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A SIMPLE PROCEDURE FOR COMBINED GAS CHROMATOGRAPHIC ANALYSIS OF NEUTRAL SUGARS, HEXOSAMINES AND ALDITOLS

DETERMINATION OF DEGREE OF POLYMERIZATION OF OLIGO- AND POLYSACCHARIDES AND CHAIN WEIGHTS OF GLYCOSAMINOGLYCANS

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SUMMARY

A reliable and reproducible method that allows the combined, simultaneous gas chromatographic (GC) determination of neutral sugars, hexosamines, alditols, identification and quantitation of the reducing aldose end-group in oligo- and polysaccharides and glycosaminoglycans has been described. It involves the following steps: release of the reducing end-group from its protein linkage in glycosaminoglycans and reduction of this reducing end-group into alditol, release of the components of the reduced polymer by resin-catalysed hydrolysis, nitrous acid deamination of the resin-bound hexosamines in this hydrolysate into anhydroaldoses and a combined derivatization and GC determination of the neutral sugars as aldononitrile acetates, anhydroaldoses as peracetylated oximes and alditols as alditol acetates. Application of the method to determination of degree of polymerization of oligo- and polysaccharides and chain weights of proteoglycans has been described. This method has several advantages over the previous methods.

INTRODUCTION

Gas–liquid chromatography (GLC) is widely used for determination of neutral sugars and hexosamines, which are important constituents of glycosaminoglycans and glycoproteins. The determination of the terminal reducing end-group has been used for calculating the degree of polymerization of a polysaccharide and the chain weights of proteoglycans. Among the various chemical end-group analysis methods[1] reduction of the end-group sugar with $NaBH_4$ or tritiated borohydride[2] and determination of the alditol formed gives a more sensitive analysis of the end-group.

In the case of proteoglycans it is necessary to release the reducing sugar end-group of its polysaccharide portion from its covalent linkage to protein prior to any reducing end-group determination. The alternate approach in these cases has been determination of the neutral sugar components of the linkage region of the intact

proteoglycan[3,4]. GLC analyses give more accurate determinations of the end-groups than the majority of the chemical end-group methods. While a number of different derivatives[5,6] have been used for GLC analyses of neutral sugars in such situations, their determination as aldononitrile acetates[7–9] and peracetylated oximes of anhydrosugars[10] formed by deamination of hexosamines has several advantages including their easy preparation, lower retention times, single peak per sugar and greater stability of the derivatives. In the case of trimethylsilyl (TMS) derivatives of the sugars, multiple peaks are obtained due to anomerization and ring isomerization making the chromatogram difficult to interpret. Also, xylans cannot be analyzed when TMS derivatives are used, since one of the xylose peaks and xylitol overlap. In the analysis of alditols as TMS derivatives or acetates a single peak is obtained per alditol, but arabinose and lyxose give the same alditol. Also, there are problems in the simultaneous resolution of the sugar pairs: fucose–rhamnose, arabinose–ribose, lyxose–arabinose, glucose–galactose and glucosamine–galactosamine[5,6]. The use of the aldononitrile acetate derivatives helps identification and determination of the parent sugar with greater reliability and overcomes the aforementioned problems of the resolution.

In previous publications we reported the resolution of twelve neutral sugars as their aldononitrile acetates on a single column and its application to polysaccharides[7], and analysis of neutral sugars[8] and hexosamines[9] from glycoproteins and mucopolysaccharides. In a recent publication[11] we reported the simultaneous determination of neutral sugars and hexosamines from glycoproteins and mucopolysaccharides of tissues and biological fluids. Baird *et al.*[12] have reported the applicability of aldononitrile acetates to the analysis of Smith degradation products. Morrison[13] has recommended the use of these derivatives to the analysis of the degree of polymerization of oligo- and polysaccharides. This paper reports the simultaneous GLC resolution of neutral sugars, hexosamines and alditols and its application to the determination of the degree of polymerization of oligosaccharides and polysaccharides and the chain weights of the glycosaminoglycans.

