, diol and Si sorbents; however, xylose was retained only on Si. The amino phase proved unsuitable because the sorbent leached from the cartridge giving rise to a peak that co-eluted with mannitol on the PA-100 column. However, solid-phase extraction was not pursued because the amino-trap and PA-10 columns, in combination, gave robust chromatography free from interference after direct injection of urine diluted 1 + 99 in 0.025 M NaOH (Fig. 6).

3.4. Accuracy and precision of the LCEC method

Published data for intestinal permeability tests in humans indicate that 10.0–16.5% of a 5 g oral

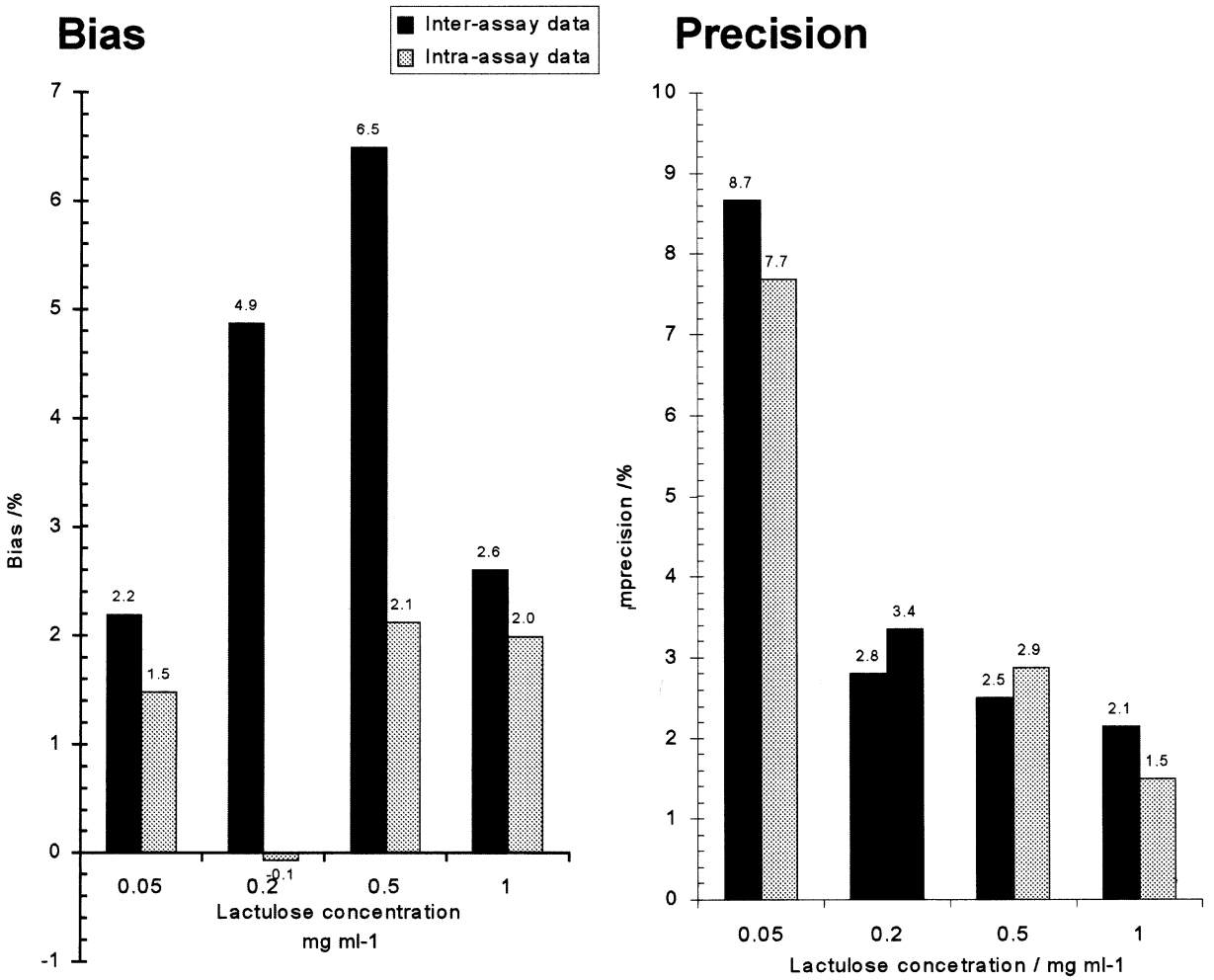

Fig. 8. Summary inter- and intra-assay bias and precision data for the determination of lactulose in human urine; $n = 6$ for each concentration level for inter- and intra-assay studies.

dose of mannitol is excreted in urine over the 5 h period following administration; 0.231–2.5% of a 10 g lactulose dose is collected. We envisage that the urine volume will be in the range 250–500 ml. This indicates that the predicted urinary concentrations of mannitol and lactulose are between 0.25–3.0 and 0.05–1.0 mg ml^{-1}, respectively. Accordingly, urine samples were prepared by spiking aliquots of pooled fasting human urine with 0.25–2.5 mg ml^{-1} mannitol and 0.05–1.0 mg ml^{-1} lactulose. Summary inter- and intra-assay bias and imprecision data for the analysis of these samples are presented in Figs. 7 and 8. Over the concentration ranges studied intra- and inter-assay bias is $< \pm 6.5\%$ and imprecision is $< 9\%$ for each carbohydrate.

Samples should be diluted in either 0.025 M NaOH or pooled control urine, if necessary, to extend the upper concentration range.

4. Conclusions

The systematic voltammetric studies described demonstrate that mannitol, xylose and lactulose are irreversibly oxidized by an electrocatalytic

mechanism at copper electrodes. The electro-oxidation process has been successfully utilized for the development of a sensitive and reliable chromatographic method that has been applied for the bioanalysis of mannitol and lactulose in samples of fasting human urine. The method should prove valuable for supporting clinical studies to determine intestinal permeability.

5. Addendum

A recent report by Vera et al [31] concludes that urinary sucrose excretion is a marker for gastric mucosal damage in children and may be used as a screening test. Preliminary studies indicate that sucrose may also be determined using the method described above.

Acknowledgements

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