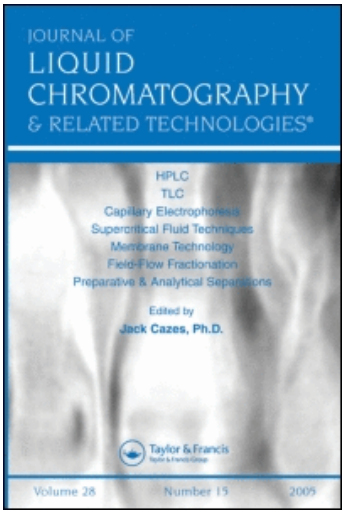

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A Rapid HPLC Method for the Determination of Free Fucose in Urine. A Marker of Malignancy in Children

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A RAPID HPLC METHOD FOR THE DETERMINATION OF FREE FUCOSE IN URINE. A MARKER OF MALIGNANCY IN CHILDREN

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ABSTRACT

The concentration of free fucose in urine has been shown to be elevated in adults with malignancy. Current analytical methods include enzymatic analysis and high pressure liquid chromatography (HPLC) with fluorescence detection. We describe a simple HPLC method using anion exchange chromatography with pulsed electrochemical detection for the determination of free fucose in urine of children with malignancy. Separation of sugars is achieved within 7 minutes. The method is linear to 200 μmol/l fucose with a detection limit of 0.5 μmol/l in urine. Mean recovery was 96.6 % ± 11.8%. Precision was good with coefficient of variation (CV) of 4.3% (25 μmol/l), 3.7% (50 μmol/l) and 4.9% (100 μmol/l). The median fucose : creatinine ratio in healthy children was 13.6 μmol / mmol (5.5 - 128.5). Children with malignancy had a median fucose : creatinine ratio of 8.1 μmol / mmol (2.6 - 66.4) which was significantly lower than the control subjects (p=0.038). The method enables rapid determination of free fucose in urine with minimal sample preparation and without the need for derivatization. Urine free fucose was lower in children with malignancy than in their age-matched controls and requires further investigation.

INTRODUCTION

L-fucose is a monosaccharide found at the non-reducing end of oligosaccharides on glycoproteins and is important in their function. It has been reported that plasma and urinary free fucose concentrations are elevated in patients with diabetes mellitus, cystic fibrosis and a variety of malignancies [1-6]. Increased excretion of fucose has been demonstrated in a wide range of cancers including liver, pancreatic, breast and oesophagus [5,6].

Analytical methods used for the assay of fucose include enzymatic [4,5] and high pressure liquid chromatography [6]. The HPLC method of Suzuki et. al. [6] employs fluorescence detection with the derivatization of fucose with 2-aminopyridine. This requires time-consuming sample preparation coupled with an elution time of 100 minutes for each sample.

The use of anion exchange chromatography together with pulsed electrochemical detection [7] allows the sensitive estimation of sugars within biological matrices without the need for extensive sample preparation or derivatization [8].

It has been postulated that elevated urinary fucose excretion may provide a suitable marker for some forms of cancer. We have applied this technique to investigate the concentration of urinary free fucose in children with malignancy, a group not previously studied.

MATERIALS

Fucose,3-O-methyl glucose, mannitol, dulcitol, arabinose and α-methyl glucose were obtained from Sigma Chemical Co. (Poole, UK.) Sodium hydroxide (500g/l), Amberlite IR 120 H^+ and IRA 400 Cl^- were obtained from BDH. (Poole, UK.) Deionized water (18Mohm cm^{-1}) was prepared by an in-house deionizer (Millipore, MA, USA).

Equipment

High pressure liquid chromatography equipment was composed of a quaternary gradient pump, Carbopac PA1 40mm x 250 mm anion exchange column with associated guard column, and pulsed electrochemical detector all supplied by Dionex UK Ltd. (Camberley, UK). Signal recording was provided by a Dionex 4100 integrator.

