Journal of Chromatography, 134 (1977) 199–203

CHROM. 9812

Note

Separation of hexosamines, hexosaminitols and hexosamine-containing di- and trisaccharides on an amino acid analyser

A. S. R. DONALD

Division of Immunochemical Genetics, M.R.C. Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (Great Britain)

(Received October 11th, 1976)

The separation and identification of oligosaccharide fragments containing amino sugars are important techniques for use in structural determinations on glycoproteins, glycosaminoglycans, glycolipids, and oligosaccharides. The degradation of such materials into their constituent di- and trisaccharides may provide evidence concerning the order in which the sugars occur and indicate the position and anomeric nature of the glycosidic linkages joining the residues. Of the methods used at present for identification of such di- and trisaccharides, paper chromatography has found the widest application (see ref. 1, for example), but this procedure has the disadvantages of poor resolution of the hexosamine-containing disaccharides, and lack of precision in relative mobility measurement. Other methods of actual or potential use in this field are gas–liquid chromatography (GLC)[2], various forms of liquid chromatography (for reviews, see refs. 3 and 4) including separation of sugar–borate complexes on anion-exchange resins[5], and partition chromatography on either anion- or cation-exchange resins[6].

This paper describes a novel method whereby hexosamine-containing oligosaccharides are reduced and de-N-acetylated; the resulting amino alditols are separated on an amino acid analyser and borate–citrate buffers are used for elution.

EXPERIMENTAL

The amino acid analyser was manufactured by Evans Electroselenium (Halstead, Great Britain), and was modified by the addition of an automatic sample loader and buffer change valve supplied by the Locarte (London, Great Britain). All separations were performed on a column (40 × 1 cm I.D.) of Locarte No. 12 resin (a nominally X8 cross-linked sulphonated polystyrene) at 50°. The buffer and ninhydrin flow-rates were 45 and 22.5 ml/h, respectively. A 20-mm flow-through cuvette was used and the absorbance at 570 nm was recorded. The pH 2.2 buffer was that of Moore *et al.*[7]. Other buffers were prepared as follows: (I) 0.1 *M* in Na^+, contained trisodium citrate·$2H_2O$ (49.0 g), boric acid (1.55 g), 33% (w/v) Brij-35 solution (15 ml), water to 5 l, and HCl to pH 7.5. (II) 0.2 *M* in Na^+, prepared from trisodium citrate·$2H_2O$ (91.88 g), sodium tetraborate·10 H_2O (11.9 g), 33% (w/v) Brij-35 solution (15 ml), water to 5 l, and HCl to pH 8.0.

The disaccharide Galβ(1→6)GlcNAc and milk oligosaccharides were obtained from the laboratories of the late Professor R. Kuhn (Max-Planck Institute, Heidelburg, G.F.R.). GlcNAcβ(1→3)Gal was prepared by partial acid hydrolysis of lacto-N-tetraose and isolation of the disaccharide by paper chromatography. Other oligosaccharides were prepared in the laboratories of Professors Winifred M. Watkins and W. T. J. Morgan at the Lister Institute (London, Great Britain). Glucosamine, galactosamine, glucosaminitol, and galactosaminitol were obtained as described previously[8]. N-Acetylmannosamine was purchased from Koch-Light (Colnbrook, Great Britain). Mannosamine solutions were prepared by acid hydrolysis of N-acetylmannosamine, and mannosaminitol solutions by $NaBH_4$ reduction and acid hydrolysis of N-acetylmannosamine.

Oligosaccharides were reduced and de-N-acetylated as follows: To the sugar solution (20 μg–1 mg in 90 μl water) in Pyrex tubes (5 × 0.4 cm I.D.) was added 10% (w/v) $NaBH_4$ (10 μl). After 3 h, 6 *M* NaOH (20 μl) was added; the samples were frozen in acetone–solid CO_2 and then sealed under vacuum. The tubes were heated for 16 h at 100°, then opened, and the contents made up to 1 ml with pH 2.2 buffer. Aliquots containing 5–50 μg of the reduced de-N-acetylated sugars were transferred to the autoloader of the amino acid analyser, washed in with the pH 2.2 buffer, and run with the following programme: Buffer I (320 min), buffer II (200 min), 0.5 *M* NaOH (40 min), buffer I (120 min).

RESULTS AND DISCUSSION

In order to generate free amino groups capable of reacting with the analyser cation-exchange resin, the N-acetyl hexosamine-containing oligosaccharides were rendered alkali stable by $NaBH_4$ reduction and then de-N-acetylated with aqueous NaOH. The resulting amino alditols were run on the amino acid analyser using borate–citrate buffers for elution and the ninhydrin reaction for detection. The principle used for the separations is that the formation of borate complexes should lower the affinity of the amino sugars for a cation-exchange resin in a manner dependent upon the acidity of such complexes, which is in turn a function of the number and steric arrangements of the various –OH and $-NH_2$ groups present in a particular sugar.

