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SIMULTANEOUS DETERMINATION OF NEUTRAL SUGARS AND HEXOSAMINES IN GLYCOPROTEINS AND ACID MUCOPOLYSACCHARIDES (GLYCOSAMINOGLYCANS) BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A reliable and reproducible method for the simultaneous determination of neutral sugars and hexosamines in glycoproteins and acid mucopolysaccharides has been described. It involves the following steps: the release of neutral sugars and hexosamines from biopolymers by resin-catalysed hydrolysis, the nitrous acid deamination of resin-bound hexosamines in this hydrolysate to aldoses, and the determination of these newly formed anhydroaldoses together with the pre-existing neutral sugars as aldononitrile acetates by gas chromatography. Applications of this method to analyses of glycoprotein from urine of schizophrenic patients, bovine thyroglobulin, human brain glycoprotein, chondroitin sulfates from bovine vitreous humor, and veal brain, and human umbilical cord hyaluronic acid are given.

INTRODUCTION

Neutral sugars and hexosamines are important constituents of glycoproteins and acid mucopolysaccharides. Gas chromatography offers a sensitive and convenient method for the identification and determination of these components. The advantages and limitations of the use of various volatile derivatives for gas chromatography of carbohydrates have been reviewed recently[1,2]. The trimethylsilyl derivatives are more commonly employed for determination of neutral sugars and hexosamines. The trimethylsilyl derivatives of the aldoses and hexosamines give multiple peaks per sugar due to anomerization and ring isomerization making interpretation of the chromatogram difficult. However, the acetylated and trimethylsilylated alditols give single peaks, but the pair arabinose–lyxose yields the same alditol. Also, no single liquid phase or a combination of different liquid phases packed in a single column is available for simultaneous resolution of the alditol acetates of the sugar pairs fucose–rhamnose, arabinose–ribose, lyxose–arabinose, glucose–galactose, and glucosamine–galactosamine[1,2].

A recent publication[3] utilizing the application of nitrous acid deamination of glycoprotein hydrolysates for the simultaneous determination of neutral sugars and

hexosamines as alditol acetates is of limited use. The peaks for xylitol and 2,5-anhydromannitol formed from xylose and glucosamine, respectively, overlap and hence this method cannot be used for analyses of glycosaminoglycans (many of which contain both xylose and glucosamine[4]), and for glycoproteins in which the pairs xylose–glucosamine[5] and fucose–rhamnose[6] occur together. The gas chromatogram given in this publication[3] shows the separation of only five standard neutral sugars and excludes some commonly occurring monosaccharides.

In a previous publication[7] we reported the separation of twelve neutral sugars by gas–liquid chromatography (GLC) of their aldononitrile acetates on a single column and resolved the afore-mentioned pairs of neutral sugars. The successful applications of this aldononitrile acetate method to the analysis of neutral sugars[8] and hexosamines[9] from glycoproteins and acid mucopolysaccharides were the subjects of our later publications. The chromatographic conditions used in these procedures did not allow the simultaneous separation of deamination products of hexosamines and other neutral sugars. We now report a procedure for the simultaneous determination of neutral sugars and hexosamines from both glycoproteins and acid mucopolysaccharides. The method described here involves the following steps: the release of neutral sugars and hexosamines from biopolymers by resin-catalysed hydrolysis, the nitrous acid deamination of the resin-bound hexosamines in this hydrolysate to aldoses and the simultaneous determination of these newly formed aldoses and the pre-existing neutral sugars as their aldononitrile acetates by GLC. This method overcomes the afore-mentioned difficulties encountered with the previous methods in the resolution of the sugar pairs fucose–rhamnose, arabinose–lyxose, arabinose–ribose, glucose–galactose, and galactosamine–mannosamine. The separation of xylose and 2,5-anhydromannose (from deamination of glucosamine) is fair, which allows the quantitation of these components when present together in a biopolymer. Also, the aldononitrile acetates are stable derivatives and are more easily prepared than alditol acetates, which need time-consuming repeated evaporations of the reduced mixture for removal of borate as methyl borate.