EXPERIMENTAL

Materials

The materials were obtained or prepared as described previously[11].

Alkaline-borohydride reduction

A 2–5-mg sample of chondroitin-4-sulfate was dissolved in 1.75 ml of 0.2 *N* NaOH and treated with 11–15 mg of $NaBH_4$ for 48 h at 25° in a tightly stoppered 25-ml evaporating flask[14]. In the case of oligosaccharides 1–3-mg samples were dissolved in 0.5 ml water or 0.5 ml of 0.2 *N* NaOH and treated with $NaBH_4$ (10 moles per mole of reducing sugar) at room temperature for 1 h[13]. The solution was neutralized carefully to pH 5 with 3 *M* acetic acid and evaporated to dryness in a rotary evaporator at room temperature. The excess of boric acid was removed by repeated evaporations with methanol. The solid was dissolved in a small quantity of water and transferred quantitatively into a 10-ml glass ampoule containing 20–50 μg of a neutral sugar (as an internal standard) not present in the test material and freeze dried.

Resin-catalysed hydrolysis of reduced material

The residue in the ampoule was dissolved in 0.1 ml of water and to this 0.6–0.8 ml of a 40% (w/v) suspension of AG 50W-X8 (H^+) resin in 0.02 *N* HCl was added. The tip of the ampoule was sealed carefully and left in the oven at 100° for 48 h.

Nitrous acid deamination of resin-bound hexosamines

This step was omitted for the oligosaccharides, since they did not contain the hexosamines. The ampoule containing the glycosaminoglycan hydrolysate was cooled to room temperature, the tip was gently broken and after adding 0.15 ml of a solution containing 35 mg of $NaNO_2$, the tip of the ampoule was quickly and carefully resealed avoiding any heating of the solution. The cooled ampoule was subjected to intermittent vortexing for 30 min for completion of deamination[11].

Isolation of the mixture of aldoses, anhydroaldoses and alditols

The ampoule was opened and a pinch of AG 50W-X8 (H^+) resin was added. The contents of the ampoule were transferred with water washings to a tandem arrangement of two columns made of two 10-ml Kimble glass disposable serological pipettes (Owens-Illinois, Toledo, Ohio, U.S.A.) plugged with glass wool and packed to the 5-ml capacity with resins. The upper column containing AG 50W-X8 (H^+) drained into the lower column packed with AG 1-X2 (HCO_3^-). A 30–40 ml of eluate and washings with deionized water were collected in a 50-ml evaporating flask and evaporated to dryness in a rotary evaporator at room temperature.

Derivatization and washing of the derivatives

The solid in the evaporating flask was transferred to a 10-ml glass ampoule with 0.5–1 ml of dry pyridine. The excess pyridine was removed with a stream of nitrogen, leaving 8–10 drops of pyridine in the ampoule. To this 6–7 mg of dry hydroxylamine hydrochloride were added and further processed for derivatization as described previously[11]. After derivatization, the residue obtained by evaporation with nitrogen can be dissolved in dry chloroform and gas chromatographed. However, we have found that washing of the derivatives prior to GLC prevents any possible tailing of peaks and also prolongs the life of the column. For washing, the evaporated derivatives were transferred with 1 ml chloroform into a centrifuge tube fitted with a stopper. The chloroform solution was washed successively with 1 ml of each of cold 3 *M* HCl, deionized water, 0.5 *M* $NaHCO_3$ solution and deionized water. At each step extraction was done in the original centrifuge tube by shaking, centrifugation and careful removal of the top aqueous layers (which were discarded) from the lower chloroform layer. The final chloroform solution was dried by adding a pinch of anhydrous sodium sulfate. The dried solution and the chloroform washings were transferred to a vial, evaporated to dryness with nitrogen, dissolved in 25–50 μl of chloroform and gas chromatographed.

