CELLULOSE COLUMN CHROMATOGRAPHY FOR THE FRACTIONATION AND ISOLATION OF ACID MUCOPOLYSACCHARIDES

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A number of techniques are available for fractionation and isolation of acid mucopolysaccharides. In one procedure, AMPS** is precipitated from an aqueous solution containing different metal ions (Ca, Mg, Ba) with organic solvents[1,2]. Ion exchange chromatography with Dowex[3,4], DEAE[5] or ECTEOLA cellulose[6] have been used by other investigators. The complex formed between polyanions and quaternary ammonium salts, described by SCOTT[7,8] has since been utilized as the basis for the isolation of individual fractions by several investigators[9–11]. It has been our experience that none of these methods provides sharp separations.

ANTONOPOULOS and co-workers[12] recommended a method combining the SCOTT procedure with column chromatography. This provided clean separations of HA, the CS group and Hep, but was not capable of distinguishing members of the chondroitin sulfate group on Dowex 1, DEAE or ECTEOLA columns[13–15].

To permit identification of members of the chondroitin sulfate group in investigations of arterial AMPS, a two-step procedure was adopted. The first step served to separate HA, HMS, and the CS group by elution from a Dowex 1 column[4,15] or the CP–AMPS complexes from a cellulose column[16], in the second step, the CS group was subjected to alcohol fractionation on a Hyflo supercel column, an adaptation of KAPLAN AND MEYER's technique[17]. It proved feasible to combine these two steps on a single cellulose column for the sharp separation of individual components from the mixture in isolated aortic AMPS.

MATERIALS

Reference standards consisted of: HA (Sigma Co., St. Louis, Mo., Grade I) purified by CPC fractionation: CS-A (Nutritional Biochemicals Co., Cleveland, Ohio) purified twice by alcohol fractionation; CS-B was kindly furnished by Dr. J. A. CIFONELLI, Chicago University; CS-C (Kakenaku-Kako Co. Ltd., Kyoto, Japan); HMS extracted from human aorta and purified by alcohol fractionation after Dowex 1 column fractionation; Hep (Upjohn Co., Kalamazoo, Mich.) purified by Dowex 1 column fractionation.

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** Abbreviations used: AMPS = acid mucopolysaccharides; HA = hyaluronic acid; CS = chondroitin sulfate; HMS = heparitin sulfate; Hep = heparin; CP(C) = cetyl pyridinium (chloride).

(*a*) *Preparation of the column*

The cellulose column was a modification of those described by GARDELL[18] and ANTONOPOULOS *et al.*[12,10]. Glass tubes with an inner diameter of 1.0 cm and a length of 20 cm extending from a pear-shaped 200 ml reservoir, were filled with cellulose powder (Standard grade, Whatman Co.) using a slurry that had been washed four times in distilled water.

(*b*) *Fractionation*

Two to five mg of AMPS dissolved in 20 ml of 0.05 *M* NaCl, were precipitated quantitatively with 2–3 ml of 2 % aqueous CPC (K & K Laboratory Co.) (see Table I).

After the addition of about 0.3 g of Celite (Johns-Manville) the mixture was allowed to stand overnight at 25°; the CP–AMPS complex was then harvested by centrifugation, resuspended in washing solution (0.05 *M* NaCl in 0.01 % CPC aqueous

TABLE I

FRACTIONATION AND ISOLATION OF ACID MUCOPOLYSACCHARIDES

1. Dissolve sample in 0.05 *M* NaCl.
2. Add 2 % CPC aqueous solution.
3. Add 0.3 g of Celite.
4. Allow to precipitate overnight at 25°.
5. Centrifuge, discard supernatant.
6. Apply precipitate to cellulose column.
   Elute in sequence with:
7. 0.35 *M* NaCl (40 ml).
8. 0.6 *M* NaCl (40 ml).
9. Abs. ethanol (5 ml) and 90 % ethanol sat. Ca acetate (35 ml).
10. 30 % ethanol with 5 % Ca acetate 3 % acetic acid (40 ml).
11. 10 % ethanol with 5 % Ca acetate 3 % acetic acid (40 ml).
12. Dialyse each fraction against distilled water, 2 days.
13. Add 4 volumes of 95 % ethanol containing 1 % K acetate and 1 % acetic acid.
14. Store 2 days at 20°.
15. Centrifuge at 2,000 r.p.m. for 20 min.
16. Wash 2 times with 95 % ethanol.
17. Air dry.

