Journal of Chromatography, 196 (1980) 171–175
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,874

Note

Separation of partially methylated sugars by reversed-phase high-performance liquid chromatography

N. W. H. CHEETHAM* and PADMINI SIRIMANNE

School of Chemistry, The University of New South Wales, P.O. Box 1, Kensington, 2033 N.S.W. (Australia)

(First received October 24th, 1979; revised manuscript received April 2nd, 1980)

High-performance liquid chromatography (HPLC) has been well developed for the separation of unsubstituted sugars and oligosaccharides[1–7]. Separations of derivatives have been reported[8–12] but these have often been used to enhance the sensitivity to detect the parent sugars. In order to extend the use of HPLC to the structural analysis of polysaccharides we now report some initial results on methylated sugars using reversed-phase C_{18} bonded columns. Use of chemically bonded amine columns and "carbohydrate analysis" columns was avoided, as these tend to deteriorate with long use, *e.g.* by loss of amine function possibly due to formation of Schiff bases[13].

EXPERIMENTAL

The equipment consisted of the following Waters Assoc. instruments: M6000 pump; U6K injector: U6K refractive index detector. Columns were Waters μBondapak C_{18}, 30 × 0.38 cm I.D. (analytical) and 30 × 0.76 cm I.D. (semipreparative). Nuclear magnetic resonance (NMR) studies were performed in C^2HCl_3 at 100 MHz on a JEOL JNMFX-100 spectrometer.

Various aqueous eluent compositions (0.1–2%, w/v) of sodium acetate (pH 5.5–8.1) ammonium acetate (pH 6.9) and sodium phosphate (pH 8.0), and also distilled water, had little effect on the chromatographic behaviour of the sugars analysed. Ammonium acetate (1%, pH 6.9) was used routinely as the aqueous component, with added ethanol where necessary to elute the more highly methylated compounds in a reasonable time. Sugar samples were dissolved (≈ 100 mg/ml) in the degassed (water pump vacuum and sonication) eluting solvent, filtered and injected (10–25 μl) at flow-rates from 1.5–2.5 ml/min.

RESULTS AND DISCUSSION

Table I summarizes the results for the standard sugars available. One is struck immediately by the appearance of two peaks for most sugars. Initially this was thought to be caused by impurities but subsequent work on pure crystalline samples of 2,3 di- and 2,3,6-tri-O-methyl glucose showed them to be the α- and β-anomeric forms.

Evidence for this conclusion is illustrated in Fig. 1; Fig. 1a shows the chroma-

0021-9673/80/0000–0000/$02.25 © 1980 Elsevier Scientific Publishing Company

TABLE I

RETENTION TIMES OF METHYLATED SUGARS IN 1% AMMONIUM ACETATE–ETHANOL (9:1)

Flow-rate, 2.5 ml/min.

Sugar	*Retention time (min)*
D-*glucose*	
2-O-Methyl	0.75
3-O-Methyl	0.75
6-O-Methyl	0.75
2,3-Di-O-methyl	1.30 (pure α); 1.15, 1.30 (equil.)
2,3,4-Tri-O-methyl	1.75, 2.05 (α)*
2,3,6-Tri-O-methyl	1.35 (pure α); 1.1, 1.35 (equil.)
2,3,4,6-Tetra-O-methyl	3.3; 3.6 (α)*
D-galactose	
2,3-Di-O-methyl	1.4; 1.95 (α)*
2,3,4-Tri-O-methyl	1.30, 2.25
2,3,4,6-Tetra-O-methyl	1.1; 1.75 (α)*
D-xylose	
2,3,4-Tri-O-methyl	1.15, 1.6
D-mannose	
2,3,6-Tri-O-methyl	1.15; 1.65 (α)*
2,3,4,6-Tetra-O-methyl	1.1; 1.75 (α)*

* Determined by NMR, see Results and discussion.

togram of 2,3-di-O-methyl-α-D-glucopyranose freshly dissolved in water and injected as soon as possible ($\approx$ 5 min), while Fig. 1b and c show the results of injecting further samples after 15 and 75 min respectively. Using the semipreparative column with water as eluent, samples of both peaks were collected, immediately frozen (acetone–solid carbondioxide) and lyophilized. Peak 2 yielded crystalline material while peak 1 material was syrupy. Chromatography of peak 2 material yielded a chromatogram identical with that in Fig. 1a, while peak 1 material showed two peaks with the one of shorter retention time predominating (2:1). Each isolated sample finally reached a pattern essentially identical to Fig. 1c.

