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Note

A gas chromatographic method for determination of hexosamines in glycoproteins and acid mucopolysaccharides

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Hexosamines constitute an appreciable portion of the carbohydrates in glycoproteins and acid mucopolysaccharides. The two common hexosamines, glucosamine (2-amino-2-deoxyglucose) and galactosamine (2-amino-2-deoxygalactose), may occur singly or may coexist in the same polysaccharide as in orosomucoid[1]. Mannosamine (2-amino-2-deoxymannose) occurs rarely and has been reported only in a few glycoproteins especially from brain[2].

The commonly used spectrophotometric methods based on the Elson–Morgan reaction[3] for free hexosamines or the Morgan–Elson reaction[4] for their N-acetyl derivatives, and the indole–HCl reaction[5] measure only the "total" hexosamines. In the Morgan–Elson reaction[4] the color yield from galactosamine is only one-third of that obtained from glucosamine. The indole–HCl reaction gives only 55 % of the value found with the Elson–Morgan reaction when applied to acid mucopolysaccharides like hyaluronic acid and chondroitin sulfates[6]. Also, the indole–HCl reaction, which measures the 2,5-anhydrosugars obtained by deamination of hexosamines, is unable to determine mannosamine since this hexosamine upon deamination gives glucose[7] and not a 2,5-anhydrosugar.

The most commonly used analytical procedures for separation and determination of the various hexosamines present in the hydrolysates of biological materials are paper chromatography[8,9], gas chromatography, automatic amino acid analyzer[10–12] and cation-exchange column chromatography followed by determination of each pooled hexosamine fraction by the Elson–Morgan reaction[13] or by some other[14] reaction. The gas chromatographic determination makes use of a number of volatile derivatives of hexosamines[15–20] or their deamination products[21,22]. The commonly used trimethylsilyl (TMS) derivatives are not stable and therefore cannot be preserved if the analysis is to be deferred for a few days. Also, except for the alditol derivatives, all other derivatives of hexosamines give multiple peaks which makes quantitation difficult. Further, the alditol acetates of hexosamines have a limited use since the alditol acetates of mannosamine and galactosamine are not resolved[22]. The present method overcomes these difficulties.

This note reports a simple and accurate procedure for the gas chromatographic determination of hexosamines in glycoprotein and acid mucopolysaccharide hydrolysates. The hexosamines from the hydrolysates are retained on a small Bio-Rad AG 50W-X8 (H^+) column, eluted with HCl and then deaminated with nitrous acid. The

2,5-anhydromannose, 2,5-anhydrotalose and glucose formed by deamination of glucosamine, galactosamine and mannosamine, respectively, are derivatized to their aldononitrile acetates[23], which are very stable and give single, well separated peaks, and are determined by gas chromatography as described by Varma *et al.*[23,24]. The sensitivity of this method is comparable to other conventional methods.

The modified deamination procedure used in this study is simpler and shorter as compared to those hitherto published[5,22,25–28]. The molar concentration of sodium nitrite used in other methods is 50–10,000-fold excess of that used in this method. Further, the use of ammonium sulfamate, needed in the other methods for decomposition of excess of nitrous acid following deamination, is eliminated in our procedure. A trace of inorganic salt formed in the present method need not be removed since it does not cause any loss of sugar due to entrapping among the salt crystals and also, its presence does not interfere with derivatization to the aldononitrile acetates. In other deamination procedures, a loss of sugar may occur due to the presence of a large amount of salt and also due to the use of large quantities of resins[29] needed for adjustment of pH and deionization.

Deamination is complete in 10–15 min as compared to the reduction procedure for alditols which takes place overnight and needs repeated time-consuming evaporations with methanol for removal of boric acid prior to acetylation. The use of alditol acetates of the deamination products of hexosamines reported by Niedermeier[22] makes the analyses even more time-consuming, and the resolution of galactosamine from mannosamine is not reported in his paper.