Preparation of standards

Standards containing 100 μg of each of the neutral aldoses, 150 μg of each of hexosamines and 100–150 μg of each alditol were prepared as described previously[11].

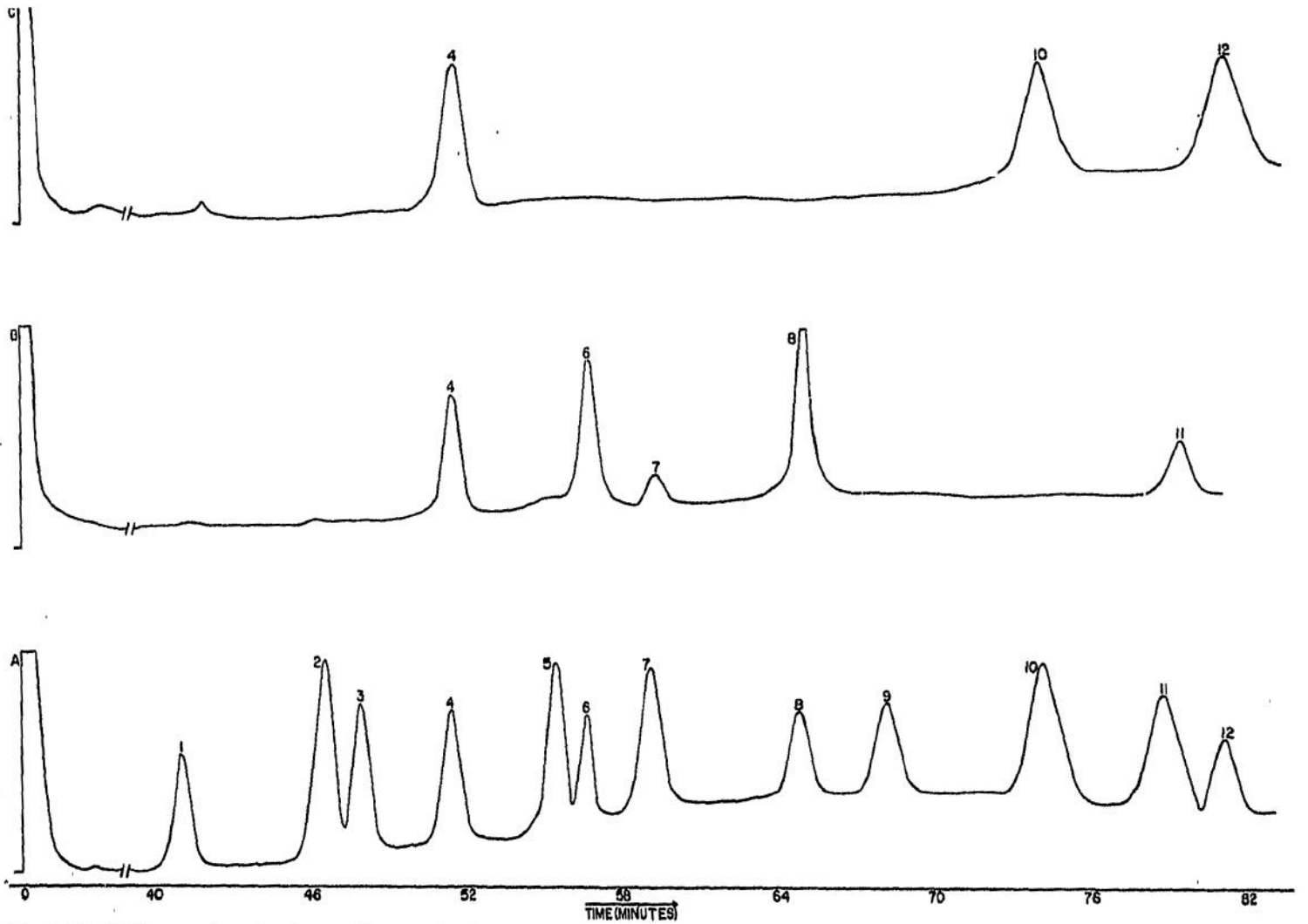

Fig. 1. (A), GLC separation of a mixture of standards: aldoses as aldononitrile acetates, hexosamines as peracetylated oximes of anhydroaldoses and alditols as alditol acetates. 1 = Rhamnose; 2 = fucose; 3 = fucitol; 4 = arabinose; 5 = xylose; 6 = glucosamine; 7 = xylitol; 8 = galactosamine; 9 = mannose 10 = glucose; 11 = galactose; 12 = glucitol. (B), GLC analysis of sugars from borohydride-reduced bovine vitreous humor chondroitin-4-sulfate. Internal standard, arabinose. Peak numbers as in (A). A smaller amount of sample was injected for the analysis of galactosamine. (C), GLC analysis of sugars; from borohydride-reduced maltose. Internal standard, arabinose. Peak numbers as in (A).

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 (100–120 mesh) was used. The gas chromatograph was initially programmed from 130°–195° at 1°/min and later operated isothermally till the last component (sorbitol) had eluted. Nitrogen was used as the carrier gas at a flow-rate of 36 ml/min. The injector and detector temperatures were maintained at 130° and 230°, respectively.

RESULTS

Fig. 1A illustrates the resolution of a standard mixture of aldononitrile acetates of neutral aldoses, alditol acetates and peracetylated oximes of anhydrosugars formed by nitrous acid deamination of hexosamines. The retention times for all these derivatives are given in Table I. Fig. 1B shows a typical chromatogram for the separation of arabinose (internal standard) and the interior chain components (galactose, glucosamine and galactosamine) of bovine vitreous humor chondroitin-4-sulfate and xylitol formed by reduction of its terminal reducing end-group xylose exposed upon β-elimination reaction in the alkaline medium. A similar chromatogram was obtained for the veal brain chondroitin-4-sulfate.

TABLE I

RETENTION TIMES AND RECOVERIES OF NEUTRAL SUGARS, HEXOSAMINES AND ALDITOLS

Sugar	*Retention time (min)*	*% Recovery (mean ± S.D., n = 7)*
Rhamnose	41.1	93 ± 5
Fucose	46.6	92 ± 5
Fucitol	47.9	94 ± 5
Arabinose	51.4	93 ± 5
Xylose	55.4	94 ± 6
Glucosamine	56.6	93 ± 5
Xylitol	59.0	94 ± 5
Galactosamine	64.7	93 ± 5
Mannose	68.1	94 ± 5
Glucose	74.0	94 ± 4
Galactose	78.8	93 ± 6
Glucitol	81.1	92 ± 5

Fig. 1C shows the separation of arabinose (internal standard), glucose and sorbitol obtained from the hydrolysate of borohydride reduced maltose. Maltotriose gave a similar chromatogram. The values obtained by the present method were in close agreement with the theoretical values for maltose and maltotriose (Table II). The percentage of each sugar component was calculated using the equation given elsewhere[11]. The procedure for polysaccharide is the same as for oligosaccharides and the applicability to polysaccharides is not given here since it has been given earlier[13]. The degree of polymerization of an oligosaccharide or a polysaccharide can be deter-

mined from the ratio of the interior chain residues of the homopolymer to the terminal reducing sugar group measured in the present method as aldononitrile acetates and alditol acetates, respectively. The ratios of the percentages of glucose and glucitol obtained by this method agree with the calculated theoretical values for maltose and maltotriose (Table II).

