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MODIFICATION OF THE SIMULTANEOUS DETERMINATION OF ALDITOL ACETATES OF NEUTRAL AND AMINOSUGARS BY GAS—LIQUID CHROMATOGRAPHY

APPLICATION TO THE FRACTIONATION OF SIALOGLYCOPROTEINS FROM BONE

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SUMMARY

A modification of a gas—liquid chromatographic method is described that allows better simultaneous separations of the neutral and aminosugar alditol acetate derivatives as single peaks. Using 3% SP-2340 on 100—200 mesh Supelcoport, retention times were relatively short and baseline separation between glucose and galactose was achieved. The method is particularly suitable for monitoring the fractionation of complex mixtures of glycoproteins and glycosaminoglycans, and its application is illustrated in the fractionation of bone matrix extracts subjected to ion-exchange chromatography. A convenient procedure allowing the separation and estimation of sialic acid in the same aliquot is also described and evaluated.

INTRODUCTION

The advantages and limitations of methods used for cleavage of the glycoconjugates of glycoproteins and the estimation of the various volatile carbohydrate derivatives by gas—liquid chromatography (GLC) have been extensively reviewed [1—4]. A number of the currently described GLC methods yield multiple peaks for each component making quantitation complex. A procedure resulting in single peaks for each monosaccharide [3] avoids this problem by employing a resin-catalyzed hydrolysis of neutral sugars and aminosugars, followed by nitrous acid deamination of the resin bound hexosamine to neutral 2,5-anhydrohexoses and subsequent chromatography of all the corresponding neutral alditol acetates. However, in this procedure, separation between the

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alditol acetates of glucose and galactose is not complete and this often presents a problem during purification procedures of glycoproteins due to exogenous sources of glucose-containing contaminants. A method giving single aldonitrile derivatives has also been reported [5], but retention times are relatively long and these derivatives have appreciable water solubility.

In this communication we report on an improved GLC method, with relatively short retention times and better separations of the alditol acetates of the constituent monosaccharides, including a baseline separation between glucose and galactose.

The application of this method is illustrated by the analysis of isolated glycoproteins and glycosaminoglycans and has proved particularly useful in monitoring the fractionation of glycoprotein-rich extracts from bone by ion-exchange chromatography. GLC methodology had not been previously applied to glycoproteins from bone [6—8] and it would appear that this method is also suited to the analysis of small samples of bone, such as are available from bone biopsy and reconstructive surgery for arthritic conditions. The procedure has also been modified so that an initial separation and estimation of the bound sialic acid is conveniently performed in the same aliquot prior to analysis of the remaining monosaccharides. This method could therefore be applied to the study of both glycoproteins and proteoglycans in pathological processes of bone or in the growth and development of bony tissue.

MATERIALS AND METHODS

Reagents

Acetic acid, acetic anhydride, methanol, hydrochloric acid, and sodium borohydrate were certified Fisher grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Pyridine, solution grade, was obtained from Pierce (Rockford, IL, U.S.A.). The resins AG 50W-X8 (H^+), 200—400 mesh, and AG 1-X2 (Cl^-) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). AG 1-X2 (HCO_3^-) was prepared by passing a 2 mol/l $NaHCO_3$ solution through a column of AG 1-X2 (Cl^-) and washing thoroughly with deionized water. DEAE-Sephadex, A-25 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), converted to the acetate form using 0.5 mol/l sodium acetate and equilibrated with 0.05 mol/l Tris acetate buffer, pH 8.0.

Biological materials

Chondroitin sulfate A was a standard obtained from Drs. Mathews and Cifonelli, University of Chicago (see also Table II). Fetuin was obtained from Sigma (St. Louis, MO, U.S.A.) (Type IV, lot No. F3004). Solubilized bone matrix (SBM) enriched in glycoproteins was obtained from rat bone by a method previously described [9], Briefly, decalcified bone matrix was extracted with 4 mol/l guanidinium chloride and the extract dialyzed against water. The retentate was then extracted with isotonic (0.154 mol/l) sodium chloride, which was again dialyzed against water. The retentate from this step was soluble in salt solutions of relatively low ionic strength and was termed SBM.

