A MICROMETHOD FOR THE DETERMINATION OF ACID MUCOPOLYSACCHARIDES IN VASCULAR TISSUE

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INTRODUCTION

Accurate isolation and quantitative measurement of the different acid mucopolysaccharides (AMPS*)[1-8] of human and animal tissues is complicated by the structural similarity of the different macromolecules, by variations in size (*e.g.* HA from Rous sarcoma has a smaller particle size than HA from normal tissue[9]); and by differences in the relative composition of individual AMPS isolated from different tissues[10,11]. These difficulties are magnified when attempts are made to isolate and fractionate AMPS quantitatively from minute samples of tissue which contain only minor amounts of these substances.

KAPLAN AND MEYER[12] identified the constituents of human aortic AMPS as HA, HS, ChS-B (dermatan sulfate, B-heparin) and ChS-C (chondroitin 6-sulfate). They observed alterations in the relative proportions (though not the total amount) of the four AMPS with age, but this technique required large pooled samples, and, therefore, was not suitable for the measuring of the AMPS pattern of individual aortas. Micromethods by others, such as MANLEY[3] and MANLEY AND KENT[4] did not provide sufficient information to indicate the quantitative effectiveness of their fractionation. The same criticism can be applied to TRUNDLE AND MANN'S[5] report of a semi-micro (5 mg) procedure which is a modification of RINGERTZ AND REICHARD'S[13], using in addition electrophoresis on cellulose acetate followed by quantitation by densitometry after staining with acridine orange.

Quantities containing as little as 2–5 μg of hexosamine were assayed by ANTONOPOULOS *et al.*[1]; but the method is relatively involved and does not distinguish members of the chondroitin sulfate group. Moreover, in its application to human aorta[2] the complete separation of components was achieved only by sacrificing quantitative recovery.

TANAKA AND GORE[6] fractionated an aortic AMPS mixture by successive elution of the cetylpyridinium complexes from a cellulose column with 0.35 and 0.6 *M* sodium chloride solutions; the residual chondroitin sulfate complexes were then disrupted with absolute ethanol and elution continued with diminishing concentrations of ethanol in water. Having established the identity of the fractions separated by electrophoresis on cellulose acetate, densitometric measurements served to indicate the

* Abbreviations used: AMPS = acid mucopolysaccharides; HA = hyaluronic acid; ChS-A = chondroitin sulfate A; ChS-B = chondroitin sulfate B; ChS-C = chondroitin sulfate C; HS = heparitin sulfate.

proportionate composition of AMPS. The procedure proved adequate to analyze human aortic AMPS containing "1.0–2.0 mg, of orcinol reacting uronic acid", or approximately twice that quantity of pure AMPS.

The present report describes a highly reproducible micro procedure for the determination of AMPS in aortic tissue, which seems to overcome some of the limitations inherent in reported methods.

METHODS

Fresh or frozen aortic tissue (approx. 50 mg of intima and media) was carefully freed of adventitia and kept overnight at room temperature in 10 ml of acetone in a capped tube. After slicing this tissue into 1 mm sized fragments, acetone extraction was continued for 24 h using two changes. Acetone insoluble lipids were first removed by suspending each sample in chloroform–methanol (1:1,v/v, 10 ml) at room temperature for 6 h, and then in fresh solvent overnight. The dehydrated and defatted sample was dried in air and in a vacuum oven (200 mm Hg, 25°) overnight. Each sample (approx. 10 mg) was then placed in a 5 ml tube containing 0.3 ml of 0.1 *N* NaOH and allowed to stand at room temperature overnight. Using 0.1 *N* HCl to adjust the pH to 6.4, activated papain solution* (0.3 ml) and 1 drop of *n*-octanol were added and the mixture incubated at 65° overnight.

Following proteolysis, 5 mg of Lloyd's reagent were added to each tube and thoroughly mixed. The supernatant was collected by centrifugation in the cold (+ 4°) at 1400 *g* for 20 min, to which was added a 0.6 ml distilled water rinse of the precipitate.

