CHROM. 11,917

CONVENIENT METHOD FOR THE GAS CHROMATOGRAPHIC ANALYSIS OF HEXOSAMINES IN THE PRESENCE OF NEUTRAL MONOSACCHARIDES AND URONIC ACIDS

SUSUMU HONDA, KAZUAKI KAKEHI and KŌZŌ OKADA

Faculty of Pharmaceutical Sciences, Kinki University, Kowakae, Higashi-osaka (Japan)

(Received April 20th, 1979)

SUMMARY

A convenient gas chromatographic method has been devised for the analysis of hexosamines in the presence of neutral and acidic sugars, which involves sequential derivatization reactions of nitrous acid deamination, mercaptalation, and trimethylsilylation. This method allows rapid, simultaneous determination of 0.1–1 μmole samples of hexosamines with coefficients of variation of less than 3%.

INTRODUCTION

Hexosamines are basic constituents of a variety of carbohydrate materials, including glycolipids, glycoproteins and proteoglycans, and their determination is of primary importance in the elucidation of the structures of these materials. The gas chromatographic determination of hexosamines in carbohydrate materials is most frequently performed on the trimethylsilyl derivatives of the N-acetylated methanolysates[1], for complete separation of their peaks from those of accompanying neutral and acidic monosaccharides. However, this method gives multiple peaks of hexosamine derivatives owing to different anomeric configurations, and is laborious. Borohydride reduction of the acid hydrolysates, followed by trifluoroacetylation[2], may convert hexosamines into trifluoroacetylated hexosaminitols, which give single peaks, but this procedure necessitates purification of the reduction products on a column of Sephadex prior to trifluoroacetylation. Recently a novel derivatization method[3] was reported, which involves sequential reactions of nitrous acid deamination, oximation, and acetylation. Nitrous acid deamination is a rapid reaction, but the other two are time-consuming. Therefore, we replaced these reactions by mercaptalation and trimethylsilylation[4]. In this paper we describe this simple and rapid 2,5-anhydrohexose dithioacetal trimethylsilylate method for the determination of hexosamines in the presence of neutral and acidic monosaccharides.

EXPERIMENTAL

Materials

2-Amino-2-deoxy-D-glucose and -D-galactose hydrochlorides (Wako, Osaka,

Japan) were used as samples of hexosamines. Chondroitin sulfate (whale cartilarge), hyaturonic acid (human umbilical cord), and porcine mucine were purchased from Seikagaku Kogyo (Tokyo, Japan), Tokyo Kasei (Tokyo, Japan), and Sigma (St. Louis, Mo., U.S.A.), respectively. The urinary carbohydrate materials were obtained by dialysis of urine samples for 24 h against distilled water, followed by freeze-drying of the non-dialyzable fractions. Barium nitrite was purchased from Nakarai (Kyoto, Japan). Other chemicals were also obtained from commercial sources. They were of the highest grade available.

Apparatus

Gas chromatography was performed on a Shimadzu 4BMPF instrument equipped with a hydrogen flame ionization detector. A sodium chloride-treated capillary column (50 m × 0.28 mm I.D.) coated with SF-96 was used at 225° throughout the work. This column was supplied by Gasurkuro Kogyo (Tokyo, Japan). The flow-rate of the carrier gas (nitrogen) was regulated at 1 ml/min by use of a 100:1 splitter. The eluate was continuously mixed with the scavenger gas (nitrogen), 50 ml/min, and the mixture was introduced into the detector. Peaks were integrated by a Shimadzu Chromatopak E1A integrator.

Hydrolysis of carbohydrate materials

A sample of a carbohydrate material (1–2 mg) or a mixture of carbohydrate materials contained in an ampoule was dissolved in 4 M hydrochloric acid (200 μl), and the ampoule was flushed with nitrogen, sealed, heated for 6 h on a boiling water-bath, and then opened. The solution was transferred with water washings to a reaction tube (5 cm × 5 mm I.D.), and evaporated to dryness under reduced pressure in a desiccator containing sodium hydroxide.