EXPERIMENTAL

Materials

The acetylating kit containing dry pyridine and acetic anhydride, and hydroxylamine hydrochloride were supplied by Applied Science Labs. (State College, Pa., U.S.A.). The resins AG 50W-X8 (H^+, 200–400 mesh) and AG 1-X2 (Cl^-, 200–400 mesh) were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). The resin AG 1-X2 (HCO_3^-) was prepared by passing 2 *M* sodium hydrogen carbonate solution through a column of AG 1-X2 (Cl^-) and washing thoroughly with deionized water.

Human umbilical cord hyaluronic acid was prepared by the procedure described previously[10]. All standard sugars and bovine thyroglobulin were obtained from Sigma (St. Louis, Mo., U.S.A.). The chondroitin sulfates from calf brain and bovine vitreous humor were obtained by the procedure[11] described elsewhere. Urinary glycoprotein from urine of schizophrenic patients was prepared[12] as 0.35 *M* NaCl fraction by anion-exchange chromatography. Human brain glycoprotein was obtained as 0.1 *M* NaCl fraction according to a previously published procedure[13].

Hydrolysis of glycoproteins and acid mucopolysaccharides

The glycoproteins and acid mucopolysaccharides were hydrolysed by a previously established resin-catalysed hydrolysis procedure[14]. A 2–3 mg sample of dry biological material was dissolved in 0.1 ml of water containing an appropriate amount (20–50 μg) of a neutral sugar not present in that biological material as the internal standard. The hydrolysis was carried out at 100° in a sealed glass ampoule with 0.5–0.6 ml of a 40% (w/v) suspension of the AG 50W-X8 (H^+) resin in 0.02 *M* HCl. For this quantity of the biological material, total neutral sugar and, or total hexosamine content of the material should not exceed 20–25%. The quantities of the biological material and the resin can be scaled down to one-half to one-third, if restricted by availability of the material or otherwise. Since deamination has to be later carried out in the same ampoule, care should be taken to seal only the tip of this ampoule.

Deamination

The sealed ampoule containing the hydrolysate was cooled to room temperature, the tip of the ampoule was broken, and a freshly prepared solution of 35 mg of sodium nitrite in 0.15 ml water was added. This ampoule was sealed again quickly by heating only its top portion, carefully avoiding any heating of the solution. After cooling it was subjected to intermittent vortexing at room temperature for 30 min for completion of deamination.

Isolation of the mixture of aldoses and anhydroaldoses

After deamination, the ampoule was opened and a pinch of AG 50W-X8 (H^+) resin was added to the deaminated mixture to convert the unused sodium nitrite to nitrous acid. After shaking the ampoule for a couple of minutes, its contents were transferred to a set of two separate columns, the upper column containing AG 50W-X8 (H^+) and draining into the lower one packed with AG 1-X2 (HCO_3^-). Each column was made up of a 10-ml Kimble glass disposable serological pipette (Owens-Illinois, Toledo, Ohio, U.S.A.), plugged with glass wool and packed to a 5-ml capacity with the resin. A total volume of 30–35 ml of the eluate and deionized water washings were collected and evaporated to a small volume in a rotary evaporator at room temperature. The concentrated solution was freeze-dried in a 5-ml ampoule.

Derivatization to aldononitrile acetates

To this glass ampoule were added 6–7 mg of hydroxylamine hydrochloride and 8–10 drops of dry pyridine. The tip of the ampoule was sealed and it was heated in an oven at 90° for 45 min. The ampoule was cooled to room temperature, the tip was broken, and 25–30 drops of dry acetic anhydride were added. The ampoule was resealed and heated in the oven for another 45 min. The cooled solution was evaporated to dryness with a stream of nitrogen. The residue was dissolved in 25 μl of dry chloroform and an appropriate volume was injected into the gas chromatograph.

