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Note

Analysis of carbohydrates in lens, erythrocytes, and plasma by high-performance liquid chromatography of nitrobenzoate derivatives

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Many methods for the qualitative and quantitative analysis of carbohydrates using high-performance liquid chromatography (HPLC) have been developed (see, e.g., ref. 1). Where sensitivity of detection is of prime importance, ultraviolet-absorbing derivatives [2] have been used with success.

Preparation of biological samples for analysis of free carbohydrates, by removal of protein, has been performed using various methods (Somogyi [3]; trichloroacetic acid; perchloric acid; ethanol; acetonitrile) which we have found to interfere with the reaction forming the UV-absorbing *p*-nitrobenzoate esters.

The work presented here gives a suitable method for sample preparation, and subsequent quantitative analysis of sugars and sugar alcohols in human and rat lens, erythrocytes, and plasma.

EXPERIMENTAL

Chromatographic system

A DuPont Series 850 liquid chromatograph was used (DuPont, U.K.; Stevenage, U.K.) equipped with an 850 gradient pump (used isocratically), a column compartment (kept at 35°C) with Rheodyne syringe loading injector, and an 852 variable-wavelength UV spectrophotometer (set at 260 nm).

The column was stainless steel (25 cm × 4.6 mm I.D.) packed with Zorbax SIL, particle size 6 μm (DuPont).

Chemicals

Pyridine (BDH, analytical grade) was refluxed for 3 h with sodium hydroxide pellets (BDH, analytical grade), distilled, and stored over sodium

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hydroxide pellets. *p*-Nitrobenzoyl chloride (Aldrich) was recrystallised from light petroleum (BDH, analytical grade, boiling range 60—80°C). Carbohydrate standards were obtained from Sigma. Solvents for the mobile phase were all HPLC grade (Fisons). All other chemicals were analytical grade.

Sample preparation and chromatography

Cataractous human lenses (obtained from Oxford Eye Hospital) were homogenised in 4 ml of sodium fluoride (2 mg/ml). Homogenate (1 ml) was filtered using the Centrifree system (Amicon, Stonehouse, U.K.), in an angle-head rotor at 2400 *g* for 30 min. Aliquots (200 μl of filtrate were pipetted into separate tubes, 10 μl of internal standard added (perseitol, i.e., α-mannoheptitol) (1 mg/ml), mixed, and lyophilised. Rat lenses were homogenised in 1 ml of sodium fluoride (2 mg/ml) and filtered; plasma samples (1 ml) were filtered undiluted; 600 μl of frozen and thawed erythrocytes were diluted with 400 μl of water to aid haemolysis, and filtered.

Standards were prepared by lyophilising 200 μl of aqueous solutions with internal standard.