solution) and transferred to the column. The flow rate from the column was adjusted between 0.2 and 0.4 ml/min by addition of Celite suspended in washing solution. Stepwise elution at 25° was conducted using in turn 40 ml each of 0.35 *M* and 0.6 *M* NaCl. An automatic fraction collector (LKB) was used to collect the effluent in 5 ml quantities. The column was then washed with 5 ml of absolute ethanol and with 35 ml of 90% ethanol saturated with calcium acetate, disrupting the CP–AMPS complex. Further elution was conducted using successively 40 ml quantities of 55 %, 45 %, 30 % and 10 % ethanol in a solution of 5% calcium acetate and 3% acetic acid. When kerato sulfate is known to be absent, the 55% elution may be omitted; similarly elution with 45 % ethanol is necessary only when chondroitin sulfate A is present. The presence of AMPS in the collection tubes was ascertained by the precipitation reaction with CPC. Uronic acid was measured in each positively reacting effluent tube using both the carbazole[20] and orcinol[21,22] methods. The presence of CPC, NaCl and calcium in the effluent caused errors in the uronic acid determination of less than 5%.

(c) Identification of acid mucopolysaccharide fractions

Identification of each eluent was performed by chemical analysis, acetate paper electrophoresis, infrared spectroscopy, digestion with hyaluronidase and paper chromatography of the product of acid hydrolysis.

Chemical analyses included the carbazole and orcinol reactions for uronic acid, as previously mentioned, the BOAS[22] modification of the Elson-Morgan reaction for hexosamine, and the method of EGAMI AND TAKAHASHI[24] for sulfate groups.

Electrophoresis was performed with acetate paper (Sepraphore III, Gelman Co.) in a horizontal chamber using pyridine–acetic acid–water (5:100:895, v/v) buffer pH 3.6[25] and a constant current of 0.5 mA/cm for 120 min at 25°. The air dried strip was stained with alcian blue (0.1 % alcian blue and 5 % acetic acid in 20 % ethanol). The Beckman Analytrol, Model RB, was used at 660 mμ for determination of the percentage composition of the mixture[26].

Infrared spectrum. A few drops of aqueous AMPS solution were allowed to dry on the surface of a cell plate formed of 56 % thallium iodide, 4 % thallium bromide and 40 % sodium chloride (Hitachi Co.). After further dehydration in a desiccator overnight, the infrared spectrum was recorded from 1,330 cm^{-1} to 400 cm^{-1} using a Perkin-Elmer model 337 spectrophotometer.

Hyaluronidase (EC 4.2.99.1) digestion of AMPS was performed in dialysis tubing with testicular hyaluronidase (Nutritional Biochemicals Co.) in 0.1 *M* phosphate buffer, pH 7.0 constituted with a physiologic concentration of NaCl. After 4 days of dialysis at 37° (against the same buffer), unaffected AMPS retained in the dialysis tube was measured by the orcinol method.

Identification of products of acid hydrolysis was accomplished by paper chromatography.

Hexosamine was determined in the following manner: after hydrolysis with 4 *N* HCl at 100° for 12 h in sealed tubes, the amino sugar component of each AMPS was ascertained by descending paper chromatography on Whatman No. 1 paper with *n*-butanol–pyridine–water (5:3:2, v/v) for 30 hours at 25°. The chromatograms were stained with ninhydrin (0.1 %) in acetone and with a water-saturated butanol solution (30 ml) of aniline (3 ml) and phthalic acid (4.8 g)[27], D-glucosamine and D-galactosamine hydrochlorides (Nutritional Biochemicals Co.) were used as control standards.

Uronic acids in AMPS were identified by the method of RADHAKRISHNAMURTHY AND BERENSON[28] following hydrolysis for 6 h at 100° in sealed tubes with formic acid. Descending paper chromatograms (Whatman No. 1) obtained with *n*-butanol–acetic acid–water (4:1:2, v/v for 18 h, were developed with aniline–phthalic acid solution and silver nitrate. D-Glucuronic acid (Sigma Co.) and L-iduronic acid derived from hydrolysed CS-B were used as standards.