EXPERIMENTAL

Materials

Pyridine, acetic anhydride, and hydroxylamine hydrochloride were obtained from Applied Science Labs., State College, Pa., U.S.A. D-Glucosamine hydrochloride was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; D-galactosamine hydrochloride and human 7S γ-globulin from Mann Research Labs., New York, N.Y., U.S.A.; and D-mannosamine hydrochloride and thyroglobulin from Sigma, St. Louis, Mo., U.S.A. Sodium nitrite (C.P.) was supplied by Fisher Scientific, Pittsburgh, Pa., U.S.A. Bio-Rad AG 1-X2 (Cl^-, 200–400 mesh) and AG 50W-X8 (H^+, 100–200 mesh) were obtained from Bio-Rad Labs., Richmond, Calif., U.S.A. Bio-Rad AG 1-X2 (HCO_3^-) was prepared by passing 2 *M* sodium hydrogen carbonate through a column of Bio-Rad AG 1-X2 (Cl^-) and washing thoroughly with water. Vitreous humor hyaluronate was prepared from bovine eyes as described previously[30]. Vitreous humor chondroitin 4-sulfate was prepared by the same procedure as that employed for hyaluronate except that this sulfated acid mucopolysaccharide was eluted from the Bio-Rad AG 1-X2 (Cl^-) column as the 2 *M* NaCl fraction and was further purified by gel filtration on a Bio-Gel P-100 column.

Drying procedures

Hexosamines, glycoproteins and the acid mucopolysaccharides were dried by the procedure described previously[23].

Hydrolysis of glycoproteins and acid mucopolysaccharides and isolation of hexosamines

For a maximal release and a minimum destruction of hexosamines, the hydrolytic conditions worked out by Swann and Balazs[31] were used. A 2–5-mg sample of dried acid mucopolysaccharide or glycoprotein preparation was hydrolyzed with 1 ml of 8 *M* HCl at 95° for 3 h in a sealed ampoule. The cooled hydrolysate was evaporated to dryness in a rotary evaporator at 40°. The residue was dissolved in water and placed on a column (1 × 20 cm) of Bio-Rad AG 50W-X8 (H^+, 100–200 mesh). The column was washed with 50 ml of distilled water and the washings were discarded. The hexosamines were eluted from the column with 1 *M* HCl (60–70 ml) and the effluent was evaporated to dryness in a rotary evaporator at 40° in a 25-ml evaporating flask.

Deamination of hexosamines

The residue containing hexosamines was taken up in 0.03–0.04 ml of concentrated hydrochloric acid and diluted with 1 ml of distilled water in the same 25-ml evaporating flask. The flask was cooled to 0–5° in a bath of ice–salt mixture and 2–3 mg of solid sodium nitrite (C.P.) were added. The flask was tightly stoppered and the temperature of the solution maintained at 0–5° for 10–15 min with occasional shaking. The stopper was then removed and the flask placed in a boiling water-bath for 2–3 min. The cooled solution was diluted to 10–15 ml with distilled water and its pH adjusted to between 5 and 6 with a trace amount of Bio-Rad AG 1-X2(HCO_3^-) resin and the resin was removed by filtration. The filtrate and water washings were either evaporated to dryness in a rotary evaporator at room temperature or freeze dried in an ampoule.

Derivatization to aldononitrile acetates

The aldoses obtained by deamination were derivatized to aldononitrile acetates by the procedure of Varma *et al.*[23]. The quantities of various reagents used were: pyridine, 5 drops; hydroxylamine hydrochloride, 3 mg; and acetic anhydride, 15 drops.

Preparation of standard sugars

A dried mixture containing 1 mg each of glucosamine·HCl, galactosamine·HCl, and mannosamine·HCl was dissolved in 1 ml of 8 *M* HCl and subjected to all the subsequent steps used for hydrolysis, deamination and derivatization to aldononitrile acetates.

Gas chromatography

GLC analyses of the aldononitrile acetates were carried out as described previously[24] on a 4-ft. column packed with 10% w/w LAC-4R-886 polyester wax on Chromosorb W AW (100–200 mesh)*.

RESULTS AND DISCUSSION

Fig. 1 illustrates the GLC separation of the aldononitrile acetates of a mixture containing 2,5-anhydromannose, 2,5-anhydrotalose and glucose formed by deamination of a standard mixture of glucosamine, galactosamine and mannosamine, respec-

* Much sharper peaks resulted using a column (5 ft. × 1/8 in.) containing 3% poly (neopentylglycol succinate) on Gas-Chrom W (60–80 mesh); initial temperature 160°, programmed to 235° at 2°/min.