Fractionation of the isolated AMPS was achieved on an Ecteola cellulose column prepared as follows: a glass microcolumn (length 1.3 cm, I.D. 0.3 cm) was packed with Ecteola cellulose suspended as a slurry in a solution of 0.025 *M* NaCl and 0.025 *M* HCl; the final bed volume was approximately 0.5 ml. Impurities were eluted with a solution of 0.025 *M* NaCl and 0.025 *M* HCl (1.3–1.5 ml); AMPS applied to the column were eluted with a solution of 3 *M* NaCl in 0.1 *M* HCl. Exactly 5 ml of eluate was collected; 1 ml was used for the determination of uronic acid (orcinol method[14]) and hexosamine[15]. The remaining 4 ml was transferred to Visking tubing (length, 7 cm; diameter, 3 cm) and dialysed with constant stirring for 48 h against four changes of 300 ml of distilled water at 4°. After quantitative transfer to a 20 ml flask the dialysed sample was lyophilized.

For electrophoresis, the lyophilisate was dissolved in sufficient but definitive amount of distilled water to form a solution containing approximately 1 μg/μl of uronic acid. Two microliters of each sample was then applied to a cellulose polyacetate strip (Sepraphore III, Gelman 1 in. × 8.5 in.) and subjected to electrophoretic separation at pH 3.6, using a buffer of copper acetate (20 g), glacial acetic acid (100 ml) and water (900 ml) at a constant current of 0.5 mA/cm for 2 h. The strips were then stained by dipping for 15 min in a solution of alcian blue (0.5 g of alcian blue dissolved in 50 ml of ethanol and 25 ml of glacial acetic acid and made up to a volume of 0.5 l with distilled water). After three rinses in 5 % acetic acid for 5 , 5 and 20 min, the strips

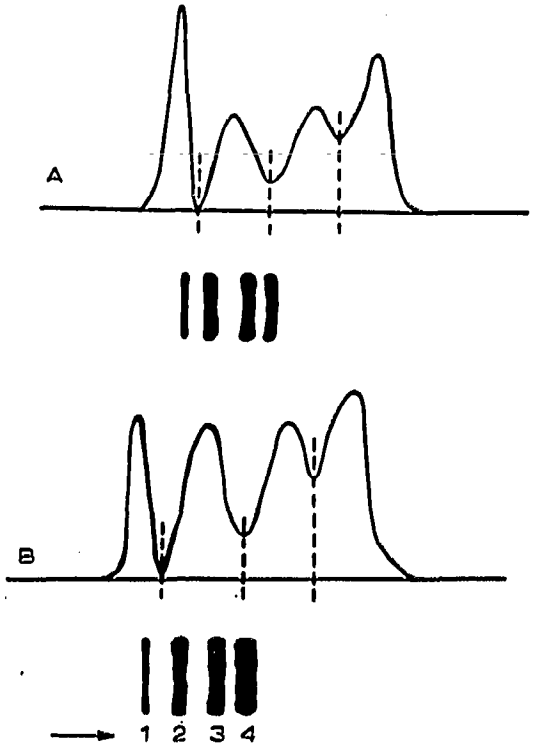
* Crystalline papain (Sigma) (0.32 mg), EDTA (0.9 mg) and cysteine·HCl (0.4 mg) were dissolved in 0.1 *M* phosphate buffer (0.3 ml), pH 6.4.

were air dried and scanned on the Beckman Analytrol RB, equipped with Scan-A-tron (Gelman), using two red color filters.

By cutting out the observed peaks from the Beckman Analytrol charts and weighing them on a Cahn micro balance the relative proportions of the individual AMPS fractions were determined (See Fig. I, for the electrophoretic pattern of AMPS of human and rabbit aortas). These values were then converted into absolute quantities by referring to the predetermined standard curve for each individual AMPS (Fig. 2). Total AMPS content would then be calculated as the sum of the values obtained for the individual AMPS, or total AMPS may be expressed as uronic acid (measured prior to lyophilization), and the values of the individual fractions as percentages of the total.