Analysis of hexosamines

A 0.1 M aqueous solution of 3-O-methyl-D-glucose (internal standard, 10–100 μl) was added to a sample of a hexosamine hydrochloride (0.1–1 μmole) or a mixture of hexosamine hydrochlorides contained in a reaction tube, or a hydrolysate obtained as above, and the mixture was evaporated to dryness under reduced pressure in a desiccator containing sodium hydroxide. To the residue were added a freshly prepared cold solution (100 μl) of 0.1 M barium nitrite and cold 0.1 M sulfuric acid (75 μl), and the mixture was kept for 1 h in an ice-bath. The mixture was evaporated in the same manner, and 20 μl of ethanethiol–trifluoroacetic acid (2:1, v/v) was added to the residue. The reaction tube was closed tightly with a polyethylene stopper, and kept for 10 min at 25°. Then pyridine (50 μl), hexamethyldisilazane (100 μl), and tri-methylchlorosilane (50 μl) were added, in that order, and the mixture was incubated for 30 min at 50° with occasional shaking. The mixture was centrifuged, and the 1-μl sample of the supernatant was analysed by gas chromatography. A mixture of authentic specimens of hexosamines was treated in the same manner, and the amounts of hexosamines in the sample were calculated by comparing the peak areas of hexosamine derivatives for the sample, relative to the internal standard, with those for the authentic specimens.

RESULTS AND DISCUSSION

Nitrous acid deamination of hexosamines is usually performed with a large excess of sodium nitrite in hydrochloric acid. However, the results indicated that the bulky crystals of resultant sodium chloride hampered the following mercaptalation process. The use of *n*-butyl nitrite in the presence of acid catalysts, such as hydrochloric acid and trifluoroacetic acid, resolved this problem, but the rate of deamination was slow even at high temperatures. Eventually, we adopted the combination of barium nitrite and sulfuric acid. The resultant precipitates of barium sulfate could be removed easily by centrifugation, but this operation could be omitted by minimizing the amounts of the nitrite and the acid used, as the loss of products was insignificant under such conditions.

Both 2,5-anhydro-D-mannose and -D-talose derivatives, formed respectively from 2-amino-2-deoxy-D-glucose and -D-galactose, gave single gas chromatographic peaks in the pentose region, and their separation was complete (Fig. 1), when they were chromatographed under the same conditions as described for the analysis of neutral monosaccharides and uronic acids (Scot SF-96 capillary column, 50 m, 225°). The resolution of 2,5-anhydro-D-mannose from D-xylose and/or D-ribose was poorer but satisfactory, unless too much of the sample was applied to the column. The use of inositol as the internal standard was inappropriate, because the peak of its derivative was superimposed on that of 2,5-anhydro-D-talose. Therefore, 3-O-methyl-D-glucose was chosen for the internal standard; its derivative appeared between the 6-deoxyhexose and uronic acid regions.
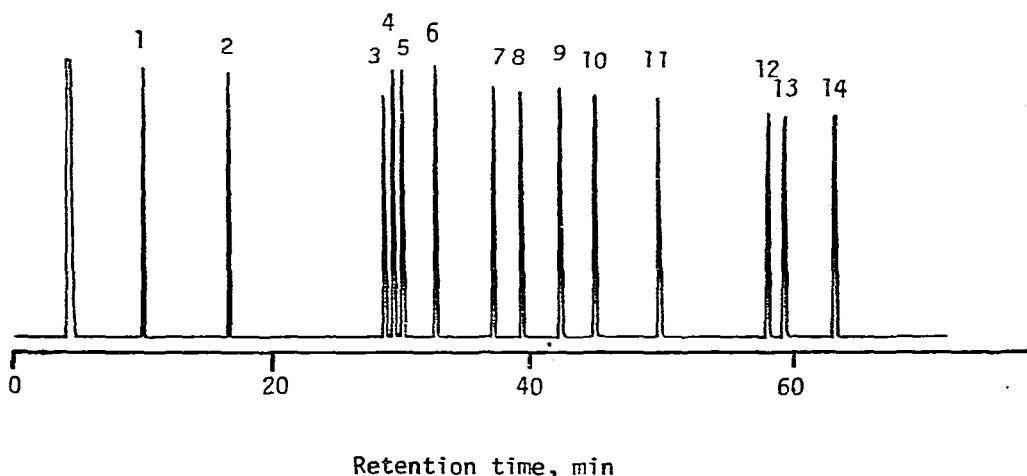

