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Note

Gas chromatographic determination of neutral sugars from glycoproteins and acid mucopolysaccharides as aldononitrile acetates*

RAJENDRA VARMA, RANBIR S. VARMA, WILLIAM S. ALLEN and AHMAD H. WARDI
Biochemical Research Department, Warren State Hospital, Warren, Pa. 16365 (U.S.A.)
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The development of gas–liquid chromatographic (GLC) methods for the detection and quantitative analysis of neutral sugars from glycoproteins and the carbohydrate–protein linkage region of acid mucopolysaccharides has been the subject of great interest and study. In a recent paper[1] we reported the GLC separation of the aldononitrile acetates of 12 neutral sugars. In this method, the reducing neutral sugars were released from polysaccharides by acid hydrolysis and converted into their aldononitrile acetates, which are very stable derivatives and give single, well separated peaks. The trimethylsilylated aldononitriles[2], which also give single peaks, were not used owing to their unstable nature.

This note reports the successful application of the aldononitrile acetate method to the identification and quantitation of neutral sugars from glycoproteins and acid mucopolysaccharides. In this method, the neutral sugars were released as their methyl glycosides using methanolysis, thereby minimizing the possibility of side reactions[3,4]. The hexosamines and the amino acids were removed by passage through Bio-Rad AG 50W-X8 (H^+). The methyl glycosides were hydrolyzed to free sugars, which were derivatized to their aldononitrile acetates and subjected to GLC analyses. Removal of hexuronic acid, which is one of the major components of acid mucopolysaccharides, is not necessary.

The common derivatives of neutral sugars used for GLC, the trimethylsilyl[5,6], acetyl and trifluoroacetyl and the trimethylsilylated and the acetylated methyl glycosides, give rise to multiple peaks for each sugar, producing a complex chromatogram, which makes quantitation difficult. The trimethylsilylated and acetylated alditols give single peaks but in the xylose series they have a limited use owing to the symmetry of xylitol[7]. Also, arabinose and lyxose cannot be resolved as they yield the same alditol. The aldononitrile acetate method overcomes these difficulties.

EXPERIMENTAL

Materials

Pyridine, acetic anhydride and hydroxylamine hydrochloride were obtained from Applied Science Labs., State College, Pa., U.S.A.

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All standard sugars and bovine thyroglobulin were obtained from Sigma, St. Louis, Mo., U.S.A. Human gamma-globulin was obtained from Mann Research Labs., New York, N.Y., U.S.A. Purified veal brain chondroitin 4-sulfate was prepared by the method of Wardi *et al.*[8]. Silver carbonate was obtained from Fisher Scientific, Pittsburgh, Pa., U.S.A. Bio-Rad AG 1-X2 (Cl^-, 200–400 mesh) and AG 50W-X8 (H^+, 200–400 mesh) were obtained from Bio-Rad Labs., Richmond, Calif., U.S.A. AG 1-X2 (HCO_3^-) was prepared by passing 2 *N* sodium hydrogen carbonate through a column of AG 1-X2 (Cl^-) and washing thoroughly with water.

Drying procedures

Chloroform, sugars, glycoproteins and acid mucopolysaccharides were dried by the procedure described previously[1]. The drying of methanol and the preparation of methanolic HCl was carried out by the procedure of Clamp *et al.*[9].

Gas chromatography

GLC analyses of aldononitrile acetates were carried out as described by Varma *et al.*[1], except that the length of the column (10% w/w LAC-4R-886 polyester wax on Chromosorb W, AW, 100–200 mesh) used was 4 ft. instead of 5 ft., as a shorter column gives a more rapid determination.

Preparation of standards

A 5-ml volume of the standard solution, containing 10 μmole/ml of each monosaccharide, was prepared from a dried mixture containing 7.5 mg of xylose, 8.2 mg of fucose and 9 mg each of mannose, glucose and galactose. A 5-ml volume of the internal standard solution was prepared from 7.5 mg of dried arabinose. Volumes of 0.5 ml of each of the internal standard and the standard solution were mixed in a 25-ml ground-glass round-bottomed flask and evaporated to dryness under reduced pressure at 40°. Then 0.8 *N* HCl in dry methanol (10 ml) was added and the mixture refluxed for 24 h. After cooling, the pH of the solution was adjusted to 4–5 with silver carbonate and the precipitate was removed by filtration through a sintered-glass funnel. The filtrate and the methanol washings were evaporated to dryness under reduced pressure at 40°. After removal of amino components through an AG 50W-X8(H^+) column (1 × 25 cm), the residue containing the methyl glycosides was hydrolyzed with 0.5 ml of 2 *N* HCl at 100° for 2 h. After cooling, the pH of the solution was adjusted to 4–5 with AG 1-X2 (HCO_3^-). The resin was removed by filtration and the filtrate and water washings were evaporated to dryness under reduced pressure at 40°. The residue was dissolved in about 1 ml of distilled water and freeze-dried in a 5-ml glass ampoule. The sugars in the freeze-dried solid were derivatized to their aldononitrile acetates by the procedure of Varma *et al.*[1].

