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CHROMATOGRAPHY OF ACIDIC GLYCOSAMINOGLYCANS ON DEAE-CELLULOSE

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SUMMARY

An ion-exchange procedure has been developed for the separation and analysis of milligram amounts of acidic glycosaminoglycans. The polysaccharides were first applied to a column of Sephadex G-50 and eluted with 0.15 M sodium chloride. The high molecular weight fraction was passed through a 1 × 5 cm DE-52 column, which retained the polyanions. These were subsequently eluted at 60° with a gradient of lithium chloride buffered at pH 4. Aliquots of the eluate were analyzed colorimetrically.

By this technique, a resolution mainly according to degree of sulfation but also according to molecular weight was achieved. Within the errors of the colorimetric analyses, quantitative yields were recorded.

INTRODUCTION

Ion-exchange chromatography of GAG* was first used by DAVIDSON AND MEYER[1] to separate a low-sulfated CS-4 fraction from the sulfated GAG in the cornea. The polysaccharides were adsorbed on a column of Dowex 1 X1 and eluted with hydrochloric acid solutions. RINGERTZ AND REICHARD[2] separated HA, CS-4 and Hep by elution with sodium chloride–hydrochloric acid · mixtures from ECTEOLA-cellulose, previously applied in the nucleic acid field[3]. The technique has been used in the study of, *e.g.*, mast cells[4], cornea[5] and cartilage[6]. Similar methods in which DEAE-cellulose[7-12], DEAE-Sephadex[13, 14], Dowex 1 X2[15, 16] or Deacidite resin FF-X8 (ref. 17) were used have also been published. Other anion exchangers have been tested[12].

A common drawback of the ion-exchange methods has been the poor recovery of the components. For instance, How *et al.*[18] reported a 30–50 % yield of sulfated GAG by elution from Deacidite resin FF-X8 with sodium chloride solutions. HA was obtained in about 70 % yield by elution from Dowex 1 X2 with sodium chloride in 8 M urea[16]. In DEAE-Sephadex chromatography, recoveries of 80 %[19] and 70 %[20]

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* GAG = glycosaminoglycans. The term is limited here to the acid "mucopolysaccharides" of mammalian connective tissues. The individual GAG are HA = hyaluronic acid, CS-4 = chondroitin 4-sulfate, CS-6 = chondroitin 6-sulfate, DS = dermatan sulfate, HS = heparan sulfate,

have been recorded for HA and GAG, respectively, from granulation tissue. The retained material may interfere with succeeding chromatograms on the same column. Furthermore, extensive tailing of the peaks may occur[18].

In the present paper, an ion-exchange procedure is described that gives virtually quantitative recoveries of the GAG applied, and peaks sharp enough for analytical purposes. The method was developed for the analysis of papain digests[21] of mammalian connective tissue specimens, but may be used also for other analytical problems.

EXPERIMENTAL

Materials

Whatman microgranular, pre-swollen DE-52 (Balston Ltd., Great Britain) was converted into the chloride form, suspended several times in 0.15 M sodium chloride and freed of fine particles. Sephadex G-50, medium grade (Pharmacia Fine Chemicals AB, Uppsala, Sweden), was allowed to swell for three days in 0.15 M sodium chloride before being packed in a column. The chemicals used were of analytical grade with the exception of lithium chloride. The latter ("purum lithium chloride"; Kebo AB, Stockholm, Sweden) was 99 % pure and contained a brown-colored impurity, which sedimented after a few days storage in solution.

HA, obtained from rooster comb by extraction with water, purified by precipitation with cetylpyridinium chloride and alcohol, and stored under refrigeration[22], was a gift from Prof. T. C. LAURENT.

HS of human aorta ("HS II"[23]), prepared by papain digestion, treatment with hyaluronidase and chondroitinase ABC and precipitation with cetylpyridinium chloride from 0.3 M sodium chloride, was a gift from Dr. P.-H. IVERIUS. HS obtained from the mother liquor of beef lung heparin ("HS I"[24]) was a gift from Dr. J. A. CIFONELLI.