Preparation of standards

The neutral sugars and hexosamines were dried and a 2-ml volume of a standard solution containing 2 mg of each of the neutral sugars, 1 mg of the desired internal standard, and 3 mg of each hexosamine was prepared. A 0.1-ml sample of this solution containing 100 μg of each of the neutral sugars and 150 μg of each hexos-

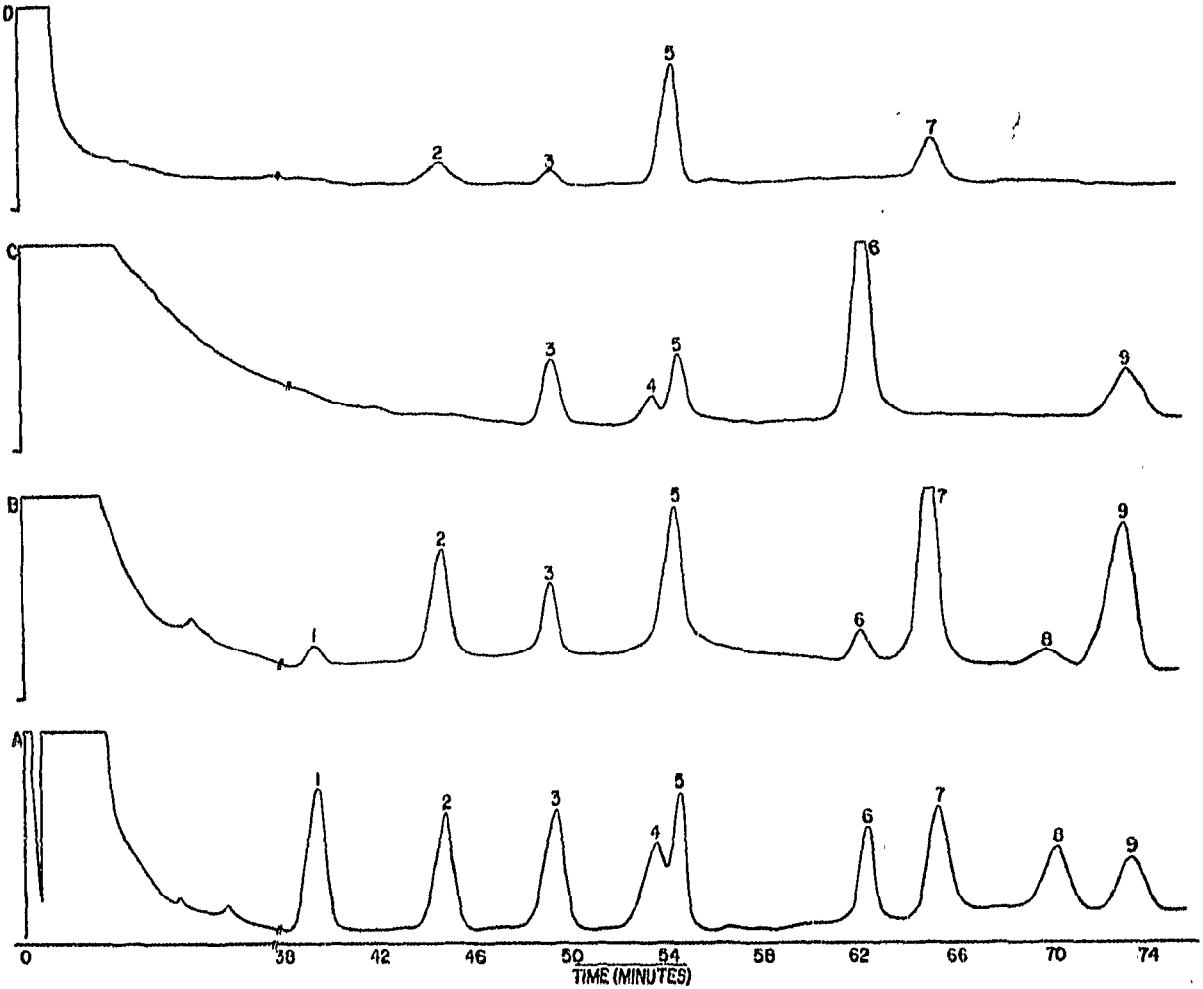

Fig. 1. (A), GLC separation of the aldononitrile acetates of standard neutral sugars and aldoses obtained by the deamination of glucosamine and galactosamine. 1 = Rhamnose; 2 = fucose; 3 = arabinose; 4 = xylose; 5 = glucosamine; 6 = galactosamine; 7 = mannose; 8 = glucose; 9 = galactose. (B), GLC analysis of the aldononitrile acetates of neutral sugars and deaminated hexosamines from the glycoprotein obtained from the urine of schizophrenic patients. 1 = Rhamnose; 2 = fucose; 3 = arabinose (internal standard); 5 = glucosamine; 6 = galactosamine; 7 = mannose; 8 = glucose; 9 = galactose. For the determination of mannose, a smaller volume of sample was injected. (C), GLC analysis of the aldononitrile acetates of the neutral sugars and deaminated hexosamines from veal brain chondroitin-4-sulfate. 3 = Arabinose (internal standard); 4 = xylose; 5 = glucosamine; 6 = galactosamine; 9 = galactose. For the determination of galactosamine, a smaller volume of the sample was injected. (D), GLC analysis of the aldononitrile acetates of neutral sugars and deaminated glucosamine from human umbilical cord hyaluronate. For the detection of glucose (< 0.02%), a large volume of the sample had to be injected. 2 = Fucose; 3 = arabinose; 5 = glucosamine; 7 = mannose (internal standard).

amine was subjected to all the afore-mentioned steps of hydrolysis, deamination, and derivatization to aldononitrile acetates.

Gas chromatography

GLC analyses were carried out on a series 1200 Varian Aerograph equipped with a flame ionization detector and a Model SRG Sargent-Welch recorder. A stainless-steel column (9 ft. × 1/8 in.) packed with 3% poly(neopentylglycol succinate) on Gas-Chrom W-AW (60–80 mesh) was used. The gas chromatograph was initially programmed from 130–195° at 1°/min for 65 min and then operated isothermally at 195°. Nitrogen was used as the carrier gas at a flow-rate of 24 ml/min. The injector and detector temperatures were maintained at 230°.

RESULTS

