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Note

Sensitive analysis of aldose sugars by reversed-phase high-performance liquid chromatography

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Liquid chromatography offers potential advantages over gas chromatographic methods for analysis of sugars. It is applicable not only to monosaccharides, but also oligosaccharides and derivatives carrying ionic groups.

Liquid chromatography of sugars has recently been reviewed[1]. Separations of unmodified sugars by high-performance liquid chromatography (HPLC) have employed packings of amine- or nitrile-bonded phases[2–6], amine-treated silicic acid[7,8] and cation-exchange materials[9]. These have used refractive index detection[7,8], ultraviolet (UV) absorption at short wavelengths[10], post-column derivatization[2], liquid scintillation counting[11,12] or mass spectrometric detection[3]. They therefore suffer from lack of sensitivity or the requirement of specialized equipment.

On the other hand, sugar derivatives carrying aromatic substituents offer excellent sensitivity by UV absorption at 254 nm[13–17] or fluorescence detection[18]. They do, however, require lengthy pre-treatment[14,17], and the presence of anomeric derivatives can complicate chromatograms[1,14,17].

In connection with structural studies of components of the Gram negative outer membrane[19,20], we require a sensitive HPLC procedure for analysis of aldoses. We report here the reversed-phase analysis of N-(*p*-methoxyphenyl)-glycosylamines, which offers the advantages of simplicity, sensitivity and resolving power.

EXPERIMENTAL

Materials

HPLC solvents were purchased from Waters Assoc. (Milford, MA, U.S.A.) and sugars from Sigma (St. Louis, MO, U.S.A.); *p*-anisidine from Fluka (Buchs, Switzerland) was decolourized with charcoal and recrystallized from water.

Equipment

Two LDC Constametric pumps (Models I and IIG) with a dynamic gradient mixer were operated in conjunction with an LDC Gradient Master. A Rheodyne Model 7120 syringe-loading injector was used with a 20-μl loop and detection was by means of an LDC UV III monitor operating at 254 nm. A Brownlee guard column and a Chrompack Economy Column were used, each packed with LiChrosorb RP-8 (10 μm). The eluting solvent was acetonitrile–water (12:88) and the flow-rate was 1 ml/min.

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An 8085-based S100 microcomputer was used to digitize and store data points at a 1-Hz rate, with 12-bit precision and auto-scaling over an eight-fold range. Timing was carried out by means of hardware interrupts.

Preparation of N-(p-methoxyphenyl)glycosylamines

Aliquots (0.020 ml) of *p*-anisidine solution (25%, w/v, in methanol) were placed carefully into the bottoms of a series of fusion tubes, followed by the sugar test solutions (0.050 ml) containing 10–500 μg total aldoses. The contents of the tubes were mixed carefully and the tubes centrifuged briefly before sealing and heating in a water bath at 60°C for the required time. The standard time was 80 min.

The tubes were then cooled and opened, water (0.200 ml) and ether (1 ml) added to each and the mixtures agitated well on a vortex mixer. After brief centrifugation to give clear separation of the layers, the organic layers were removed with a pasteur pipette and rejected. An aliquot (0.100 ml) of the aqueous layer was removed from each tube, mixed with an equal volume of 10 m*M* sodium phosphate buffer (pH 8.5) and used directly for HPLC analysis. A full (20-μl) loop was always injected.

RESULTS AND DISCUSSION

The chemistry of glycosylamines has been extensively reviewed[21,22]. Any quantitative procedure making use of these derivatives must take account of: (1) rate and equilibrium control of formation of derivatives[22–24]; (2) formation of rearrangement and decomposition products[22,25]; (3) stability of the derivatives to hydrolysis[22,26].