Hep from hog intestinal mucosa, purified by precipitation with cetylpyridinium chloride in 1.2 M sodium chloride[23, 24], was a gift from Dr. U. LINDAHL.

DS of bovine aorta ("DS III"[23]) and DS of pig skin ("DS I"[24]), prepared by papain digestion, nuclease digestion, precipitation with cetylpyridinium chloride from 0.3 M sodium chloride and alcohol fractionation, were gifts from Dr. P.-H. IVERIUS.

CS-6 of human nucleus pulposus was obtained by extraction with 1 % sodium chloride[25], digestion with papain and precipitation with cetylpyridinium chloride in excess from 0.3 M sodium chloride.

A KS fraction ("XI A"[5]), obtained by digestion of bovine cornea with trypsin and collagenase, elution from ECTEOLA-cellulose with 0.45 M sodium chloride in 0.05 M hydrochloric acid, and alcohol fractionation, was a gift from Prof. T. C. LAURENT.

Unfractionated CS-4 from bovine nasal cartilage (Tables I and II; Fig. 1 A), prepared by papain digestion and precipitation with cetylpyridinium chloride from 0.3 M sodium chloride[26], was a gift from Dr. Å. WASTESON.

CS-4 fractions of varying molecular weight and degree of sulfation (Fig. 1 B) were gifts from Prof. T. C. LAURENT (*a*), Dr. Å. WASTESON (*b*)–(*f*) and Dr. P.-H. IVERIUS

from ECTEOLA-cellulose with 0.05 M hydrochloric acid, and otherwise was prepared as described for KS. Fraction (b) was a Sephadex G-100 fraction of hyaluronidase-treated bovine nasal cartilage CS-4 ("10"[26]); fractions (c) and (d) had in addition been fractionated on Dowex 1 X2 ("9-1.25 M" and "9-1.5 M"[27]). Fractions (e) and (f) were Sephadex G-200 fractions of bovine nasal cartilage CS-4 ("8" and "3"[26]). Fraction (g) from bovine aorta ("CS-1"[23]) was obtained by alcohol fractionation of the mother liquor of DS (see above).

Chromatographic equipment

Columns of DE-52 (1 × 5 cm) and Sephadex G-50 (1.5 × 40 cm) were packed in Plexiglass tubes. Both columns were equilibrated with 0.15 M sodium chloride containing 0.02 % of sodium azide as preservative. The DE-52 column was provided with a mantle of circulating water, which could be maintained thermostatically at 60°.

A salt gradient was produced according to HINTON AND DOBROTA[28] with a peristaltic pump, type SPI A-20 (Stålprodukter AB, Uppsala, Sweden). The internal diameters of the pump tubings were chosen so as to give a flow-rate from the reservoir to the mixing vessel of one third of the flow-rate from the mixing vessel to the DE-52 column. The various connections consisted of thin polyethylene tubing.

A de-aerator (air trap) was inserted between the mixing vessel and the pump. It had a total volume of 2–3 ml and was provided with a small amount of glass-wool at the inlet (to facilitate gas liberation). Before starting the gradient, the de-aerator was filled with de-aerated 0.2 M sodium chloride and placed in the water-bath maintained thermostatically at 60°. The equipment was arranged in such a way that the de-aerator was at a level above the DE-52 column (to produce a slightly negative hydrostatic pressure in the de-aerator).

After having connected the Sephadex column to the DE-52 column, the GAG (usually in 2 ml of salt solution) were applied quantitatively to the Sephadex column. The GAG were then transferred to the DE-52 column at room temperature by elution with 0.15 M sodium chloride containing 0.02 % of sodium azide at a rate of 8 ml/h. After about 30 ml (special calibration experiments were necessary for the Sephadex column) the high molecular weight material had entered the DE-52 column. The low molecular weight material retained by the Sephadex column, as well as the high molecular weight material not retained by the DE-52 column, could, if desired, be collected for special studies.