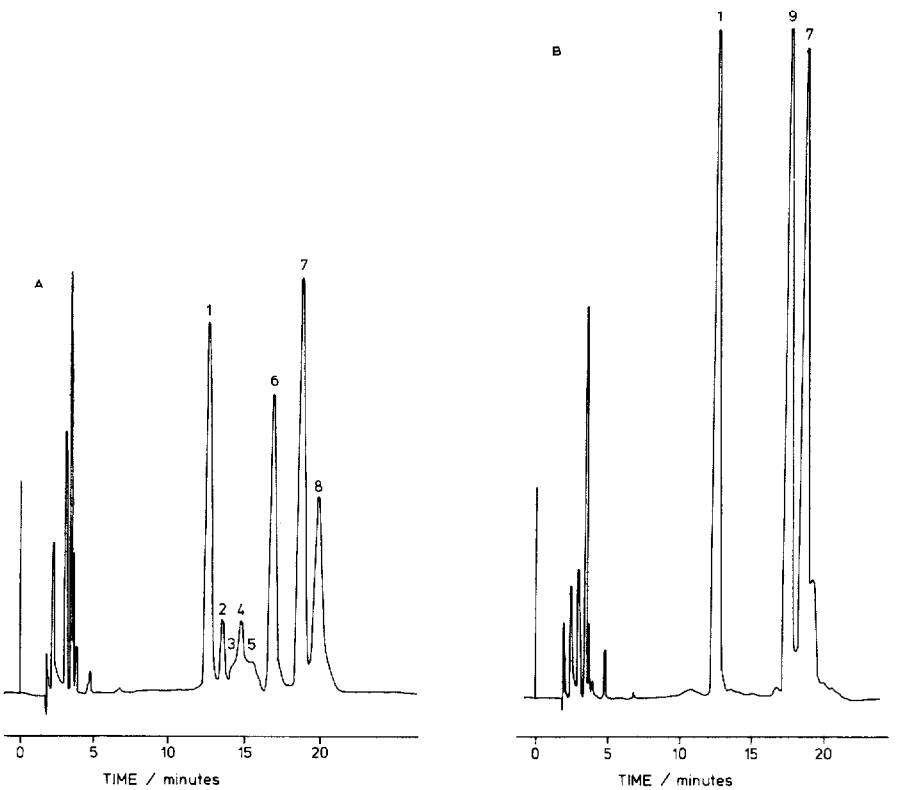
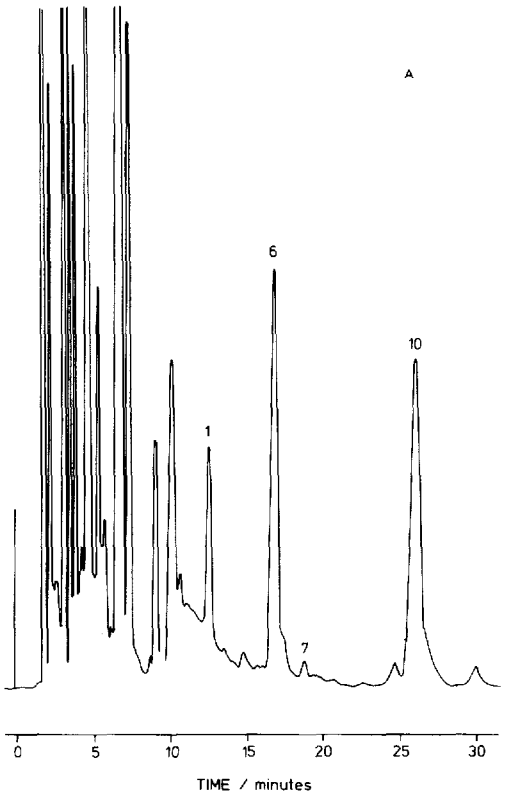


Fig. 1. HPLC of sugar standards (4-nitrobenzoate derivatives). (A) Peaks: 1, D-glucose (α- and β-); 2—5 and 8, isomers of D-fructose; 6, myo-inositol; 7, D-sorbitol; (B) separation of 9, mannitol, from 7, sorbitol. Mannitol is also resolved from inositol by this solvent mixture (see text).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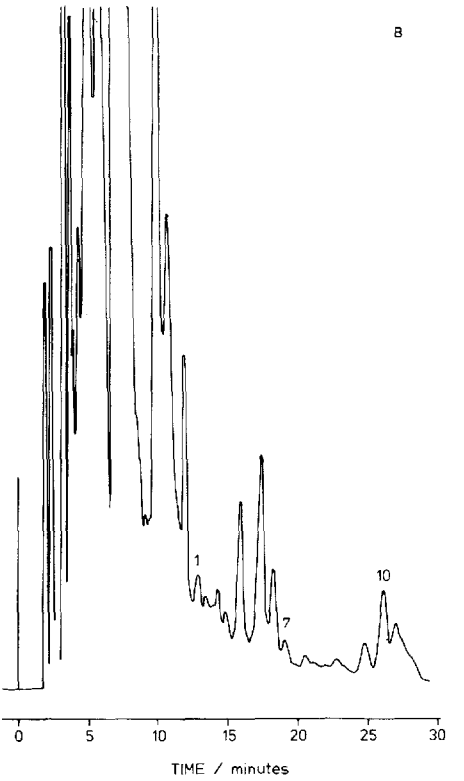
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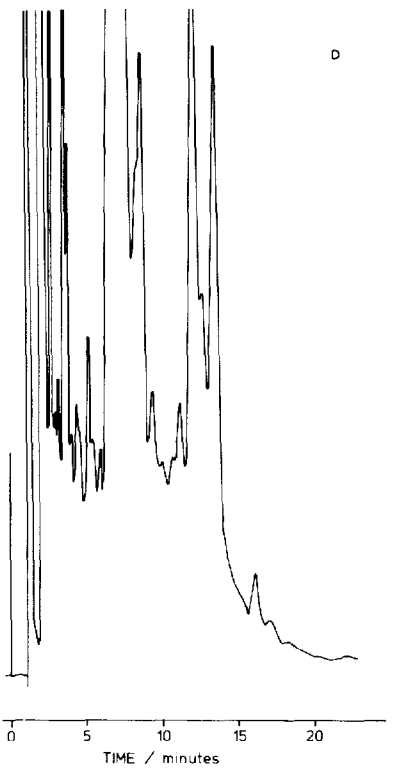
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The freeze-dried residues were taken and 200 μl of *p*-nitrobenzoyl chloride in pyridine (100 mg/ml) added, and reacted in the stoppered tubes at 60°C for 1 h. One drop of water was added to stop the reaction, and the products dissolved in 2 ml of chloroform. This was washed twice with 2 ml of sodium bicarbonate (5%) by vortex mixing for 1 min, and centrifuging for 30 sec to separate the layers prior to aspirating the aqueous layer. A further two washes, with 3 ml of 1 *M* hydrochloric acid, were performed [2].