Hydrolysis of neutral and aminosugars of the glycoconjugates

The solubilized glycoproteins were hydrolyzed by means of a resin-catalyzed hydrolysis procedure [3,10,11]. Either weight aliquots of bone matrix, or the dried content of each combined fraction was dissolved in 1 ml of 0.02 mol/l hydrochloric acid and transferred into a 16 X 125 mm glass culture tube (Kimax) containing 1 ml of the AG 50W-X8 (H^+) resin in 0.02 mol/l hydrochloric acid. The tubes were sealed with PTFE-lined screw caps and the contents hydrolyzed at 100°C for 24 h.

Deamination and isolation of carbohydrates

The samples were removed from the hydrolysis oven, allowed to cool, and centrifuged to remove condensate from septa and walls of the hydrolysis tubes. The septa were carefully removed and 50 μl of an internal standard solution containing 50 μg myoinositol were added, followed by the addition of 10 μl of a freshly prepared 5.5 mol/l solution of $NaNO_2$. The tubes were sealed again and they were subjected to intermittent vortexing at room temperature for 60 min for completion of the deamination. Following deamination, 500 μl of 40% w/v suspension of AG 50W-X8 (H^+) [3] were added to the deaminated mixture to remove excess Na^+ ions and convert $NaNO_2$ to HNO_2. After shaking the tubes for approximately 30 min, contents were transferred to a set of two separate columns, the upper column containing AG 50W-X8 (H^+) [3] and draining into a lower one packed with AG 1-X2 (HCO_3^-) resin. The columns were prepared from disposable pipettes (Oxford Macroset) plugged with glass wool, and packed to a height of 4 cm with the resin. Hydrolysis tubes were then rinsed five times with 5 ml distilled water and the washings were transferred to the columns. The eluates were collected in 30-ml tubes and evaporated to a small volume in a rotary evaporator at 30°C. The concentrated solutions were then brought to dryness in a centrifugal bio-dryer.

Reduction and derivatization

The contents of each tube were dissolved in 100 μl of water and 100 μl of 0.22 mol/l $NaBH_4$ were added to reduce the free sugars, and the reaction was allowed to proceed to completion in 1 h at room temperature. Following reduction, excess $NaBH_4$ was decomposed with glacial acetic acid. Borate was removed as volatile trimethyl borate by the addition of four 200-μl portions of methanol—hydrochloric acid (1000:1) and concentrated to dryness in the bio-dryer after each addition. The samples were acetylated for 30 min at 100°C with 100 μl of acetic anhydride and 100 μl of pyridine. They were then cooled, and 2—5 μl aliquots were injected into the gas chromatograph.

Preparation of standards

A series of standard solutions containing 5—100 μg of each of the neutral and aminosugars and 50 μg of the internal standard was prepared. A calibration curve of the ratio of areas vs. the ratio of weights was plotted for each sugar.

The results were calculated using the following relationship:

$$\text{Percentage sugar} = \left[\frac{\text{peak area of sugar derivative}}{\text{peak area of internal standard}} - IC\right] \left[\frac{\text{internal standard weight}}{S}\right] \times \frac{100}{\text{sample weight}}$$

where S is the slope and IC is the intercept of the calibration curve.

Sialic acid hydrolysis and preliminary fractionation

Sialic acid was released from the solubilized sialoglycoproteins under mild acid conditions using 5 ml of 0.1 mol/l sulfuric acid for 1 h at 90°C in 10 × 100 mm screw cap tubes. One of two procedures was then followed. When direct analysis was carried out, 0.2—0.5 ml aliquots from 1 ml of the glycoprotein-containing fraction were subjected to the mild acid hydrolysis procedure and portions of the hydrolysate were analyzed colorimetrically for sialic acid [12]. When a preliminary separation of sialic acid and other anionic components (glycosaminoglycans) was carried out, the entire sample, or a portion thereof, that had been subjected to mild acid hydrolysis, was passed through a 0.7 × 6 cm Dowex 1-X8 column, and after washing the columns with 8 ml of water to recover the desialylated glycoproteins, the bound sialic acid was eluted with 8 ml of 1 mol/l acetic acid—sodium acetate buffer, pH 4.6, as previously described [13].