Subjects

Samples of urine from 35 healthy subjects (21 female, 14 male) age range 1-17 years and 22 cancer patients (7 female, 15 male) age range 4-21 years, were obtained. All samples were taken fasting and stored at -20°C prior to analysis. Ethical permission was granted for the study by the local hospital ethical committee.

METHODS

Sample preparation

Samples of urine were diluted 1 in 10 with deionized water to a total volume of 1ml. One millilitre of the internal standard, 3-O-methyl glucose (100 μmol/l) was added. The mixture was desalted with mixed ion-exchange resin (IR 120 H^+ and IRA 400 Cl^- in a mass ratio 1:1.5). After de-salting the mixture was centrifuged and 25 μl of supernatant was injected onto the chromatographic system.

HPLC analysis

Samples were eluted with 50mM NaOH at 1 ml /min at 20°C, with an analysis time of 7 minutes. Following this the column was washed with 1M NaOH for 5 min, to ensure stability of retention times, and then was re-equilibrated

for a further 8 min in 50mM NaOH. The total time between injection of samples was 20 minutes. (Figure 1.)

Detection was by integrated amperometry using a gold working electrode with a Ag/Ag Cl reference electrode. The following potentials were utilised: detection potential +0.1V (0-0.5s) : oxidation potential +0.75V (0.5-0.65s) : reduction potential -0.75V (0.65-0.75s) with an integration period of 0.05-0.5s. Quantification was by peak height analysis with internal standardisation.

Creatinine estimation

Creatinine concentration in the undiluted urine samples was measured by alkaline picrate method as adapted for an automated discrete analyser. (Hitachi 717).

RESULTS

Complete resolution from other closely related monosaccharides and sugar alcohols was achieved using 50mM NaOH as eluent in an isocratic mode. (Figure 1)

A range of standards in deionized water was analysed from 7.8 μmol/l to 1mmol/l, with internal standardisation. The method was found to be linear to 200 μmol/l fucose. The detection limit of the assay in urine was 0.5 μmol/l.

Analytical Recovery

To assess recovery three urine samples from control subjects were assayed ten times to determine the endogenous fucose concentration. Each neat urine was diluted 1:5 with deionized water and a known concentration of fucose added at three levels (100, 50 and 25 μmol/l) to each urine. The 'spiked' samples were analysed as previously described and the measured concentration