Retention time, min

Fig. 1. Gas chromatogram of a mixture of the derivatives of hexosamines, neutral monosaccharides and uronic acids. Peaks are assigned to the trimethylsilylated diethyl dithioacetal derivatives of D-glyceraldehyde (1), D-erythrose (2), 2,5-anhydro-D-mannose derived from 2-amino-2-deoxy-D-glucose (3), D-xylose (4), L-arabinose (5), 2,5-anhydro-D-talose derived from 2-amino-2-deoxy-D-galactose (6), L-rhamnose (7), L-fucose (8), 3-O-methyl-D-glucose (internal standard) 9, D-galacturonic acid (10), D-glucuronic acid (11), D-glucose (12), D-mannose (13) and D-galactose (14).

Table I gives the comparative yields of the 2,5-anhydro-D-mannose derivative formed on deamination of 2-amino-2-deoxy-D-glucose with barium nitrite and sulfuric acid under various conditions. The maximal yield was obtained when the hexosamine

TABLE I

OPTIMIZATION OF REACTION CONDITIONS FOR DEAMINATION OF 2-AMINO-2-DEOXY-D-GLUCOSE WITH BARIUM NITRITE AND SULFURIC ACID

Barium nitrite		Sulfuric acid		Relative yield of 2,5-anhydro-D-mannose
Concentration (M)	Volume (μl)	Concentration (M)	Volume (μl)	
0.1	100	0.1	25	93
0.1	100	0.1	50	97
0.1	100	0.1	75	100
0.1	100	0.1	100	23
0.1	100	0.1	125	11
0.2	100	0.2	25	70
0.2	100	0.2	50	89
0.2	100	0.2	75	86
0.2	100	0.2	100	14
0.2	100	0.2	125	11
0.3	100	0.3	25	62
0.3	100	0.3	50	36
0.3	100	0.3	75	32
0.3	100	0.3	100	10
0.3	100	0.3	125	7

was treated with 0.1 M barium nitrite (100 μl) and a slightly smaller volume (75 μl) of 0.1 M sulfuric acid at 0°. The use of larger volumes of sulfuric acid caused a marked decrease of yield. With nitrite and acid solutions of higher concentration, the yield of
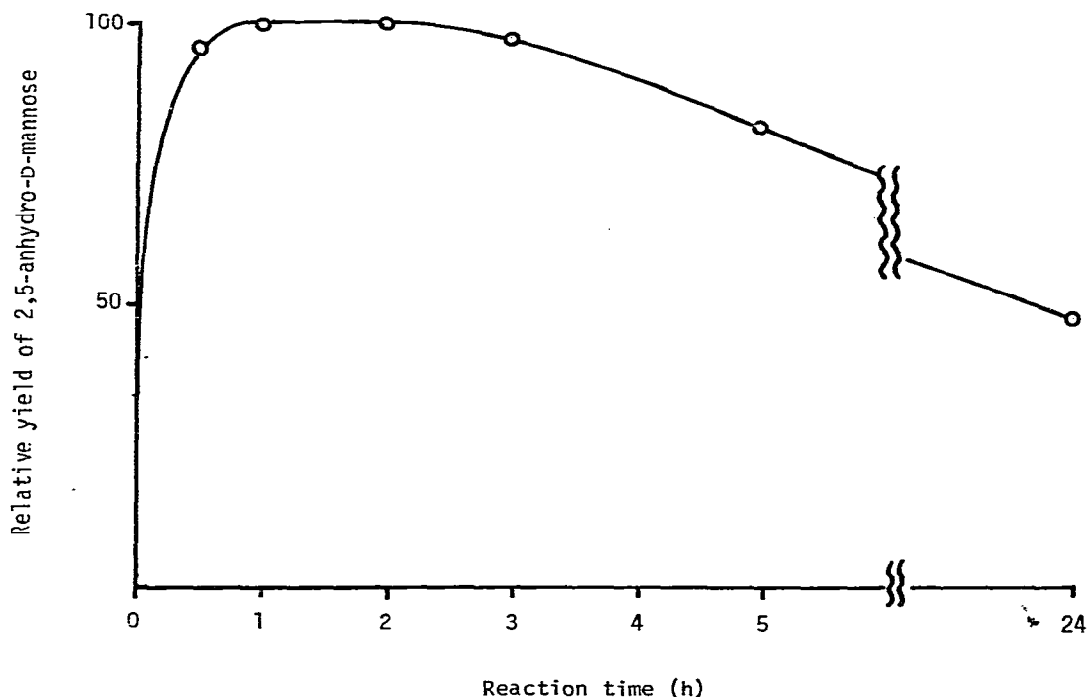