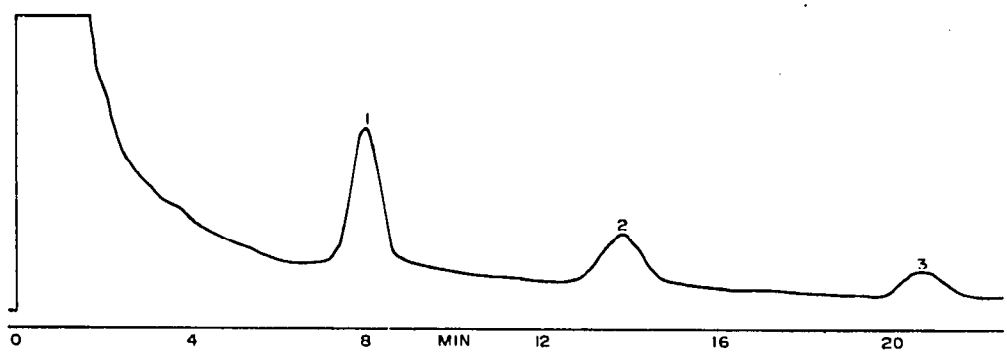

Fig. 1. GLC separation of the aldononitrile acetates of a mixture of aldoses obtained by deamination of standard hexosamines. 1 = 2,5-Anhydromannose; 2 = 2,5-anhydrotalose; 3 = glucose.

TABLE I

RETENTION TIMES OF ALDONONITRILE ACETATES OF ALDOSES FORMED BY DEAMINATION OF HEXOSAMINES

Hexosamine	*Deamination product*	*Retention time (min)*
Glucosamine	2,5-Anhydromannose	8.1
Galactosamine	2,5-Anhydrotalose	13.8
Mannosamine	Glucose	20.6

tively. The retention times of these aldononitrile acetates are shown in Table I. Figs. 2–5 represent the typical chromatograms for analyses of the hexosamines from vitreous humor chondroitin 4-sulfate, vitreous humor hyaluronate, human 7S γ-globulin and thyroglobulin, respectively. The percentage of hexosamines in these biological samples calculated by the peak area method are shown in Table II. This table shows that the results obtained by the present method are in good agreement with the results obtained by the amino acid analyzer method or with those reported in the literature.

Our studies using increasing amounts of sodium nitrite and subsequent analyses of the aldononitrile acetates of aldoses revealed that deamination was complete when the amount of this reagent was 2–3 mg for 2–5 mg of the hydrolyzed glycoprotein or

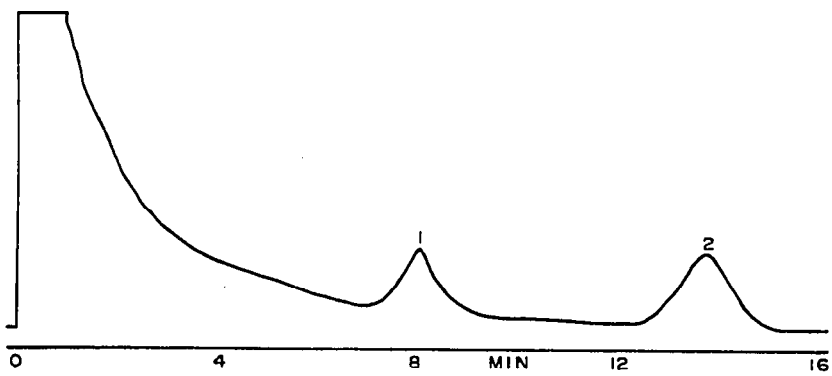

Fig. 2. GLC analysis of the aldononitrile acetates of deaminated hexosamines from vitreous humor chondroitin 4-sulfate. 1 = 2,5-Anhydromannose; 2 = 2,5-anhydrotalose.

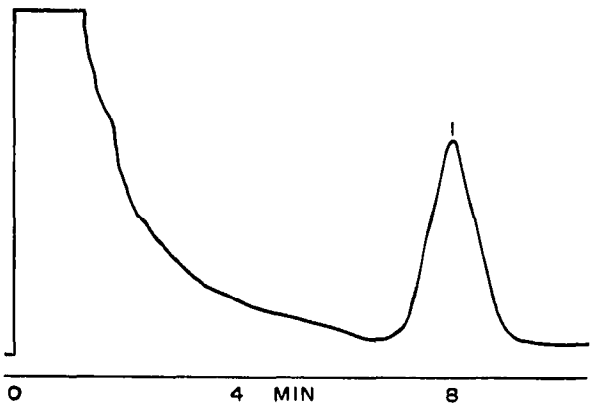

Fig. 3. GLC analysis of the aldononitrile acetate of deaminated hexosamine from vitreous humor hyaluronate. 1 = 2,5-Anhydromannose.