The separation obtained when the available di- and trisaccharides were reduced, de-N-acetylated and chromatographed in borate-containing buffers is shown in Fig. 1. The sugars eluted with buffer I contained one hexosamine residue and were mostly well resolved, although the disaccharides GlcNβ(1→3)galactitol and GlcNβ(1→6)galactitol emerged together (peaks 2 and 3 on Fig. 1). This pair of disaccharides did, however, separate when buffer I was adjusted to pH 7.0 (see Fig. 2). The resolution of the disaccharides Galβ(1→6)glucosaminitol and Galβ(1→4)glucosaminitol (peaks 7 and 8 on Fig. 1) was better at pH 7.75 but, both at this pH and at pH 7.0, adverse effects on the separations achieved in other regions of the chromatogram were observed. The excellent resolution of Galβ(1→3)glucosaminitol and Galβ(1→4)glucosaminitol (peaks 6 and 8) should be especially useful since the disaccharides from which they are derived are the backbones of the Type I and Type II chain endings of blood group specific glycoproteins (see ref. 9) and are difficult to separate by other means.

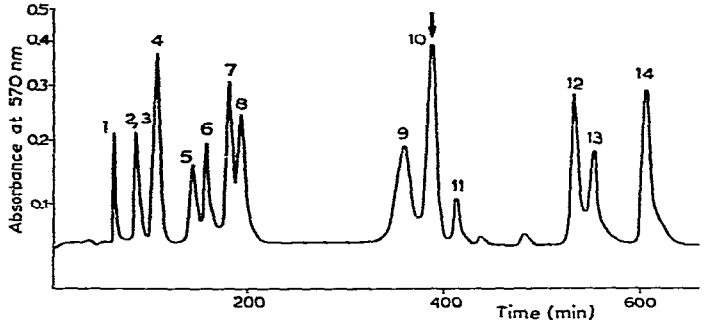

Fig. 1. Separation of reduced, de-*N*-acetylated oligosaccharides on the amino acid analyser. Elution was performed with buffers I and II, and the position at which buffer II emerges from the column is indicated by the arrow. Other conditions are given in Experimental. The peaks are numbered as follows: 1 = an unidentified contaminant which is at the void volume; 2 = GlcNβ(1→3)galactitol; 3 = GlcNβ(1→6)galactitol; 4 = GalNα(1→3)galactitol; 5 = Galβ(1→3)galactosaminitol; 6 = Galβ(1→3)glucosaminitol; 7 = Galβ(1→6)glucosaminitol; 8 = Galβ(1→4)glucosaminitol; 9 = glucosaminitol; 10 = galactosaminitol; 11 = Galβ(1→4)GlcNβ(1→6)galactosaminitol; 12 = GlcNβ(1→4)glucosaminitol; 13 = GalNα(1→3)Galβ(1→3)glucosaminitol; 14 = GalNα(1→3)Galβ(1→4)glucosaminitol. The two small peaks between 11 and 12 are unidentified contaminants from 11 and 13.

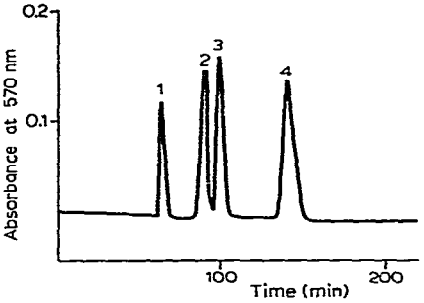

Fig. 2. Improved separation of some of the sugars at pH 7.0. Conditions and numbering of peaks as given for Fig. 1, but buffer I adjusted to pH 7.0.

Glucosaminitol (9) was eluted with buffer I and galactosaminitol (10) came off at the front when the change to buffer II occurred (Fig. 1). The other sugars which were eluted with buffer II contained two hexosamine residues and all the available examples were satisfactorily separated, including the important pair (peaks 13, 14) derived from the blood group A active trisaccharides GalNAcα(1→3)Galβ(1→3)-GlcNAc and GalNAcα(1→3)Galβ(1→4)GlcNAc. When the reduced and de-N-acetylated tetrasaccharide lacto-N-tetraose and larger milk oligosaccharides were run, they all emerged at the position of peak 1 in Fig. 1, which corresponds to the void volume of the column. Gel permeation chromatography of sugars on columns of a X8 cross-linked resin similar to the one used in the analyser column established that tetrasaccharides and higher oligomers are excluded from the pores of the resin. The use of X4 cross-linked resin may extend the separation method reported in this paper to permit sugars in the tetra- to hexasaccharide range to be resolved.

Separation of mixtures of glucosamine, galactosamine and their alcohols in borate–citrate buffer at about pH 5 has been described previously[8,10,11]. It has now been found that buffer I at pH 8.0 also gives a satisfactory separation of these sugars (Fig. 3a). Interestingly, the order of elution at pH 8.0 is glucosamine, galactosamine, glucosaminitol, galactosaminitol, which differs from the order galactosaminitol, glucosaminitol, glucosamine, galactosamine observed at pH 5. The method of separation can be extended to include mannosamine and mannosaminitol since buffer I at pH 7.5 gives adequate resolution (Fig. 3b) of all three hexosamines and their alditols.