TABLE II

CARBOHYDRATE COMPOSITION (%) OF CHONDROITIN-4-SULFATES FROM VEAL BRAIN AND BOVINE VITREOUS HUMOR, MALTOSE AND MALTOTRIOSE

Sugar	*Veal brain chondroitin sulfate*		*Vitreous humor chondroitin sulfate*		*Maltose*		*Maltotriose*	
	Previous method[11]	*Present method*	*Previous method*[11]	*Present method*	*Theoretical value*	*Present method*	*Theoretical value*	*Present method*
Xylose	0.52	—	0.86	—	—	—		
Xylitol	—	0.50	—	0.83	—	—		
Galactose	1.30	1.28	1.99	1.90	—	—		
Glucosamine	3.76	3.43	5.28	5.33	—	—		
Galactosamine	17.6	17.1	21.39	20.93	—	—		
Glucose	—	—	—	—	52.63	47.37	71.42	67.50
Glucitol	—	—	—	—	53.21	50.72	36.11	33.73

The values of xylitol determined in the borohydride-reduced chondroitin-4-sulfates from veal brain and vitreous humor are close to the values for the terminal neutral sugar xylose in the unreduced chondroitin-4-sulfates (Table II). The number-average chain weights (C_n) of chondroitin sulfates were calculated from its content of linkage region terminal neutral sugar xylose or its reduction product xylitol as the weight of chondroitin sulfate in grams, which will contain 1 mole (150.13 g) of xylose or 152.13 g (1 mole) of xylitol. The chain weight values calculated for chondroitin sulfates from veal brain and vitreous humor from xylitol values by this method were 30,487 and 18,329, respectively, which agree well with the values of 29,151 and 17,457 calculated from xylose content of the intact unreduced chondroitin sulfates.

DISCUSSION

Proteoglycans, *e.g.* chondroitin sulfates, are covalently linked protein–polysaccharide complexes. The alkali-borohydride reduction brings about the cleavage of the covalent linkage between the hydroxyamino acid serine in the protein and the terminal neutral sugar xylose in the polysaccharide chain by β-elimination reaction[15] and the xylose residue so exposed is reduced to xylitol simultaneously. In case of oligosaccharides and polysaccharides any free reducing end-group is reduced to the corresponding alditol. The resin-catalysed hydrolysis quantitatively releases[16] the hexosamines, aldoses and alditol from the reduced material. The hexosamines and amino acids in this hydrolysate stay bound to the cationic resin and upon nitrous acid deamination glucosamine and galactosamine yield 2,5-anhydromannose and 2,5-anhydrotalose, respectively, which are released from the resin[11]. Deamination of the

amino acids present in this hydrolysate gives the corresponding carboxylic acids[9] which, along with any hexuronic acid from mucopolysaccharide hydrolysis, are removed by the anionic resin AG 1-X2 (HCO_3^-) during passage through the tandem columns. Treatment of the aldoses, anhydroaldoses and alditols, isolated from the eluate, with hydroxylamine hydrochloride in pyridine converts the aldoses and anhydroaldoses into their oximes. Subsequent acetylation of the mixture yields aldononitrile acetates from aldose oximes, peracetylated anhydroaldose oximes from anhydroaldose oximes and alditol acetates from alditols. All these derivatives are determined simultaneously by GLC.

Mannosamine is of a limited occurrence[17,18] and upon deamination forms glucose[19]. If and when the pair mannosamine–glucose is, rarely, present, the resin-bound hexosamines may be separated from the neutral sugars in the hydrolysate by two slightly different approaches and determined after deamination as described previously[11].

In proteoglycans a number of polysaccharide chains may be present covalently linked to protein as complexes. The number-average chain weight (C_n), which is the molecular weight of the individual polysaccharide chain is a meaningful and useful parameter in addition to the molecular weight of the proteoglycan *per se*, which offers information on the structure of the proteoglycan on a more detailed level[20]. Bollet and Nance[21] applied this concept of the chain weight for understanding the changes in chondroitin sulfate in osteoarthritis. The determination of the xylitol following alkali-borohydride reduction gives the positive identification and a reliable quantitative value of xylose that was involved in alkali-labile protein–carbohydrate linkage. The agreement between the chain weights for chondroitin sulfates from vitreous humor and veal brain calculated from xylitol value following alkali-borohydride reduction and that calculated from xylose content of the intact unreduced samples suggests that all of the xylose present in these samples is involved in an alkali-labile covalent linkage.