A 50-μl aliquot of the chloroform layer was injected into the chromatograph, and eluted with hexane—chloroform—acetonitrile (10:3:1.9) with 0.1% water added at a flow-rate of 1.5 ml/min.

Recovery experiments were carried out by spiking lens homogenate with various amounts of glucose and sorbitol, and comparing to spiked sodium fluoride standards taken through the complete procedure.

RESULTS AND DISCUSSION

Sample preparation

The ultrafiltration method used has been found to provide a lens extract which does not interfere with the derivatising reaction by producing spurious peaks (Somogyi extract [3]), hydrolysis of reagent by excess acid (trichloroacetic acid, perchloric acid), or partial derivatisation by leaving significant quantities of protein in solution (perchloric acid, ethanol, acetonitrile). The method has also been found to be suitable for use with plasma and haemolysed erythrocytes.

A similar ultrafiltration method has been used to prepare hexoses and hexosamines from bovine lens [4].

Chromatography

Carbohydrate standards could be resolved as single peaks or anomers, dependent on the solvent used. Glucose eluted as a single peak which was more easily quantified, but fructose was separated into its anomers (Fig. 1A). Of the sugar alcohols, mannitol was completely resolved from sorbitol (Fig. 1B).

Calibration and precision

Use of perseitol as an internal standard (not found in lens) gave linear calibration graphs (r = 0.99) of peak height ratios with glucose and sorbitol over a range of initial concentrations from 3 to 60 μg/ml, the amounts detected being 15 to 120 ng, and the quantitative detection limit being in the region of 1—2 ng of these carbohydrates at 0.005 a.u.f.s.

The recovery of glucose and sorbitol from lens homogenate was found to be 105.7% (± 0.9) and 104.8% (± 5.4), respectively. No significant deviation in recovery was noted for plasma and erythrocyte samples.

Fig. 2. HPLC of biological tissue extracts. (A) Human cataractous lens homogenate; (B) human erythrocytes; (C) human plasma, showing major glucose peak (15 cm × 4.6 mm I.D. column, 2.56 a.u.f.s.); (D) human plasma, showing minor components, (0.08 a.u.f.s.). Peaks: 1, glucose; 6, inositol; 7, sorbitol; 10, perseitol.

Carbohydrate extracts

Chromatograms of typical elution profiles are shown (Fig. 2). The levels of carbohydrates in human cataractous lenses (n=8) were found to be (mg per g lens wet weight ± S.D.): glucose, 1.01 ± 0.77; sorbitol, 0.35 ± 0.21; inositol, 11.74 ± 8.16. These values are comparable to those found by previous workers using paper chromatography [5].

This technique provides a useful method for the analysis of small quantities of carbohydrates in biological tissues using HPLC, and negates any artefacts produced by chemical methods of deproteinising. The method is currently being used for analysing the effects of aldose reductase inhibitors on carbohydrate levels in tissues prone to diabetic sequelae.

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