(d) Recovery of individual standard acid mucopolysaccharides

Five mg of each AMPS (HA, HMS, CS-A, CS-B, CS-C and Hep) were dissolved in 25 ml of 0.05 *M* NaCl. Five ml of each solution were retained as a control while 2 ml of 2 % aqueous CPC and 0.3 g of Celite were added to the remaining 20 ml which was then kept overnight at 25°. The CP–AMPS precipitate separated by centrifugation was applied to a cellulose column and fractionated in sequence with NaCl and with ethanol as previously described.

TABLE II

CELLULOSE COLUMN FRACTIONATION OF ACID MUCOPOLYSACCHARIDES

Fraction	*Recovery of individual standards* (%)					
	HA	*HMS*	*CS-A*	*CS-B*	*CS-C*	*Hep*
0.35 *M* NaCl	98.2	0	0	0	0	0
0.6 *M* NaCl	0	97.3	2.0	0	3.0	2.0
55 % ethanol	—	—	—	—	—	—
45 % ethanol	0	0	22.7	0	65.5	0
30 % ethanol	0	0	74.8	2.1	30.1	0
10 % ethanol	0	0	0	96.6	0	98.7
Total recovery	98.2(± 0.5)	97.3(± 0.5)	99.5(± 1.2)	98.7(± 1.1)	98.6(± 1.2)	100.7(± 1.2)

The recovery rate for each substance exceeded 97 % as shown in Table II.

(*e*) *Recovery of standard acid mucopolysaccharides from a mixture*

Four mg of HA, HMS, CS-B and CS-C were mixed and dissolved in 40 ml of 0.05 *M* NaCl, 10 ml of 2 % aqueous CPC and 0.5 g of Celite were added. The mixture was left overnight at 25° and subjected to the fractionation procedure previously described. AMPS recovered in each fraction was measured and identified. The results shown in Table III indicate very effective separation and recovery of the individual standards.

TABLE III

RECOVERY OF STANDARDS FROM A MIXTURE OF ACID MUCOPOLYSACCHARIDES WITH CELLULOSE COLUMN FRACTIONATION

Fraction	*Substance*	*Purity as revealed by acetate paper electrophoresis* (%)	*Recovery* (%)
0.35 *M* NaCl	HA	100	98.5
0.6 *M* NaCl	HMS	100	98.3
90 % ethanol (Ca acetate saturated)	No AMPS		0
30 % ethanol	CS-C	97	98.2
	CS-B	3	
10 % ethanol	CS-B	97	97.7
	CS-C	3	

(*f*) *Human aortic acid mucopolysaccharides*

Sodium chloride was added to aqueous solutions of AMPS extracted from aortas of twenty to forty year old normal humans (20–30 ml containing 1.0 to 2.0 mg of orcinol reacting uronic acid) to a concentration of 0.05 *M* (Table IV). Two or three ml of 2 % aqueous CPC and about 0.3 g of Celite were added and allowed to react overnight at 25°. The resultant CP–AMPS complexes were isolated and fractionated. Each fraction was measured and identified by acetate paper electrophoresis (those not completely resolved were refractionated). Electrophoretically homogeneous

TABLE IV

CELLULOSE COLUMN FRACTIONATION OF ACID MUCOPOLYSACCHARIDES OF HUMAN AORTAS

Eluent	*AMPS* (%)	*Carbazole–orcinol ratio*	*Uronic acid*–hexosamine ratio*	*Sulfate–hexosamine molar ratio*	*Paper chromatography*		*Digested*** (%)	*Identity****
					Hexosamine	*Hexuronic acid*		
0.35 *M* NaCl	16.3	0.98	1.01	0.11	Glucosamine	Glucuronic acid	98	HA
0.6 *M*	11.8	1.73	0.75	0.65	Glucosamine (2% galactosamine)	Glucuronic acid	4	HMS
30% ethanol	58.9	1.04	1.15	0.97	Galactosamine (2% glucosamine)	Glucuronic acid	95	CS-C
10% ethanol	10.2	0.58	0.98	0.98	Galactosamine	Iduronic acid	0	CS-B
Recovery	97.2							

* Uronic acid determined by the orcinol method.
** With testicular hyaluronidase.
*** Verified by electrophoretic mobility and infrared spectral absorption.

fractions were further identified by paper chromatography of amino sugars and uronic acids, by sulfate group determinations and by infrared spectroscopy.