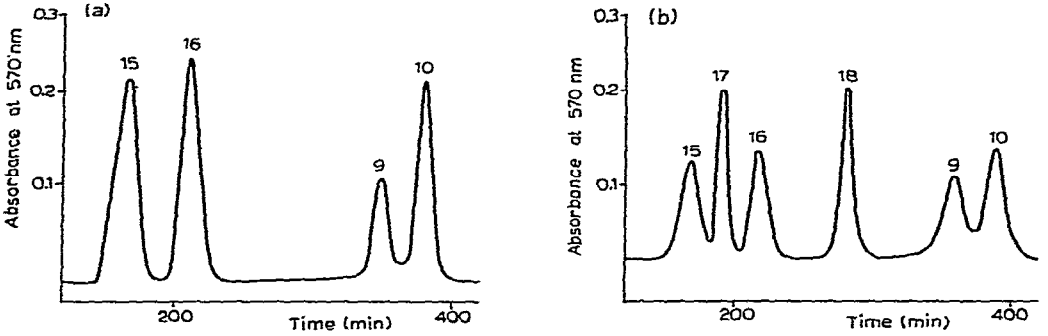

Fig. 3. Separation of hexosamines and their alditols. The amino sugars (5 μg) and alditols (10 μg) were loaded onto the analyser in pH 2.2 buffer. Peaks are numbered as follows: 9 = glucosaminitol; 10 = galactosaminitol; 15 = glucosamine; 16 = galactosamine; 17 = mannosamine; 18 = mannosaminitol. For (b) conditions are as in Fig. 1, but for (a) buffer I was adjusted to pH 8.0.

Standard mixtures of amino acids were run on the analyser under conditions identical to those used for the sugars. The acidic and neutral amino acids and His ran near peaks 1 and 2 of Fig. 1, while Lys emerged between peaks 11 and 12. The presence of any interfering amino acids may be established by a control run using any of the normal analytical procedures for these compounds.

That borate complex formation plays a part in the separations described is demonstrated by the fact that all the mono-, di- and trisaccharides ran faster in borate buffers than in buffers of identical Na^+ concentration and pH, but lacking borate. The presence of borate does, however, produce some reduction in the colour yield of the amino sugars in the ninhydrin reaction. Another factor which may affect the overall colour yield given by the sugars is possible variation in the extent of de-N-acetylation due to the presence of different substituents and steric configurations in the vicinity of the N-acetyl groups[12,13]. Nevertheless all the saccharides examined were readily detectable at the 5-μg level, and the ability to identify such small amounts of sugars should be of considerable value in studies on hexosamine-containing materials. The method, at an earlier stage of its development, provided evidence concerning the structure of a blood group HI glycolipid[14].

REFERENCES

1 A. M. S. Marr, A. S. R. Donald, W. M. Watkins and W. T. J. Morgan, *Nature (London)*, 215 (1967) 1345.

2 R. Chambers, J. R. Clamp, B. Bayard and J. Montreuil, *Biochimie*, 55 (1973) 1195.
3 K. Čapek and J. Staněk, Jr., in Z. Deyl, K. Macek and J. Janák (Editors), *Journal of Chromatography Library Vol. 3, Liquid Column Chromatography —A Survey of Modern Techniques and Applications*, Elsevier, Amsterdam, Oxford, New York, 1975, p. 465.
4 Z. J. Zmrhal, J. G. Heathcote and R. J. Washington, in Z. Deyl, K. Macek and J. Janák (Editors), *Journal of Chromatography Library, Vol. 3, Liquid Column Chromatography —A Survey of Modern Techniques and Applications*, Elsevier, Amsterdam, Oxford, New York, 1975, p. 665.
5 N. K. Kochetkov, V. A. Derevitskaya and N. P. Arbatsky, *Eur. J. Biochem.*, 67 (1976) 129.
6 J. Havlicek and O. Samuelson, *Anal. Chem.*, 47 (1975) 1854.
7 S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, 30 (1958) 1185.
8 A. S. R. Donald, *J. Chromatogr.*, 35 (1968) 106.
9 W. M. Watkins, in A. Gottschalk (Editor), *Glycoproteins, Structure and Function*, Elsevier, Amsterdam, 1972, p. 830.
10 A. M. Bella, Jr. and Y. S. Kim, *J. Chromatogr.*, 51 (1970) 314.
11 R. G. Spiro, *Methods Enzymol.*, 28B (1972) 3.
12 D. H. Horton and D. R. Lineback, *Methods Carbohyd. Chem.*, 5 (1965) 403.
13 S. Hanessian, *Methods Carbohyd. Chem.*, 6 (1972) 208.
14 A. Gardas, *Eur. J. Biochem.*, 68 (1976) 177.