Fig. 2. Course of the deamination of 2-amino-2-deoxy-D-glucose.

the 2,5-anhydro-D-mannose derivative was lower, probably owing to some of the product being adsorbed on the surface of the precipitates.

Fig. 2 shows the course of the deamination of 2-amino-2-deoxy-D-glucose under the optimum conditions mentioned above. The reaction was very rapid, and a plateau was reached in 1 h. After 5 h the product decomposed gradually, and the yield of 2,5-anhydro-D-mannose was reduced to 48% after 24 h. No remarkable difference in yield was observed between the reactions in open and capped vessels at 0°, but higher reaction temperatures were unprofitable. The conditions for mercaptalation and trimethylsilylation have been already established[4,5].

On the basis of the results obtained above, a procedure was devised for the analysis of hexosamines, which are simpler and more rapid than any other reported methods[1-3]. Although the authentic specimens of 2,5-anhydrohexoses were unavailable and their absolute yields were unknown, the gas chromatogram obtained under the optimum conditions indicated that there were no peaks other than those of 2,5-anhydrohexose derivatives for both hexosamines. Both hexosamines gave their 2,5-anhydrohexose derivatives, which had the same molar response factor (0.73)
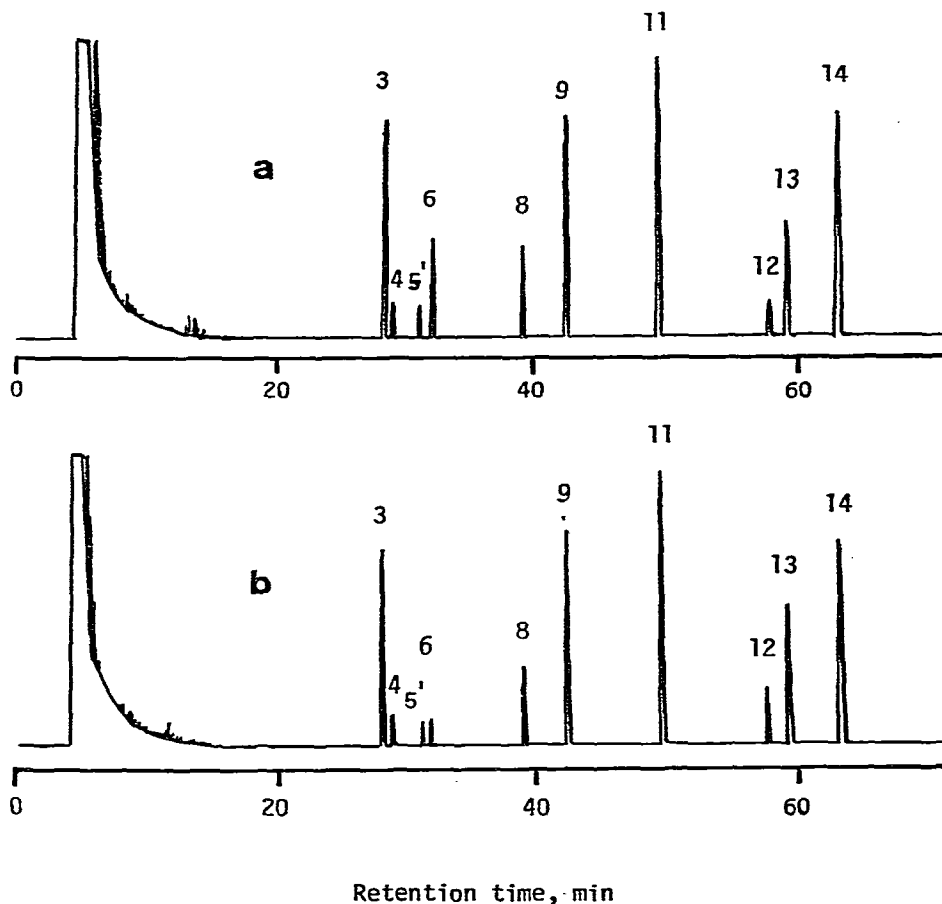