Fig. 1A illustrates the separation of a mixture of standard neutral sugars and anhydrohexoses from deamination of hexosamines. The retention times of the aldononitrile acetates of these aldoses and anhydroaldoses are shown in Table I. Fig. 1B shows a typical chromatogram for the separation of neutral sugars and hexosamines in urinary glycoprotein from schizophrenic patients. This chromatogram is given for representing the applicability of this method to glycoproteins because of the presence in it of a wide variety of neutral and amino sugar components, *i.e.*, rhamnose, fucose, mannose, glucose, galactose, glucosamine, and galactosamine, in addition to arabinose present as the internal standard.

TABLE I

RETENTION TIMES OF ALDONONITRILE ACETATES OF NEUTRAL SUGARS AND DEAMINATION PRODUCTS OF HEXOSAMINES COMMONLY OCCURRING IN GLYCOPROTEINS AND ACID MUCOPOLYSACCHARIDES

Sugar	*Retention time (min)*
Rhamnose	39.4
Fucose	44.8
Arabinose	49.4
Xylose	53.6
2,5-Anhydromannose (from glucosamine)	54.6
2,5-Anhydrotalose (from galactosamine)	62.4
Mannose	65.3
Glucose	70.2
Galactose	73.4

The results given in Tables II and III were calculated using the following relationship:

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

where X = the carbohydrate for which the analysis is being carried out, A_x = the area of the peak representing the carbohydrate for which the analysis is being carried

out, and A_{is} = the area of the peak representing the carbohydrate used as the internal standard.

The percentage analyses of neutral sugars and hexosamines in schizophrenic urine, bovine thyroglobulin and human brain glycoprotein by the present method and the previously published methods[8,9] are given in Table II. The results are comparable.

TABLE II

ANALYSES OF NEUTRAL SUGARS AND HEXOSAMINES IN GLYCOPROTEINS

Carbohydrate component	*Schizophrenic urine glycoprotein (%)*		*Bovine thyroglobulin (%)*		*Human brain glycoprotein (%)*	
	Present method	*Previous method**	*Present method*	*Previous method**	*Present method*	*Previous method**
Rhamnose	0.32	0.31	—	—	—	—
Fucose	2.50	2.57	0.44	0.43	6.24	6.19
Mannose	5.50	5.57	2.30	2.31	13.52	13.43
Glucose	0.45	0.43	—	—	small amount	
Galactose	8.31	8.40	1.35	1.34	10.51	10.41
Glucosamine	11.60	11.57	2.80	2.79	18.90	18.81
Galactosamine	2.77	2.78	—	—	1.93	1.93

* For neutral sugars, see ref. 8. For hexosamines, see ref. 9. Comparable values were obtained by the amino acid analyzer method.