DISCUSSION

As the accompanying Table II shows, the recovery of any of six individual acid mucopolysaccharides from the fractionation procedure exceeds 97 %; with a mixture of four, such as those normally present in arteries[17,20,30], highly effective separation is accomplished with no impairment of the yield. Acetate paper electrophoresis indicated mutual contamination of CS-C and CS-B of about 3 %.

Additional steps are necessary if the AMPS mixture included CS-B and Hep, both of which are eluted in the 10 % ethanol fraction, and if CS-A and CS-C are present together. Since the salt solubilities of the CP-complexes of CS-B and Hep are different[4,31], they can be easily separated using a cellulose column and two eluting solutions differing in salt concentration. The incomplete partition of CS-A and CS-C in the 45 % and 30 % ethanol fractions can be rectified by precipitation of CS-A with 45 % ethanol containing 5 % calcium acetate and 3 % acetic acid.

The results of the fractionation of human aortic AMPS are summarized in Table IV and Fig. 1. A small amount of chondroitin sulfate eluted with 0.6 *M* NaCl accounted for the presence of 2% galactosamine in the HMS fraction. A greater

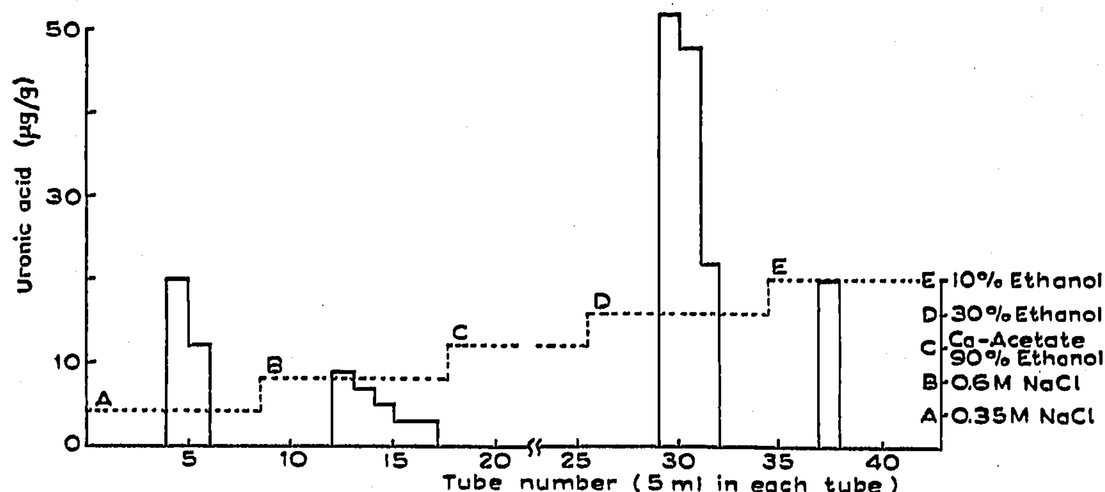

Fig. 1. Fractionation of aortic acid mucopolysaccharides.

potential error may arise because quantities of HMS at times as much as 10 %, are incompletely eluted with 0.6 *M* NaCl and then appear with CS-B in the 10 % ethanol fraction. Electrophoresis and paper chromatography permit such occurrences to be recognized. They are then corrected by careful refractionation of the CP-complex with 0.6 *M* NaCl. This difficulty seems attributable to reported variations in sulfate content and the degree of polymerization[32–34]. LAURENT AND SCOTT[35] observed that CS–CP complexes of low molecular weight are more readily soluble in salt solutions than complexes of high molecular weight. It is to be noted that neither heparin nor kerato sulfate were identified in analyses accounting for more than 97 % of human aortic AMPS contrary to other published reports[20,36].

SUMMARY

An acid mucopolysaccharide (AMPS) mixture may be separated into its individual constituents by eluting the cetyl pyridinium complexes from a cellulose column with 0.35 *M* and 0.6 *M* NaCl, disrupting the complexes with 90% ethyl alcohol and eluting further with 55, 45, 30 and 10% concentrations of ethanol. Depending upon the specific AMPS in the mixture, the procedure can be simplified further. Human arterial AMPS, for example, can be adequately partitioned with only two concentrations of alcohol, 30 and 10%. Acetate paper electrophoresis, infrared spectra, chemical analysis, hydrolysis with hyaluronidase and paper chromatography of products of acid hydrolysis of AMPS are used to identify the separated components and to ascertain the adequacy of separation. Different situations encountered during separation of various mixtures of AMPS are discussed.

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