Retention time, min

Fig. 3. Gas chromatogram for the hydrolysate of the non-dialysable fraction of the urine sample from a patient bearing breast cancer (a) and that from normal female adult (b). The numbering is the same as in Fig. 1 except for 5' (unknown peak).
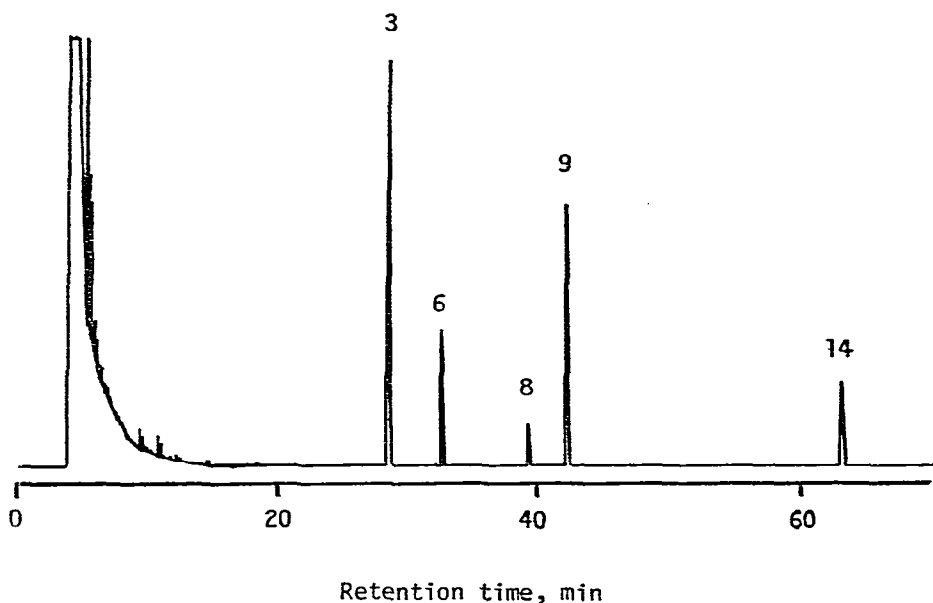
Retention time, min

Fig. 4. Gas chromatogram for the hydrolysate of porcine mucine. The numbering is the same as in Fig. 1.