The amounts of the various reagents used were: pyridine 0.5 ml, hydroxylamine hydrochloride 9 mg and acetic anhydride 1.5 ml. The residue at the end of the reaction was dissolved in 0.1–0.2 ml of dry chloroform and a 2–4-μl sample was used for GLC analysis.

Preparation of samples for chromatography

A 10-mg amount of the dried glycoprotein or the acid mucopolysaccharide was weighed into a 25-ml flask and 0.5 ml of the internal standard solution of arabinose (7.5 mg per 5 ml) was added. The mixture was evaporated to dryness under

reduced pressure at 40° and the residue was dissolved in 10 ml of 0.8 *N* HCl in dry methanol and subjected to all the subsequent steps used for the standard sugars. The amounts of the various reagents used for the derivatization to aldononitrile acetates were: pyridine 0.5 ml, hydroxylamine hydrochloride 2 mg and acetic anhydride 1.5 ml. The residue was dissolved in 0.02–0.03 ml of dry chloroform and a 5–10-μl sample was used for GLC analysis.

RESULTS AND DISCUSSION

The results given in Table I were calculated using the following relationship:

$$\text{X in sample (\%)} = \frac{(\mu\text{mole of X in standard}) \cdot \left(\frac{A_x}{A_{is}} \text{ in sample}\right) \cdot (\text{mol. wt. of X})}{(\text{mg of sample}) \cdot \left(\frac{A_x}{A_{is}} \text{ in standard}\right) \cdot 10}$$

where X=carbohydrate for which the analysis is being carried out, A_x=area of peak representing carbohydrate for which the analysis is being carried out and A_{is}= area of peak representing the internal standard sugar. These results were identical

TABLE I

PERCENTAGE OF NEUTRAL SUGARS IN BOVINE THYROGLOBULIN, HUMAN GAMMA-GLOBULIN AND VEAL BRAIN CHONDROITIN 4-SULFATE OBTAINED BY THE ALDONONITRILE ACETATE METHOD AND THE REPORTED VALUES

Sugar	*Thyroglobulin*		*Gamma-Globulin*		*Chondroitin 4-Sulfate**
	Reported values[10]	*Aldononitrile acetate method*	*Reported values*[11]	*Aldononitrile acetate method*	*Aldononitrile acetate method*
Fucose	0.41	0.43	0.29	0.28	–
Xylose	–	–	–	–	0.89
Mannose	2.32	2.31	0.75	0.75	–
Galactose	1.34	1.34	0.45	0.45	2.07

* No reported values are available. However, the molar ratio xylose: galactose is 1:2, as determined from the experimental percentage.

with the values calculated by the use of molar adjustment factors of individual sugars, which were determined by the procedure of Clamp *et al.*[9]. The reported percentage of fucose, mannose and galactose in thyroglobulin[10] and gamma-globulin[11] and those determined by the use of aldononitrile acetates are in good agreement with each other. No literature values are available for the percentage of xylose and galactose in veal brain chondroitin 4-sulfate; the experimental values are listed in Table I.

Fig. 1 illustrates the GLC separation of the aldononitrile acetates of a mixture

containing the standard sugars that commonly occur in glycoproteins and acid mucopolysaccharides. The retention times of the aldononitrile acetates are shown in Table II. Figs. 2, 3 and 4 represent typical chromatograms for the GLC separation of the aldononitrile acetates of neutral sugars from bovine thyroglobulin, human gamma-globulin and veal brain chondroitin 4-sulfate, respectively.

Glycoproteins and acid mucopolysaccharides were hydrolyzed by methanolysis[4,9], as this procedure protects the liberated reducing groups as the methyl

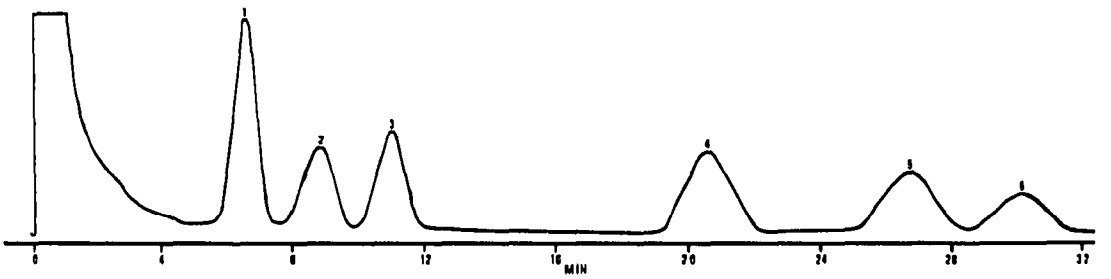

Fig. 1. GLC separation of the aldononitrile acetates of a mixture of the standard sugars. (1) Fucose; (2) arabinose (internal standard); (3) xylose; (4) mannose; (5) glucose; (6) galactose.