Apparatus

A Varian Model 2800 gas chromatograph equipped with dual flame ionization detectors, temperature programmer and coiled glass columns (158.4 cm × 4 mm I.D.) was employed. Two different column packing materials were used. Column packing No. 1 was composed of 0.75% HIEFF - IBP, 0.25% EGSS - X, and 0.1% 144-B on 60—80 mesh Gas-Chrom Q, as had been previously suggested [3]. Column packing No. 2 consisted of 3% SP-2340 on 100—120 mesh Supelcoport. Chromatography was conducted with temperature programming beginning at 150°C with a program rate of 2°C/min to a final temperature of 220°C. The injection port temperature was 220°C and the detector temperature was 310°C. The carrier gas was nitrogen at a flow-rate of 40 ml/min. Hydrogen and air flow-rates to each detector were 30 ml/min and 300 ml/min, respectively. Peak areas were measured with a Gould 110 Recorder equipped with an electronic chart integrator.

Fractionation of solubilized bone matrix enriched in glycoproteins

For the separation of solubilized glycoproteins a column of DEAE-Sephadex A-25, 20 cm × 2 cm, prepared as described above, was used. A sample containing 10—50 mg of solubilized rat bone matrix was dissolved in 3—5 ml of the Tris acetate buffer and was carefully layered on top of the column bed. After the sample had drained into the bed, the gel surface and the column wall were washed with 5 ml of the buffer solution. The column was eluted first isocratically with 100 ml of Tris acetate buffer and then switched to gradient

elution. The following gradients were used sequentially: 0—0.13 mol/l, 0.13—0.8 mol/l and 0.8—2 mol/l sodium chloride, all made up in Tris acetate buffer, pH 8.0. Total volume of each gradient was automatically controlled by means of a solenoid valve operated through a thermistor sensing circuit. At the end of the gradient elution the column was washed with 100 ml of 2 mol/l sodium chloride in the same buffer solution.

Fractions containing 5 ml each were collected on an LKB fraction collector. Single column fractions were pooled into another set of combined fractions representing a volume eluted under an individual peak or band with a maximal absorbance at 280 nm (see Fig. 2). Carbohydrate-containing material as reducing sugar equivalent [14] was also determined in the eluted fractions. These fractions were then dialyzed against double-distilled deionized water until free of chlorine. The dialysants were then concentrated to a smaller volume in a desiccator under vacuum and lyophilized. The entire amount or a portion of each combined fraction was used for GLC analysis for the constituent neutral and aminosugars as described above, and spectrophotometric analysis for sialic acid [12].

General methods

UV absorption of eluted fractions from DEAE-Sephadex was determined routinely at 230 and 280 nm using a Shimadzu Spectronic 200 UV spectrophotometer. Hydroxyproline was determined in hydrolysates (6 mol/l hydrochloric acid for 24 h at 100°C) of dialyzed and lyophilized fractions with a modification of a colorimetric method [15] or by amino acid analysis.

RESULTS

Chromatographic resolution of alditol acetates

The retention times of the alditol acetate derivatives of neutral and aminosugars are shown in Table I. The resolution of a mixture containing neutral and aminosugars on two different columns is shown in Fig. 1A and B. Preliminary work was carried out on column No. 3 [3]. However, a column packed with SP 2340, packing No. 2, was found to be superior. On this column, base-

TABLE I

RETENTION TIMES FOR ALDITOL ACETATE DERIVATIVES OF NEUTRAL AND AMINOSUGARS

Sugar	Retention time (min)
L-Fucose	12.5
D-Arabinose	17.9
D-Glucosamine	22.3
D-Xylose	23.3
D-Galactosamine	28.7
D-Mannose	30.3
D-Galactose	32.6
D-Glucose	35.7
Myoinositol	39.2