The GAG, which had been retained by the DE-52 column, were eluted at 60° with a lithium chloride gradient buffered at pH 4 at a rate of 5 ml/h. The mixing vessel contained 100 ml of 0.2 M lithium chloride + 0.05 M sodium acetate buffer of pH 4. This solution was freshly prepared by dilution of 5 ml of stock solution with de-aerated distilled water. The reservoir contained 3 M lithium chloride. The gradient, the fraction collector and the thermostat were started simultaneously. The final temperature of 60° was reached after about 1 h.

Analysis of the fractions

Aliquots (0.5 ml) of each fraction were analyzed for uronic acid by the carbazole reaction as described by BITTER AND MUIR[29], using 3 ml of sulfuric acid reagent. However, the borax concentration was increased to 0.05 M and 30 ml/l

Galactose (in KS) was analyzed by a modification of the primary cystein hydrochloride reaction of DISCHE AND DANILCHENKO[30]. Aliquots (0.5 ml) were boiled for 10 min with 3 ml of the sulfuric acid reagent described above. After cooling, 0.1 ml of 1.5% cystein hydrochloride in water was added. The colour was read after 3 h.

Chloride was determined titrimetrically with 0.1 M silver nitrate using potassium chromate as indicator.

RESULTS

A typical chromatogram of HA, CS-4 and Hep is shown in Fig. 1A. The positions in the chromatogram of other GAG are also indicated. As CS-4, CS-6, D S and HS with similar degrees of sulfation and molecular weights show the same c hromatographic behaviour, the polysaccharide backbone structure is apparently
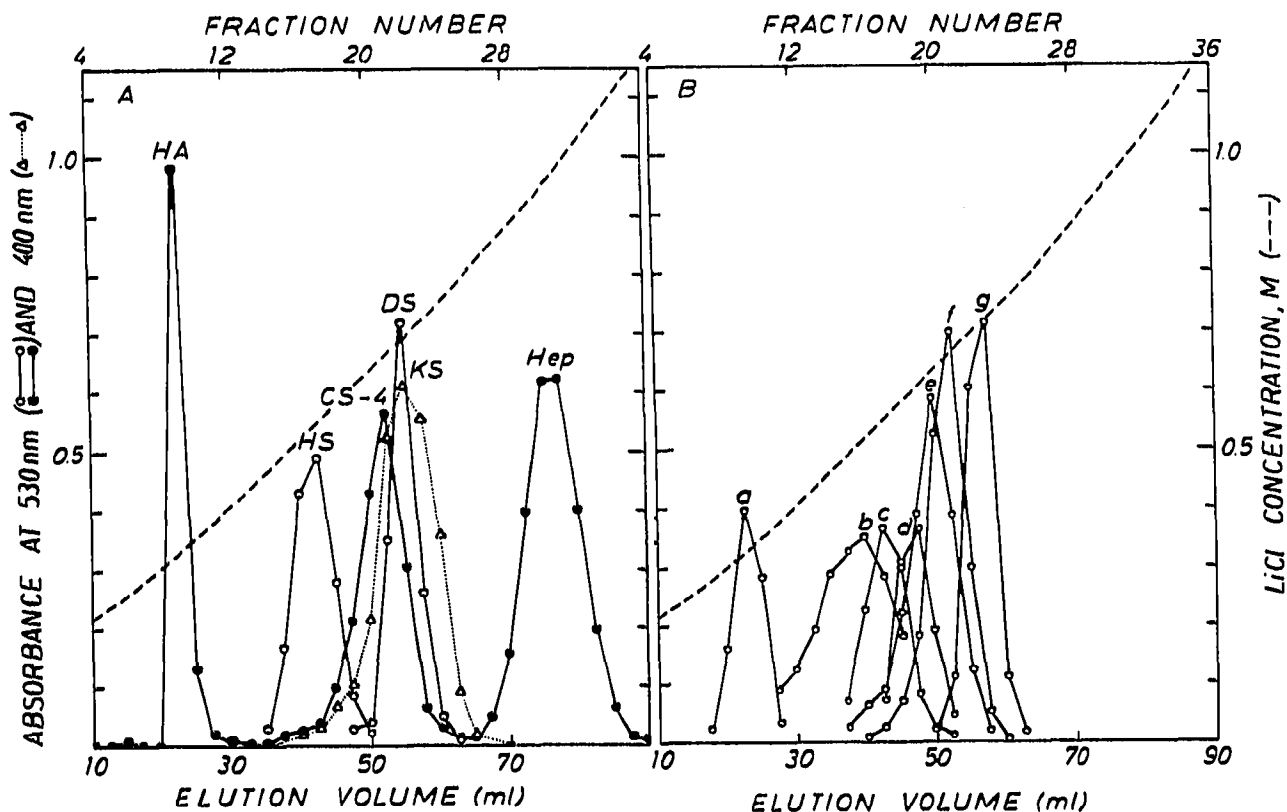