The present method has several advantages over the other methods. It allows the determination of the neutral sugars, hexosamines, alditols and identification and determination of the terminal reducing aldose and the number-average chain weight (C_n) in a single GLC analysis. Since the complete analysis is carried out in the same run, more accurate and reproducible results are obtained. Recoveries of various neutral sugars, hexosamines and alditols carried through the entire experimental procedure as described earlier are shown in Table I. Reproducibility of the present method was determined by carrying out analyses on several different starting concentrations of maltose, since this disaccharide was available in abundance, and each analysis done in duplicate. The mean and standard deviation of the difference between each pair of values was 0.28 ± 0.21 for glucose and 0.23 ± 0.18 for glucitol. The aldononitrile acetates, peracetylated oximes of anhydrosugars and alditol acetates are stable derivatives, give single peaks and a simple, easily-interpretable chromatogram. Also, the present method overcomes the difficulties encountered with the other derivatives[5,6] in the resolution of the pairs: fucose–rhamnose, arabinose–ribose, lyxose–arabinose, glucose–galactose, glucosamine–galactosamine, xylose–xylitol, xylitol–2,5-anhydromannitol.

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REFERENCES

1 F. Smith and R. Montgomery, *Methods Biochem. Anal.*, 3 (1956) 153.
2 G. N. Richards and W. J. Whelan, *Carbohyd. Res.*, 27 (1973) 185.
3 A. Wasteson and E. Wessler, *Biochim. Biophys. Acta*, 252 (1971) 13.
4 R. Varma, W. S. Allen and A. H. Wardi, *J. Neurochem.*, 20 (1973) 241.
5 G. G. S. Dutton, *Advan. Carbohyd. Chem. Biochem.*, 28 (1973) 11.
6 G. G. S. Dutton, *Advan. Carbohyd. Chem. Biochem.*, 30 (1974) 9.
7 R. Varma, R. S. Varma and A. H. Wardi, *J. Chromatogr.*, 77 (1973) 222.
8 R. Varma, R. S. Varma, W. S. Allen and A. H. Wardi, *J. Chromatogr.*, 86 (1973) 205.
9 R. S. Varma, R. Varma, W. S. Allen and A. H. Wardi, *J. Chromatogr.*, 93 (1974) 221.
10 R. Varma, J. R. Vercellotti and R. S. Varma, *Biochim. Biophys. Acta*, 497 (1977) 608.
11 R. Varma and R. S. Varma, *J. Chromatogr.*, 128 (1976) 45
12 J. K. Baird, M. J. Holroyde and D. C. Ellwood, *Carbohyd. Res.*, 27 (1973) 464.
13 I. M. Morrison, *J. Chromatogr.*, 108 (1975) 361.
14 R. U. Margolis, R. K. Margolis and D. M. Atherton, *J. Neurochem.*, 19 (1972) 2317.
15 R. D. Marshall and A. Neuberger, *Advan. Carbohyd. Chem. Biochem.*, 25 (1970) 407.
16 W. F. Lehnhardt and R. J. Winzler, *J. Chromatogr.*, 34 (1968) 471.
17 S. Bogoch, *Biochemistry of Memory*, Oxford Univ. Press, London, 1966, p. 199.
18 E. G. Brunngraber, A. Aro and B. D. Brown, *Clin. Chim. Acta*, 29 (1970) 333.
19 P. W. Kent and M. W. Whitehouse, *Biochemistry of Amino Sugars*, Academic Press, New York, 1955, p. 213.
20 H. Lyons and J. A. Singer, *J. Biol. Chem.*, 25 (1971) 277.
21 A. J. Bollet and J. L. Nance, *J. Clin. Invest.*, 45 (1966) 1170.