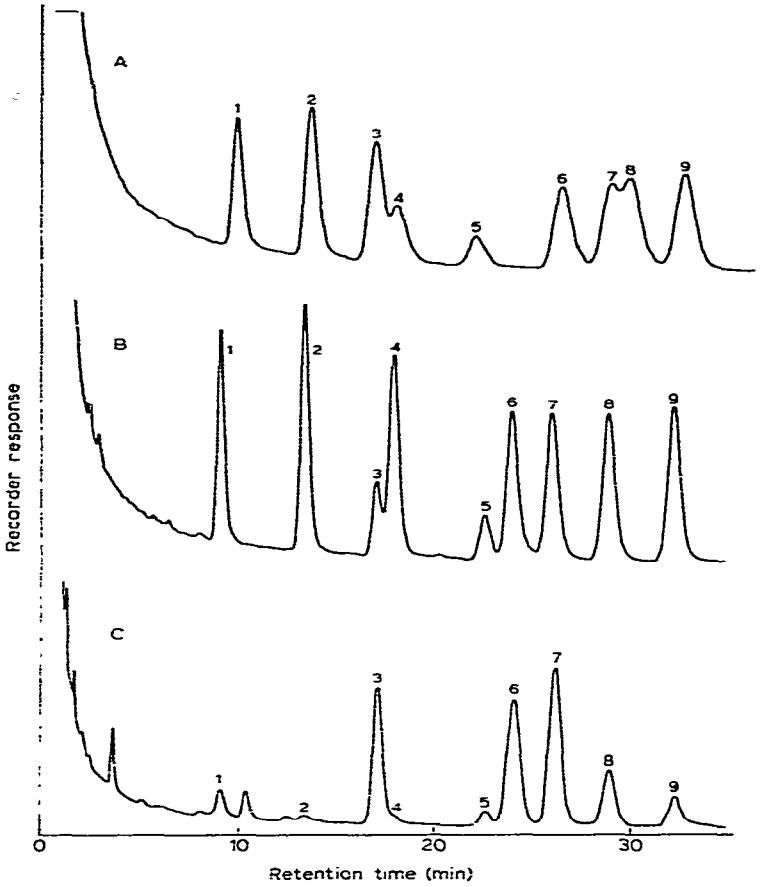

Fig. 1. Separation of sugar alditol acetate derivatives on two different GLC columns. (A) Column packed with 0.75% HIEFF-IBP, 0.25% EGSS-X and 0.1% 144B on 60—80 mesh Gas-Chrom Q. The alditol acetate derivatives of the neutral sugars and aminosugars shown above are numbered as follows: (1) fucose, (2) arabinose, (3) xylose, (4) glucosamine, (5) galactosamine, (6) mannose, (7) galactose, (8) glucose, (9) inositol. Peaks 4 and 5 probably represent the respective 2,5-anhydrohexositol acetate derivatives. (B) Column packed with SP-2340 on 100—200 mesh Supelcoport. The alditol acetate peaks are numbered as follows: (1) fucose, (2) arabinose, (3) glucosamine, (4) xylose, (5) galactosamine, (6) mannose, (7) galactose, (8) glucose, (9) inositol. (C) Gas chromatogram of solubilized bone matrix, utilizing the second column packing described above (Fig. 1B) and in the text. The chromatogram was obtained from solubilized bone matrix fractionated on DEAE-Sephadex, using a steep linear sodium chloride gradient, pH 7.0. Under these conditions most of the glycoprotein coelutes with the soluble collagen at the beginning of the gradient and the chromatogram shown is from this fraction. Note the baseline separation between glucose and galactose.

line separation of glucose and galactose was achieved. Also, adequate separation of xylitol and 2,5-anhydromannitol peaks formed from xylose and glucosamine was obtained. Fig. 1C illustrates that satisfactory baseline separations of the sugars are obtained also from fractions containing complex mixtures of glycoproteins and collagen from bone matrix.