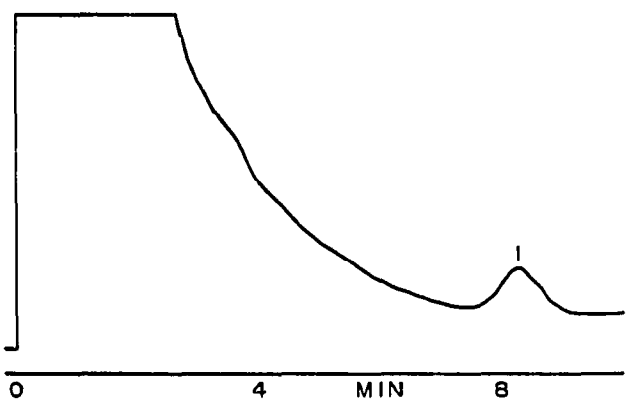

Fig. 4. GLC analysis of the aldononitrile acetate of hexosamine from human γ-globulin. 1 = 2,5-Anhydromannose.

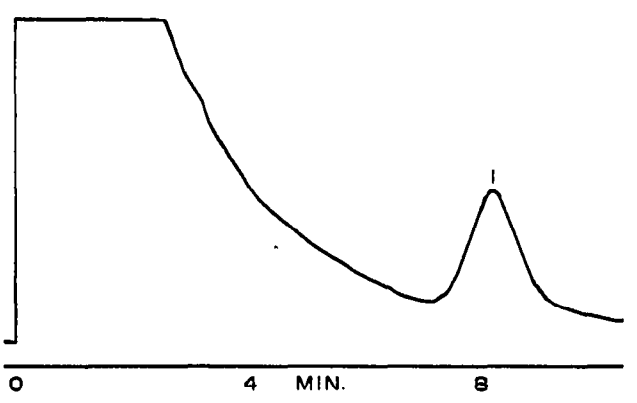

Fig. 5. GLC analysis of the aldononitrile acetate of hexosamine from thyroglobulin. 1 = 2,5-Anhydromannose.

TABLE II

PERCENTAGE OF HEXOSAMINES IN BOVINE VITREOUS HUMOR HYALURONATE, BOVINE VITREOUS HUMOR CHONDROITIN 4-SULFATE, HUMAN γ-GLOBULIN AND BOVINE THYROGLOBULIN

Hexosamines	*Vitreous humor hyaluronate*		*Vitreous humor chondroitin 4-sulfate*		*Human γ-globulin*		*Thyroglobulin*	
	Aldononitrile acetate method	*Amino acid analyzer method*[*]	*Aldononitrile acetate method*	*Amino acid analyzer method*[*]	*Aldononitrile acetate method*	*Reported values (refs. 33–35)*	*Aldononitrile acetate method*	*Reported values*[36]
Glucosamine	40.50	40.50	5.37	5.31	1.17	1.14, 1.20, 1.00	2.79	2.61
Galactosamine	–	Trace	21.25	21.70	–	–	–	–

[*] Determination of hexosamines by amino acid analyzer method was carried out by the procedure described in ref. 32.

acid mucopolysaccharide. Thus, the present method eliminates the reported use of much larger amounts of the deaminating agent and the use of ammonium sulfamate, which cause the formation of an appreciable amount of inorganic salt, which is very difficult to remove, and may cause loss of sugars.

The hexosamines are not amenable to analysis as aldononitrile acetates since, unlike neutral sugars, they do not form these derivatives under the normal experimental conditions[23]. This difficulty has been circumvented by deamination of hexosamines to aldoses. The formation of aldoses from hexosamines proceeds only when the aldehyde group of the hexosamine is free and the amino group is not acetylated. This was achieved by acid hydrolysis for which the previously described conditions[31] for a maximal release and a minimum destruction of hexosamines were used. The hexosamines released by hydrolysis were retained on a small column of a cation-exchange resin, eluted with acid and then deaminated with nitrous acid using a simple, modified procedure. The 2,5-anhydromannose, 2,5-anhydrotalose and glucose formed by deamination of glucosamine, galactosamine and mannosamine, respectively, were then derivatized to the aldononitrile acetates and determined by gas chromatography. A good resolution of the three hexosamines was obtained. Amino acids eluted from a Bio-Rad AG 50W-X8 (H^+) column also undergo deamination, whereby the amino group is replaced by a hydroxyl group. When subjected to the conditions for derivatization to aldononitrile acetates, the hydroxyl groups are acetylated but the carboxyl groups remain free making them non-volatile and hence undetectable in the gas chromatogram. For the same reason glucuronic acid is also not detected[23,24]. This gas chromatographic method gave results comparable in every respect with those obtained by conventional methods and is therefore an independent and a reliable technique for characterization and determination of hexosamines from glycoproteins and acid mucopolysaccharides.

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