relative to 3-O-methyl-D-glucose, with coefficients of variation less than 3% for 1-μmole samples. The presence of neutral monosaccharides and uronic acids had no influence on the determination of hexosamines. On the other hand, the determination of neutral monosaccharides and uronic acids was not affected by pretreatment with the deaminating reagents.
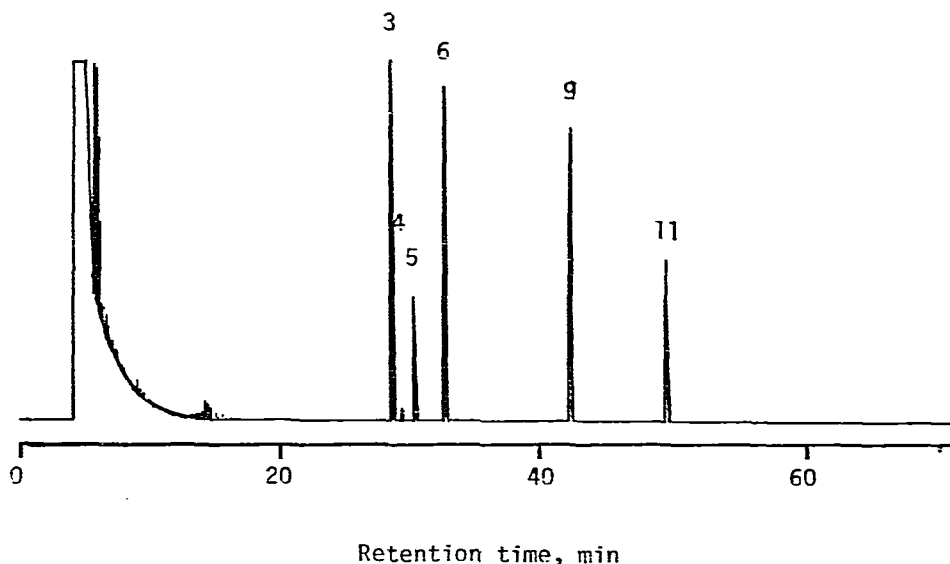

Retention time, min

Fig. 5. Gas chromatogram for the hydrolysate of an artificial mixture of chondroitin sulfate A from whale cartilage and hyaluronic acid from human umbilical cord. The numbering is the same as in Fig. 1.

Fig. 3 shows an example of the application of the method to the determination of hexosamines in the non-dialysable fractions of human urines. The samples were hydrolysed for 6 h in 4 M hydrochloric acid at 100° in a nitrogen atmosphere. Under these conditions the amounts of hexosamines released reached the maximal values. It was noticeable that the molar ratio of 2-amino-2-deoxy-D-galactose to 2-amino-2-deoxy-D-glucose for the urine sample of a patient bearing breast cancer (a) was considerably higher than that for a normal urine sample (b). This may indicate possible diagnostic value.

Fig. 4 shows the chromatogram for the hydrolysate of porcine mucine. Both the 2,5-anhydro-D-mannose and -D-talose derivatives were detected, together with the derivatives of neutral monosaccharides. Fig. 5 shows the chromatogram for the hydrolysate of an artificial mixture of chondroitin sulfate A and hyaluronic acid. A considerable part of D-glucuronic acid, the common component uronic acid, decomposed during hydrolysis to yield D-arabinose, but approximately equal amounts of 2-amino-2-deoxy-D-glucose in hyaluronic acid and 2-amino-2-deoxy-D-galactose in chondroitin sulfate A were detected as the corresponding 2,5-anhydrohexose derivatives. A small amount of D-xylose, resulting from the carbohydrate–peptide interfacial positions in these proteoglycans, was also detected.

The foregoing examples demonstrate the usefulness of this procedure for the determination of the constituent hexosamines in carbohydrate materials.

REFERENCES

1 J. R. Clamp, G. Dawson and L. Hough, *Biochim. Biophys. Acta*, 148 (1967) 342.
2 Z. Tamura, T. Imanari and Y. Arakawa, *Chem. Pharm. Bull.*, 16 (1968) 1864.
3 R. Varma and R. S. Varma, *J. Chromatogr.*, 139 (1977) 303.
4 S. Honda, N. Yamauchi and K. Kakehi, *J. Chromatogr.*, 169 (1979) 287.
5 S. Honda, Y. Fukuhara and K. Kakehi, *Anal. Chem.*, 55 (1978) 55.