Separation of sialic acid and its simultaneous estimation

A procedure for separation of sialic acid that permits its estimation as well as the estimation of neutral and aminosugars in the same sample was evaluated in the following manner (Table II). Weighed samples of fetuin were subjected to mild acid hydrolysis and the hydrolyzed samples were applied to a Dowex 1-X8 column which retained the sialic acid, as described in Materials and Methods. The remaining asialoglycoprotein was recovered in the water eluate from the Dowex 1-X8 column and was subjected to resin hydrolysis, deamination, reduction and GLC analysis. The sialic acid was then eluted with acetic acid—sodium acetate buffer and estimated colorimetrically, as described in Materials and Methods. Recovery of the sialic acid from the Dowex 1-X8 column was between 92—98% in terms of the sialic acid released after mild acid hydrolysis from fetuin or the bone matrix fractions. Recoveries from the Dowex 1-X8 column for the neutral and aminosugars were quantitative and agree well with published values for these constituents obtained by colorimetric methods after strong acid hydrolysis of fetuin.

TABLE II

FRACTIONATION OF CHONDROITIN SULFATE (CS), FETUIN AND SOLUBILIZED BONE MATRIX (SBM) ON DOWEX 1-X8 BEFORE AND AFTER MILD ACID HYDROLYSIS

Chondroitin sulfate A (Drs. M.B. Mathews and J.A. Cifonelli, University of Chicago) had a standard hexosamine content of 24.94% by the Elson Morgan colorimetric method [19]. Fetuin was a Sigma product (see Materials and Methods) with a sialic acid content of 6.2%. Previous estimates of the content of fetuin monosaccharides by colorimetric methods [2] were, mannose, 3.0%, glucosamine, 4.9%, galactosamine, 0.6%, galactose, 4.6%.

Sugar in eluate	Sugar content of water eluate (percentage of dry weight of fetuin, CS, and SBM)					
	Material applied to Dowex 1-X8					
	Fetuin* (2 mg)	Fetuin** (2 mg)	CS* (1 mg)	CS** (1 mg)	SBM* (5 mg)	SBM** (5 mg)
L-Fucose	—	—	—	—	(t)§	(t)
Xylose	—	—	(c)***	—	—	—
Mannose	3.3	3.1	—	—	1.1	1.1
Glucosamine	6.1	5.8	(c)	—	2.8	2.8
Galactosamine	0.4	0.3	26.6	—	1.8	—
Galactose	4.9	4.7	(c)	—	1.6	1.6

*Samples analyzed directly, without prior desialylation.

**Samples passed through a column of Dowex 1-X8 (acetate form) after desialylation procedure. Sialic acid recoveries from the Dowex 1-X8 column were between 92 and 98% (see text for details).

***(c) Constituent carbohydrate was present in low concentrations and was not quantitated.

§ (t) Constituent carbohydrate was present in trace amounts.

The behaviour of chondroitin sulfate A (chondroitin 4-sulfate), the major glycosaminoglycan in bone, on the Dowex 1-X8 column was also evaluated. This compound is entirely retained by the resin. Direct analysis gave values for galactosamine that were comparable to those obtained by a colorimetric

method following strong acid hydrolysis for the glycosaminoglycan standard (Table II). When whole solubilized bone matrix was applied to the Dowex 1-X8 column, all of the galactosamine-containing material was retained, but recovery of the other neutral sugars and glucosamine was complete in the water eluate from the column (Table II). This suggests that the preliminary separation on Dowex 1-X8 of glycosaminoglycan and free sialic acid from the desialylated glycoproteins can be used reliably, in conjunction with the modified GLC method for the analysis of complex mixtures of glycoconjugates, utilizing the same sample.

Application of the modified GLC method to the fractionation of glycoproteins from bone

Fractionation of solubilized bone matrix on DEAE-Sephadex, utilizing a three-step linear salt gradient resulted in a significant separation of glycoproteins from collagenous proteins (Fig. 2). All of the hydroxyproline-containing material eluted in fraction 1 (F1) while the carbohydrate-containing material eluted largely in fraction 3 (F3) (see also Table III) that had been processed without previous desialylation or passage through the Dowex 1-X8 column. In these fractions, the sialic acid was estimated directly after mild acid hydrolysis in an aliquot of each fraction. Fraction 3 contained significant amounts of galactosamine. Compounds containing this aminosugar could be removed (after desialylation) by a passage of fraction 3 through a Dowex 1-X8 column, as described above for the unfractionated bone matrix, without a significant change in the estimates of the other sugars recovered in the water eluate from the column (data not shown). Retained high molecular weight material on Dowex 1-X8 could be eluted with strong acid and was shown to contain chondroitin sulfate by cellulose acetate electrophoresis [16,17].