Although formation of N-arylglycosylamines is catalysed by weak acids[27,28], the more nucleophilic amines, such as aniline and *p*-toluidine, will react with aldoses without catalysis[27]. Our preliminary experiments showed that treatment of aldoses with aniline or *p*-anisidine at 100°C is accompanied by considerable darkening and decomposition. A reaction temperature of 60°C was therefore used in subsequent experiments. In the absence of an acid catalyst, *p*-anisidine converts aldoses into N-

TABLE I

INFLUENCE OF REACTION TIME ON AREAS OF N-(*p*-METHOXYPHENYL)-GLYCOSYLAMINE PEAKS

For each sugar, peak area for reaction time of 80 min = 100. Values are accurate to ±4%.

Parent sugar	*Time (min)*					
	20	*40*	*60*	*70*	*80*	*100*
D-Glycero-D-glucoheptose	80	99	101	105	100	94
D-Galactose	92	104	104	105	100	93
D-Glucose	46	66	84	99	100	100
D-Mannose	83	98	102	101	100	95
D-Arabinose	105	113	107	97	100	97
D-Xylose	88	99	104	98	100	100
D-Ribose	120	119	112	108	100	100
L-Fucose	72	84	91	96	100	100
L-Rhamnose	82	104	107	105	100	96

TABLE II

INFLUENCE OF REACTION TIME OF RELATIVE MOLAR PEAK AREAS OF N-(*p*-METHOXYPHENYL)GLYCOSYLAMINES

For each reaction time, area of mannose derivative is taken as 1.00. Values are accurate to ±1%.

Parent sugar	*Time (min)*					
	20	*40*	*60*	*70*	*80*	*100*
D-Glycero-D-glucoheptose	1.54	1.60	1.56	1.56	1.57	1.57
D-Galactose	0.94	0.97	0.97	0.97	0.98	0.94
D-Glucose	0.69	0.84	1.01	1.21	1.24	1.30
D-Mannose	1.00	1.00	1.00	1.00	1.00	1.00
D-Arabinose	0.45	0.44	0.40	0.40	0.40	0.40
D-Xylose	0.94	0.90	0.90	0.89	0.89	0.87
L-Fucose	1.05	1.05	1.09	1.27	0.26	1.26
L-Rhamnose	0.89	1.03	1.03	1.03	1.04	1.03

(*p*-methoxyphenyl)glycosylamines (Table I). With the exception of arabinose and ribose, optimal yields are obtained after *ca.* 80 min. This reaction time was adopted in the standard procedure. The relative molar responses (Table II) are constant for reaction times in excess of 70 min.

It should be noted that ketoses do not form glycosylamines without acid catalysis[29]. Our method is specific for aldoses.

In view of their known hydrolytic lability in dilute acid[22,26], we examined the stability of N-(*p*-methoxyphenyl)glycosylamines in slightly alkaline buffer. Hydrol-