Fig. 1. (A). HA (mol.wt. ca. 10[6]), CS-4 ($\bar{M}_W = 20,000$; $\bar{M}_N = 15,500$)and Hep ($\bar{M}_W = 13,000$; $\bar{M}_N = 11,000$) chromatographed on a 1 × 5 cm DE-52 column. Elution was performed at 60° with a lithium chloride gradient buffered at pH 4. Superimposed peaks show the positions in the chromatogram of human aortic HS ($\bar{M}_W = 58,000$; open circles), bovine aortic DS ($\bar{M}_W = 41,000$; open circles) and a bovine corneal KS fraction ($\bar{M}_W = 17,500$; open triangles). Skin DS, nucleus pulposus CS-6 and heparin side fraction HS (all with mol. wts. ca. 20,000) were eluted as CS-4. HA contain 0.0, aortic HS 0.5 and Hep 2.4 sulfate groups per disaccharide unit; the other GAG contains approximately one sulfate group per disaccharide unit. (B). Chromatographic behaviour of various CS-4 preparations differing in molecular weight ($\bar{M}_W$) and degree of sulfation (number of sulfate groups per disaccharide unit). (a) = 39,000, 0.1; (b) = 5,000, 0.96 (inhomogeneous); (c) = 8,500, 0.90; (d) = 8,500, 1.14; (e) = 12,500, 0.96; (f) = 19,500, 0.95; (g) = 37,000,

of less importance. It is evident that GAG with a higher degree of sulfation or a higher molecular weight are preferentially retarded on the anion-exchange column. This is also supported by the experiments summarized in Fig. 1B, where the chromatographic behaviour of various CS-4 preparations are compared. It should be noted, however, that KS (Fig. 1A), which, contrary to the other GAG studied, does not contain carboxyl groups, is eluted at a higher salt concentration than the CS-4 with a comparable molecular weight and degree of sulfation[5,31].

To test the reproducibility and the resolution of the procedure, the experiments summarized in Table I were performed. When the amount of CS-4 applied increases, the peak appears at a slightly lower salt concentration. With optimal amounts of CS-4 (0.5–2 mg; maximum 5 mg) and provided that the gradient rates and fraction volumes are constant, the reproducibility of the chromatographic behaviour is reasonable.

TABLE I

DISTRIBUTION OF BOVINE NASAL CARTILAGE CS-4 IN THE 2.5-ml FRACTIONS WHEN DIFFERENT AMOUNTS HAD BEEN CHROMATOGRAPHED

Distribution on re-chromatography of individual fractions.

Material analyzed	Percentage of the material obtained in fraction No. (chloride concentration in brackets)									
	15 (0.49)	16 (0.52)	17 (0.55)	18 (0.58)	19 (0.60)	20 (0.63)	21 (0.66)	22 (0.69)	23 (0.72)	24 (0.75)
CS-4 0.2 mg				3	8	21	36	25	7	
CS-4 0.4 mg			2	5	10	22	37	19	4	1
CS-4 1.0 mg		1	2	5	11	24	33	19	4	1
CS-4 2.0 mg		1	2	5	12	25	32	18	4	1
CS-4 5.1 mg[a]	1	1	3	8	18	29	26	12	2	
CS-4 10.2 mg	1	1	4	11	22	27	22	10	2	
Fraction[a]										
No. 16 + 17	2	17	40	34	7					
No. 18		1	5	39	46	8	1			
No. 19			3	35	50	11	1			
No. 20				5	51	40	4			
No. 21					11	64	24		1	
No. 22					1	27	61	9	2	
No. 23						10	52	35	3	

[a] 2 × 0.2 ml of each fraction of the 5 mg experiment were analyzed for uronic acid after dilution with 0.3 ml of water. The remainder of the fractions (2.1 ml) were re-chromatographed individually.