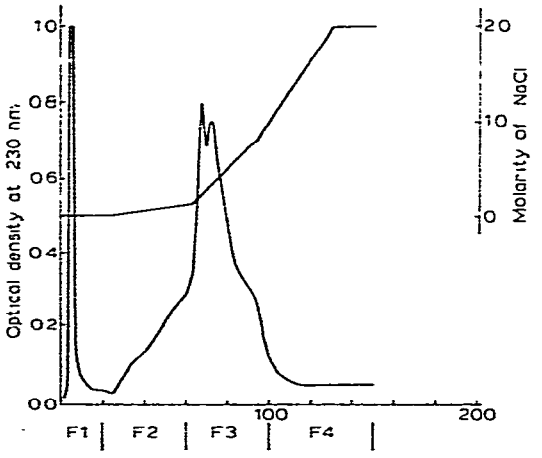

Fig. 2. Separation of solubilized bone matrix on DEAE-Sephadex, using a three-step linear sodium chloride gradient, in Tris acetate buffer, 0.05 mol/l, pH 8.0. The left-hand vertical axis shows the elution pattern as monitored by UV absorbance at 230 nm and the right-hand vertical axis indicates the molarity of the eluting sodium chloride gradient. Aminosugar, neutral sugar, and sialic acid contents are given in Table III.

TABLE III

SUGAR CONTENT IN FRACTIONATED SOLUBILIZED BONE MATRIX

A sample of solubilized bone matrix (30 mg) was fractionated on DEAE-Sephadex column and the fractions were processed and analyzed directly for neutral sugars and aminosugars, as described in Materials and Methods. For sialic acid analysis, 0.2-ml aliquots out of 1-ml final volume for each fraction were subjected to mild acid hydrolysis and the sialic acid was estimated [12] directly.

Components identified	Sugar fractions (μg)				Total sugar (% dry weight)
	F1	F2	F3	F4	
Mannose	1.7	13.5	280.0	1.7	0.98
Glucosamine	—	22.2	840.0	—	2.80
Galactosamine	—	—	455.0	58.0	1.80
Galactose	1.7	—	425.3	6.8	1.60
Sialic acid	—	—	810.0	—	2.7

The reproducibility of this method was also examined, using five replicate (from the same batch) 5-mg samples of solubilized bone matrix material. Values obtained were within a range of 5% or better. Reproducibility and recoveries of the method with known standards of neutral and aminosugars is 100 ± 3%.

DISCUSSION

A practical problem in the alditol acetate method that yields single derivatives for each monosaccharide [3] was that the separation of certain pairs of sugars (the derivatives of glucose and galactose and the derivatives of xylose and glucosamine) was incomplete. A method giving single aldonitrile acetate derivatives has also been reported [5], but this procedure does not resolve the problem of partial separations between xylose and glucosamine, and furthermore, retention times are comparatively long (e.g. 54 min for glucosamine and 70 and 75 min for glucose and galactose, respectively), which limits the application of this method for multiple sample analysis. Aldonitrile acetates of aminosugars also have appreciable water solubility, thus making derivative clean-up procedures difficult and may exhibit erratic chromatographic properties [18]. In the modification of the alditol acetate method [3] that we describe, utilizing column packing No. 2 (see Materials and Methods), separation between glucose and galactose is complete, the separation between xylose and glucosamine is improved, and the retention times are comparatively short (Table I).

The baseline separation of the glucose and galactose alditol acetate derivatives is critical during fractionation procedures of glycoconjugates extracted from tissues, since exogenous sources such as dialysis bags and Sephadex columns were shown to make variable contributions to detectable glucose (data not shown). In addition, deamination of mannosamine also yields glucose [3,19].

The preliminary separation of glycosaminoglycan (or proteoglycan) and the hydrolyzed sialic acid on the Dowex 1-X8 column adds considerable flexibility to fractionation and allows quantitative estimates of the neutral and amino-sugars of the asialoglycoproteins and the sialic acid to be carried out in the same aliquot.

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