TABLE II

RETENTION TIMES OF ALDONONITRILE ACETATES OF NEUTRAL SUGARS THAT COMMONLY OCCUR IN GLYCOPROTEINS AND ACID MUCOPOLYSACCHARIDES

Sugar	*Retention time (min)*
Fucose	6.6
Arabinose	8.8
Xylose	11.0
Mannose	20.6
Glucose	26.8
Galactose	30.2

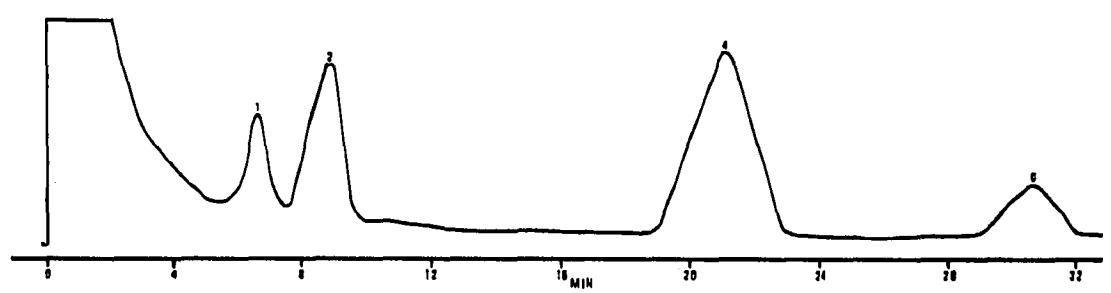

Fig. 2. GLC analysis of the aldononitrile acetates of neutral sugars from bovine thyroglobulin. (1) Fucose; (2) arabinose (internal standard); (4) mannose; (6) galactose.

glycosides, thereby minimizing the possibility of side reactions[3,4]. For the hydrolysis of glycoproteins and acid mucopolysaccharides, our studies (varying time and acidity) showed that the best procedure for optimal release of neutral sugars was to reflux with 0.8 *N* methanolic HCl for 24 h. The methanolyzed mixtures were neutralized, the solutions evaporated to dryness, the residues dissolved in water and the final solutions passed through AG 50W-X8 (H^+, 200–400 mesh) in order to remove the amino acids and the methyl glycoside of hexosamine. However, glucuronic acid did not interfere and was not removed. Finally, the methyl glycosides were hydrolyzed and the free sugars were derivatized to their aldononitrile acetates.

Polarimetric studies on the hydrolysis of standard methyl glycosides showed that the best conditions for the maximal release and minimal destruction of free sugars were heating at 100° with 2 *N* HCl for 2 h.

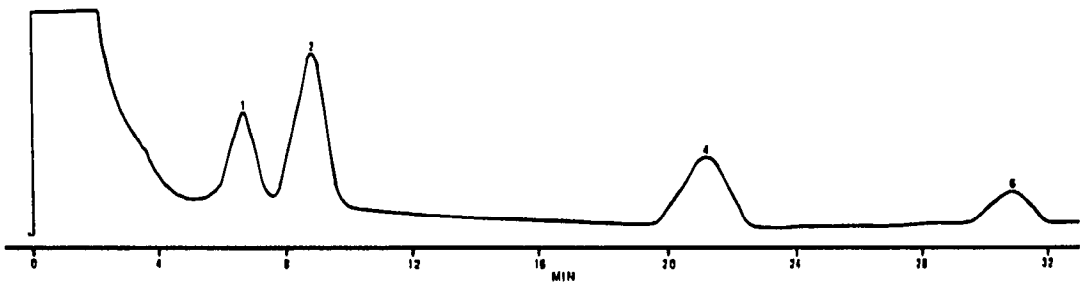

Fig. 3. GLC analysis of the aldonitrile acetates of neutral sugars from human gamma-globulin. (1) Fucose; (2) arabinose (internal standard); (4) mannose; (6) galactose.

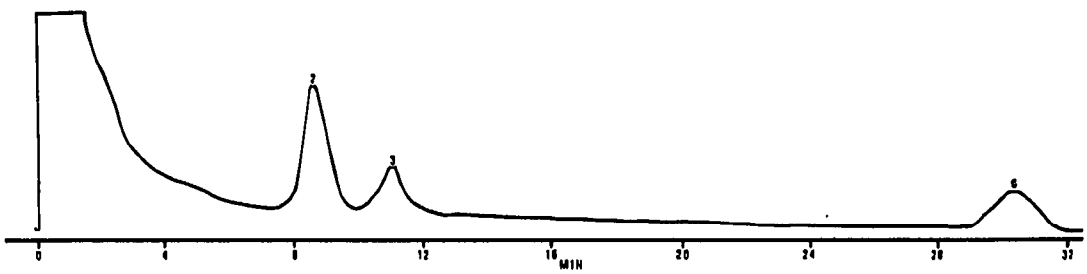

Fig. 4. GLC analysis of the aldononitrile acetates of the neutral sugars from veal brain chondroitin 4-sulfate. (2) Arabinose (internal standard); (3) xylose; (6) galactose.

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