When individual 2.5-ml fractions of the DE-52 chromatogram are re-chromatographed (Table I), it appears that there has been a complete and reproducible separation between the beginning and the end of the CS-4 peak. It is also clear that about 60% of the material appeared within 2.5 ml of the gradient, and about 90% within 5 ml. This indicates that an appreciably higher resolution in the chromatogram cannot be obtained by decreasing the fraction volume below 2.5 ml.

As is evident from Table II, in which a series of recovery experiments are

TABLE II

RECOVERIES IN THE CHROMATOGRAPHY OF HA (0.5 mg), CS-4 (1 mg) AND Hep (2 mg)

1-ml solutions of GAG containing specified amounts were applied to the columns. Standards for the colorimetric analysis were prepared from the same solutions by dilution with 0.3, 0.7 and 1.0 M lithium chloride, respectively.

Run No.	Component applied	Percentage recovery in fraction No.		
		9–12	16–25	26–36
1	HA	96	—	—
2	CS-4	1[a]	103	—
3	HA	100	—	—
4	CS-4	0.5[a]	101	—
5	Hep	—	—	97
6	HA	100	—	—
7	—	1[a]	—	—
8[b]	HA + CS-4 + Hep	96	103	100
9	—	1[a]	—	—

[a] A small amount of HA was retained on the DE-52 column and appeared in the succeeding chromatogram.

[b] Chromatogram shown in Fig. 1A.

exception is HA, of which a small amount was retained on the anion-exchange column and appeared in the succeeding chromatogram.

Further experiments* showed that no GAG passes the DE-52 column during the preliminary gel chromatographic step, even when as much as 20 mg of HA + 50 mg of CS-4 is applied. No material is eluted in the first 10 ml of the chromatogram (when equilibration of the DE-52 column to pH 4, 0.2 M lithium chloride and 60° occurs), or in the low molecular weight fraction of the Sephadex G-50 column.

To study the material retained by the DE-52 column, a mixture of HA (10 mg) and CS-4 (20 mg) was applied and eluted by the standard procedure. In four consecutive chromatograms, 2.0, 0.6, 0.3 and 0.1%, respectively, of the HA originally applied to the column was eluted at a salt concentration characteristic of this polysaccharide. In contrast, no CS-4 was detected in these chromatograms.

DISCUSSION

Preliminary chromatographic experiments with fibrous DEAE-cellulose gave a number of rather controversial results. It was found that only about 50% of HA was eluted with a sodium chloride gradient. The retained material could not be eluted by increasing the ionic strength of the eluting agent. The yield of HA and the sharpness of the peak were improved by increasing the temperature and running the gradient slowly. Inclusion of sulfate in the gradient solution increased the yield of HA, but tended to decrease the yield of the sulfated GAG. Broadening of the peaks was observed if chloride was absent at any stage of adsorption or elution of the

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* In these experiments sodium azide was excluded. Azide used as preservative in the gel

GAG on the column. Thus it was found advantageous to apply the GAG in dilute sodium chloride solution rather than in pure water.

From observations made by KNIGHT[32] and by HOW et al.[18], it can be concluded that the way in which the material is applied to the ion-exchange column is very important for the chromatographic behaviour. It seems that the GAG solution should be applied on the ion-exchange column at as high a speed and as high a salt concentration as possible. In this way, the material has a minimal chance of coming into contact with difficultly accessible regions of the ion-exchange particles or becoming trapped because of their shrinkage during the elution step. In the present procedure, this has been achieved by introducing the gel chromatographic step for application of the GAG.