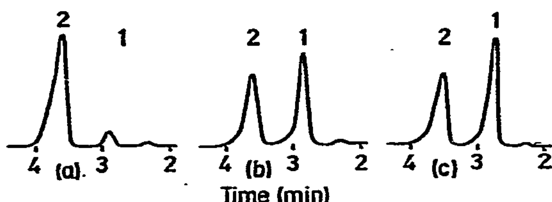

Fig. 1. Equilibration of 2,3-di-O-methyl-α-D-glucose in water. Flow-rate 2.0 ml/min. (a) Freshly dissolved (5 min); (b) after 15 min; (c) after 75 min.

The separations make feasible the preparation of pure and perhaps crystalline α and β anomers of a number of methylated sugars, some of which have not been isolated previously *e.g.* the anomers of 2,3,4-tri-O-methyl-D-glucose[14]; and the study of the rate and extent of equilibration between α and β forms of methylated sugars. The results could be compared with those obtained by NMR and gas–liquid

chromatographic methods[15]. Experiments to this end are envisaged. Various solvent systems between pH 3 and pH 8 (the stability limits of the column silica) can be examined.

A disadvantage for rate studies is the continuing equilibration once a subsample has been taken. This would require, at least, identical temperatures in the reaction vessel and the column solvent system.

A conclusion can be drawn regarding the relationship between elution times and anomeric structure. For both 2,3-di- and 2,3,6-tri-O-methyl-D-glucose, the α anomer presumably in the C_1 conformation, elutes slowest and suggests a greater interaction between axial hydroxyl groups and the matrix than between equatorial groups and the column matrix. This elution behaviour was confirmed by NMR for most of the compounds studied (Table I). Pure anomeric forms were not available for most of the methylated sugars. However samples when freshly dissolved and chromatographed showed one predominating anomeric peak. This peak was taken to correspond to the predominant proton signal in the anomeric region of the NMR spectrum. Protons were identified as belonging to α or β anomers by relative chemical shift and coupling constants[16]. In each case examined, the chromatogram peak of longest retention time corresponded to the α anomer as determined by NMR.

Other points on the separations are illustrated in Figs. 2 and 3. Fig. 2 shows the effect of adding ethanol to a purely aqueous solvent. The sample in Fig. 2 a was run in 1% ammonium acetate at 2.5 ml/min, and the 2,3,6-tri-O-methyl glucose peak developed a shoulder. Fig. 2b was run at 2.0 ml/min and though the 3-O-methyl glucose has a longer retention time at this slightly slower flow-rate, the more highly substituted sugars emerged faster. Resolution is still good, and the 2,3,6 material elutes as a single peak (though a second peak develops if the sample is allowed to

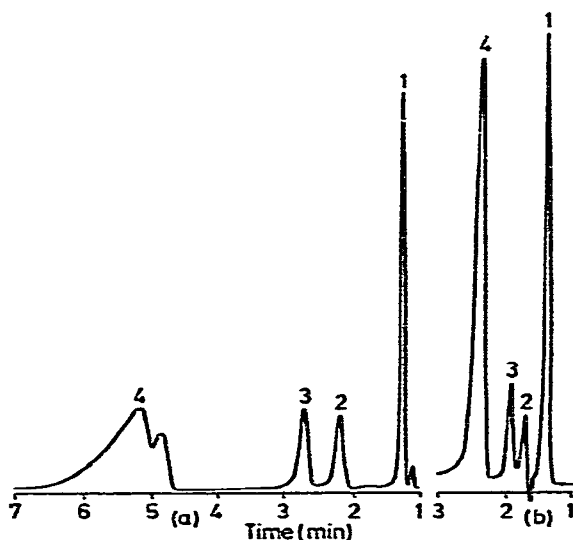

Fig. 2. Separation of 3-O-methyl-D-glucose (1), 2,3-di-O-methyl-D-glucose (2 an d3) and 2,3,6-tri-O-methyl-D-glucose (4). (a) In 1% ammonium acetate solution, flow-rate 2.5 ml/min; (b) the same sample separated in 1% ammonium acetate solution–ethanol (9:1); flow-rate 2.0 ml/min.