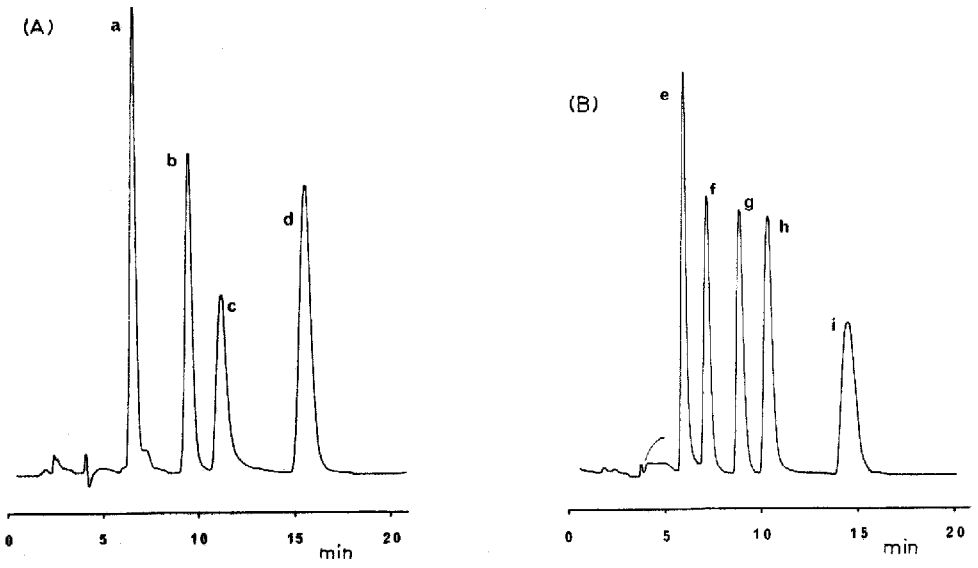

Fig. 1. Separation of aldoses by HPLC of N-(*p*-methoxyphenyl)-glycosylamines. Operating conditions: pre-column and 25-cm column, RP-8 (10 μm); mobile phase, acetonitrile–water (12:88); flow-rate, 1 ml/min. Peaks: a = D-galactose; b = D-arabinose; c = D-ribose; d = L-rhamnose; e = D-glycero-D-glucoheptose; f = D-glucose; g = D-mannose; h = D-xylose; i = L-fucose. Mixtures in panels A and B were selected to show clearly the peak shapes for each derivative.

TABLE III

HPLC PROPERTIES OF N-(*p*-METHOXYPHENYL)GLYCOSYLAMINES ON LICHROSORB RP-8

Elution solvent was acetonitrile–water (12:88).

Parent sugar	*Retention volume (ml)*
D-Glycero-D-glucoheptose	6.13
D-Galactose	6.62
D-Glucose	7.30
D-Mannose	8.95
D-Talose	9.43
D-Arabinose	9.45
D-Lyxose	10.22
D-Xylose	10.35
D-Ribose	11.17
L-Fucose	14.37
D-Quinovose	15.43
L-Rhamnose	15.75

ysis has been shown to be quite slow at pH values above 8.0 (ref. 22). A value of 8.5 was selected as a compromise between stability of the derivatives and that of the column packing. A low ionic strength was chosen because buffer catalysis is known to occur in hydrolysis and anomerization of glycosylamines[26]. The derivatives of arabinose and ribose hydrolyse to the extent of 10–15% over 5 h at 0°C in 5 m*M* sodium phosphate buffer, pH 8.5. The other derivatives are completely stable under these conditions.

Although crude N-arylglycosylamines are not anomerically pure, the β-isomer generally predominates[30]. The presence of anomeric forms would be expected to complicate chromatographic analysis[1]. In general, however, the anomers of the N-(*p*-methoxyphenyl)glycosylamines have very similar properties on reversed-phase chromatography. Under the conditions described, only galactose shows distinct shoulders. In the other cases, the existence of anomeric mixtures can be demonstrated by elution with a lower proportion of acetonitrile.

The selectivity shown in this procedure is rather better than might be expected for a reversed-phase method (Fig. 1). Apart from the general separation on the basis of sugar class, good resolution of diastereomers is obtained (Table III), presumably as a result of differential solvation. Interestingly, the order of elution in our procedure is exactly opposite to that for underivatized sugars on an amine-modified silicic acid column[1].

Our method has some similarity to that of Hase *et al.*[18], which involves conjugation of sugars with 2-aminopyridine followed by reduction. Their chromatography was carried out at acid pH, however, and retention appears to be based on the hydrophobicity of the sugars themselves rather than that of the aromatic group.

The standard procedure has not been optimized for sensitivity. Typically, however, glycosylamine equivalent to 1 μg of hexose gives a peak with full-scale response on a detector setting of 0.032 a.u.f.s. The method is therefore much more sensitive than those employing refractive index detection[1]. Moreover, without modification of the method, satisfactory derivatization and analysis can be carried out on samples

containing as little as 10 μg of an individual sugar. In our experience, this provides greater overall sensitivity than gas chromatographic methods[31] and is experimentally simpler and less time-consuming.

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