The microgranular DE-52 (ref. 32) was found to be superior to the other anion exchangers tested (ECTEOLA-cellulose, fibrous DEAE-cellulose, DEAE-Sephadex, AG 1-X2) in giving sharp peaks and high recoveries. This is in accordance with the work of BOHN AND KALBHEN[12], who made an extensive comparison between several methods for separation of GAG.

The position of the components in the chromatogram was influenced by pH, although not in such a critical way as is experienced with proteins. By excluding the acetate buffer of pH 4 and running the gradient at an ambient pH of about 5, virtually the same chromatogram was obtained, with the exception that the Hep peak was shifted to two earlier fractions. However, in runs at ambient pH, it was on some occasions noted that the pH increased to 7–8. Under such conditions, HA was eluted at a higher, and CS-4 and Hep at a lower, salt concentration. For reproducible results, it is therefore advisable to control the pH. The choice of acidic conditions had several advantages. The resolution between the components improved, the nucleic acids (as obtained in proteolytic digests of connective tissues) moved ahead of the monosulfated GAG and the ion-exchange column did not accumulate bicarbonate.

Although lithium chloride, previously used by SILBERT[9], was found to be the most convenient to use for the present purpose, similar chromatograms were obtained with sodium chloride and magnesium chloride at similar chloride concentrations. However, with sodium chloride, slightly poorer resolution was obtained. With magnesium chloride (run without buffer), aortic HS was eluted relatively closer to HA, possibly indicating a stereospecific effect in the binding of the divalent cation to the former polysaccharide.

Running the gradient slowly and increasing the temperature sharpened the peaks and increased the yields in the preliminary experiments. However, after introduction of the gel chromatographic step, these parameters became less critical. By running the gradient at room temperature instead of 60°, the yield of HA decreased to about 90 %, whereas the yield of the sulfated GAG seemed to remain quantitative. At lower temperatures, the peaks were shifted to a lower salt concentration, a well known phenomenon[10,21].

In the development of this method, the HA caused the greatest problems. In fact, these problems have not been completely solved, as a small amount (1–3 %) remains on the ion-exchange column and interferes in the following chromatograms. The high molecular weight of HA compared with the other GAG studied may account

to elute non-branched glucuronoglycans from DEAE-cellulose at pH 4, and BENDICH *et al.*[3] reported low recoveries of nucleic acids at pH 7 with this ion exchanger.

There is a close parallel between the chromatographic behaviour of the GAG on ion-exchange columns and the dissociation of, *e.g.*, cetylpyridinium–polyanion complexes in electrolyte solutions as described by SCOTT and co-workers[21,33,34]. In both instances there is a dissociation at certain critical electrolyte concentrations (CEC) typical of each polyanion species, the best resolution being obtained by using small, highly hydrated cations. A mathematical model based on the law of mass action was developed by LAURENT AND SCOTT[34]. For a given polyanion species, there was shown to be a linear relationship between the inverse of the molecular weight and the logarithm of the CEC. This seems to apply to the present method also. Using the available data and defining CEC as the lithium chloride concentration at the peak maximum, the monosulfated glycosaminoglycuronans fitted the equation:

$$1/MW = -1.19 \cdot 10^{-3} \log CEC - 1.6 \cdot 10^{-4}$$

From this equation, it can be concluded that the molecular weight fractionation effect for molecular weights above 20,000 is relatively small. The chromatographic behaviour is then influenced mainly by the degree of sulfation. It can furthermore be concluded that even if the molecular weight is very high (approaching infinity in the equation above), the monosulfated glycosaminoglycuronans will be eluted at a lithium chloride concentration below 0.75 M in the present system.

When compared with other ion-exchange procedures for the separation of GAG[4-17], it seems that the present method is advantageous with regard to recovery and peak sharpness. In these respects, the procedure described here is comparable with methods based on cetylpyridinium fractionations, *e.g.*, as described by ANTONOPOULOS *et al.*[33]. By application of the GAG to the ion-exchange column via a gel chromatographic step, using a gradient for elution of the GAG and analysing aliquots with strong sulfuric acid reagents, a minimum of handling is involved.

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