The applicability of this method to the simultaneous determination of neutral sugars and hexosamines in acid mucopolysaccharides (also called glycosaminoglycans) is typified in Figs. 1C and 1D, which give gas chromatograms for veal brain chondroitin-4-sulfate and human umbilical cord hyaluronic acid, respectively. The values obtained for the components of these acid mucopolysaccharides by the present method and other methods[8,9] are given in Table III and are in close agreement.

TABLE III

ANALYSES OF NEUTRAL SUGARS AND HEXOSAMINES IN ACID MUCOPOLYSACCHARIDES (GLYCOSAMINOGLYCANS)

Carbohydrate component	*Vitreous humor chondroitin-4-sulfate (%)*		*Veal brain chondroitin-4-sulfate (%)*		*Human umbilical cord hyaluronic acid (%)*	
	Present method	*Previous method**	*Present method*	*Previous method**	*Present method*	*Previous method**
Fucose	—	—	—	—	0.20	0.21
Xylose	0.86	0.89	0.52	0.52	—	—
Arabinose	—	—	—	—	0.05	0.05
Glucose	—	—	—	—	<0.02	<0.02
Galactose	1.99	2.07	1.30	1.29	—	—
Glucosamine	5.28	5.31	3.76	3.74	37.00	37.20
Galactosamine	21.39	21.70	17.61	17.30	—	—

* For neutral sugars, see ref. 8. For hexosamines, see ref. 9. Values obtained by the amino acid analyzer method were comparable.

DISCUSSION

The hexosamines and neutral sugars were quantitatively released from the biopolymers by a previously established resin-catalysed hydrolysis procedure[14]. In the hydrolysates so obtained, the hexosamines and amino acids are present bound to the cationic resin, AG 50W-X8 (H^+). Nitrous acid deamination of the resin-bound hexosamines glucosamine, galactosamine and mannosamine produces 2,5-anhydromannose, 2,5- anhydrotalose, and glucose, respectively, which are released from the resin. This deamination is quantitative and is complete in 30 min under these previously established[3] conditions used in this method. In the course of this deamination process, the resin-bound amino acids are also deaminated whereby their amino groups are replaced by hydroxyl groups and these are released from the resin.

Upon passage through the tandem arrangement of columns, the cationic resin in the upper column removes Na^+ ions and the anionic resin, AG 1-X2 (HCO_3^-), present in the lower column removes hexuronic acid (from glycosaminoglycans), sialic acid (from glycoproteins), and the carboxylic acids derived from the deamination of amino acids.

Glucosamine and galactosamine are the most universally found hexosamines in nature; mannosamine is of only limited occurrence in glycoproteins from brains of some animal species[15,16] and upon deamination forms glucose[17]. However, mannosamine and glucose are not known to occur together in the same glycoprotein or mucopolysaccharide. In the case of such a rare coexistence, if any, the hexosamines may be analyzed separately by transferring the resin hydrolysate of the biopolymer to a small column of disposable pipette plugged with glass wool, collecting the eluate and washings with deionized water for neutral sugars. The hexosamines can then be eluted from the cationic resin in the column with 1 *M* HCl and deaminated by adding solid sodium nitrite to the concentrated eluate as described elsewhere[9]. Alternatively, the ampoule containing the resin hydrolysate can be put in a centrifuge tube, and centrifuged. The supernatant and two further supernatants from two washings with deionized water can be collected for the determination of neutral sugars. The hexosamines bound to the resin in the pellet can then be deaminated and determined by the procedure described above.

The reproducibility of this method was checked by repeated analyses of some of the samples. The values were within a range of 2–3%. This GLC method gave results which were comparable with those obtained by the previously published methods and, therefore, it represents an independent and reliable method having several advantages over the commonly used methods. The aldononitrile acetates are quite stable derivatives and are more easily prepared than the corresponding alditols, which need time-consuming repeated evaporations with methanol for removal of borate as methyl borate. Also, the use of aldononitrile acetates provides a solution to the aforementioned difficulties in the resolution of some sugar pairs encountered in the use of alditol acetates.

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