equilibrate (Table I). A single peak per sugar sample is obviously desirable for quantitative work. In an effort to achieve this, ethanol and methanol up to 70% (v/v) were used in combination with ammonium acetate solution or water as trial solvents. Unfortunately the amount of organic solvent required to produce a single peak from a sugar sample also decreased the resolution between individual sugar samples to unacceptable levels. This might have been expected from the large resolution between anomers relative to that between certain samples, *e.g.* 2,3-di-O-methyl and 2,3,6 tri-O-methyl glucose (Table I). Fig. 3 serves as illustration of the potential to prepare partially methylated sugars for reference purposes. A sample of deliberately undermethylated sucrose containing up to tetramethyl derivatives was run in 1% ammonium acetate. Standards of glucose, fructose, sucrose and a monomethylated fraction allowed the identification of various sections of the chromatogram. Semipreparative scale up of the procedures is envisaged to obtain individual samples. Extension to other deliberately undermethylated sugar samples should allow a stock of standard compounds to be built up in a relatively short time.

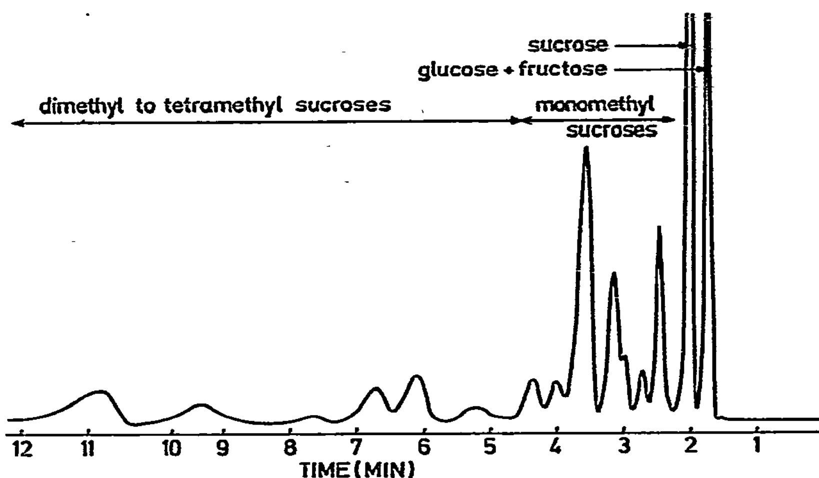

Fig. 3. Separation of partially methylated sucrose derivatives in 1% ammonium acetate; flow-rate 1.5 ml/min.

ACKNOWLEDGMENT

The authors wish to thank Professor G. N. Richards and S. J. Angyal for samples of methylated sugars, Professor G. W. K. Cavill for permission to use the HPLC equipment and Mr. V. Pickles and Mrs. H. Stender for running NMR spectra.

REFERENCES

1 R. Schwarzenbach, *J. Chromatogr.*, 117 (1976) 206.
2 J. Hettinger and R. E. Majors, *Varian Instrum. Appl.*, 10 (1976) 2, 6.
3 A. D. Jones, I. W. Burns, S. G. Sellings and J. A. Cox, *J. Chromatogr.*, 144 (1977) 169.
4 *Application Highlights, AH338*, Waters Assoc., Milford, MA, 1974.

5 R. B. Meagher S.J. and A. Furst, *J. Chromatogr.*, 117 (1976) 211.
6 *Rapid and Reliable Analysis of Beverages, Brochure N67*, Waters Assoc., Milford, MA, 1976.
7 J. F. Kennedy and J. E. Fox, *Carbohyd. Res.*, 54 (1977) 13.
8 J. Lehrfeld, *J. Chromatogr.*, 120 (1976) 141.
9 F. Nachtmann, *Z. Anal. Chem.*, 282 (1976) 209.
10 G. D. McGinnis and P. Fang, *J. Chromatogr.*, 130 (1977) 181.
11 F. Nachtmann and K. W. Budna, *J. Chromatogr.*, 136 (1977) 279.
12 R. M. Thompson, *J. Chromatogr.*, 166 (1978) 201.
13 K. Aitzetmüller, *J. Chromatogr.*, 156 (1978) 354.
14 E. J. Bourne and S. Peat, *Advan. Carbohyd. Chem.*, 5 (1950) 145.
15 I. M. Campbell and R. Bentley, in R. F. Gould (Editor), *Carbohydrates in Solution* (Advances in Chemistry Series 117), American Chemical Society, Washington, DC, 1973, Ch. 1, p. 1.
16 D. R. Bundle and R. U. Lemieux, *Methods Carbohyd. Chem.*, 7 (1976) 79.