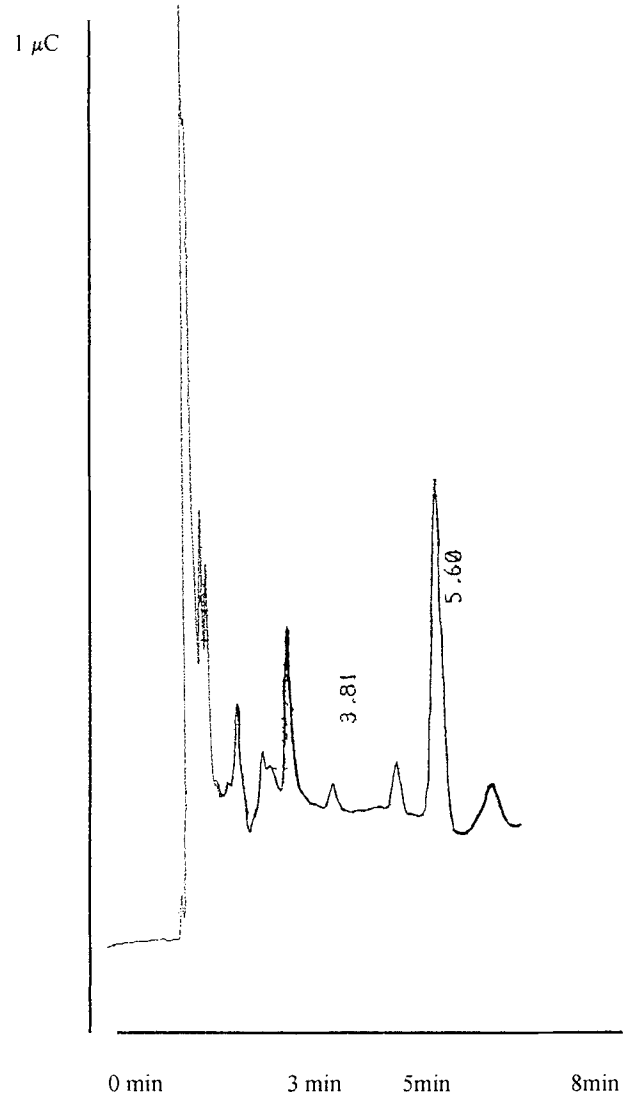

Figure 1. Chromatogram from a child with malignancy. Fucose =3.81 mins. 3-O methylglucose (internal standard) = 5.60 mins. Full scale deflection = 1μC

Table 1.

Analytical Recovery of Fucose in Urine.

Sample	Amount Added	Total (μmol/l)	% Recovery
Basal Concentration			
1. 4.2 μmol/	25μmol/l	24.8	85%
	50μmol/l	39.0	72%
	100μmol/l	99.0	95%
2. 16.5 μmol/l	25μmol/l	41.5	100%
	50μmol/l	69.8	105%
	100μmol/l	109.5	94%
3. 4.8 μmol/l	25 μmol/l	31.0	104%
	50 μmol/l	58.6	107%
	100μmol/l	113.2	108%

Overall mean recovery : 96.6% ± 11.8% (sd)

was compared to the theoretical value and the percentage recovery calculated. The results are summarised in table 1.

Precision

Imprecision of the assay was assessed by repeatedly analysing samples of known fucose concentration at three separate levels and the results are summarised in Table 2. The overall coefficient of variation did not exceed 5% at any concentration and was considered acceptable for this type of assay.

Measurement of Fucose in Urine

Fucose was measured in urine of 35 healthy children by the methods described previously. Fucose concentrations were corrected for creatinine

Table 2.
Estimate of Imprecision of fucose measurement in urine

Mean Fucose	Standard Deviation	CV %
29.9μmol/l	1.3	4.3%
62.9 μmol/l	2.4	3.7%
85.1 μμoλ/l	4.2	4.9%

concentration within each sample to yield a fucose : creatinine ratio for each child. Fucose : creatinine ratios ranged from 5.5 μmol/mmol to 128.5 μmol / mmol with a median ratio of 13.6 μmol/mmol. Forty nine samples analysed from 21 children with malignancy (7 female, 14 male) showed a median fucose : creatinine ratio of 8.1 μmol/mmol. (range 2.8 to 66.4 μmol/mmol) This was significantly lower than in the control group (p= 0.038 Mann Whitney U Test). Creatinine concentrations in urine did not differ significantly between the two groups, and thus the drop in the ratio could be attributed to the fall in the fucose concentration in the subjects with malignancy.

DISCUSSION

In a previous study by Suzuki et al [6] urinary free fucose concentrations were found to be elevated in adults with various types of malignancy. Both enzymatic and HPLC methods for the determination of free fucose in urine have been described [4-6]. The method described in this paper utilises the potential of anion exchange chromatography coupled with pulsed amperometric detection. Separation from other compounds in urine is achieved within a short analysis time, and thus many samples can be analysed within one working day. Sample preparation is simple and requires no extensive purification procedures.

The use of pulsed amperometric detection allows for sensitive and specific detection of fucose without the need to resort to fluorescene derivatization.

Urinary free fucose concentrations in children were found to be lower in subjects with malignancy when compared to healthy controls. This is in contrast to the findings in adults [6]. Little is known about the course of metabolic events during malignancy, particularly in relation to glycoproteins, however there is little to suggest that metabolism should differ so markedly between adults and children.

The method described here allows rapid, sensitive and specific measurement of free fucose in urine and will allow the further investigation of both children and adults with malignancy.

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