DISCUSSION OF RESULTS

In practice, the method we have described proved adequate for the isolation of AMPS from 10 mg of dry, defatted, aortic tissue containing 80–100 μg of AMPS and for fractionation and determination of the isolate.



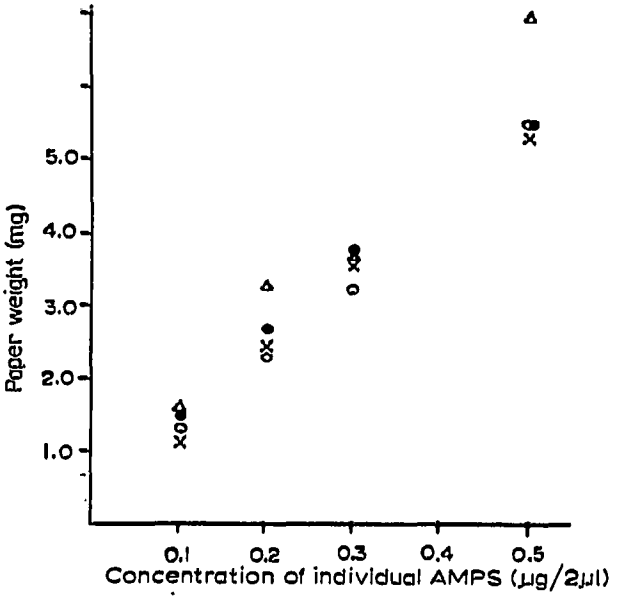


Fig. 1. Electrophoretic pattern of the AMPS from the aortic tissue of a human (A) and from a rabbit (B); the above curves were produced by densitometry. Electrophoresis and subsequent staining were performed as described under "Methods". Electrophoretic migration is indicated by the arrow: 1 = HA; 2 = HS; 3 = ChS-B; 4 = ChS-A/C.

Fig. 2. Standard curves for individual AMPS. HA was generously donated by Dr. A. TAUSSIG, ChS-C and B by Hoffmann-La Roche, N.J. and HS was prepared in our own laboratory from rabbit aorta by the method of TANAKA AND GORE[6]. (○) ChS-A; (△) ChS-B; (×) HS; (●) HA.

In determination of AMPS by the present procedure there are several possible sources of error:

(a) if not all of the AMPS are solubilized by proteolysis, erroneously low values would be obtained;

(b) a similar error would result from incomplete elution of AMPS from the Ecteola cellulose column;

(c) incomplete solubilization of the lyophilized sample;

(d) the details of the electrophoretic separation, moreover, are critical, since lack of clear separation lessens the reproducibility of the method.

To eliminate these possible sources of error, the procedure has been modified as follows:

(a) for proteolysis, as others have done, papain was chosen for its rapid action at an elevated temperature[21], which inhibits bacterial growth. AMPS was completely solubilized since five samples (10 mg) of rabbit aorta after proteolysis were found to be free of hexosamine as determined by the BOAS[15] modification of the ELSON-MORGAN reaction;

(b) to establish the completeness of elution of AMPS, uronic acid was determined before and after chromatography on Ecteola cellulose column. Satisfactory recovery was obtained amounting to 96.1 ± 1.9 % (mean ± standard error of five determinations);

(c) the recovery of AMPS after lyophylization, electrophoresis and densitometry is lower, only 83.5 ± 3.7 % (mean ± standard error of five determinations). The overall recovery of AMPS during the whole procedure is, therefore, 80.2 %;

(d) considering only the reproducibility of electrophoresis on acetylated paper, and densitometrical evaluation of the individual AMPS components of five identical samples of rabbit's aorta, satisfactory results as noted in Table I were obtained.

Electrophoresis on Sepraphore III (Gelman) acetylated paper proved slightly superior to the other brands tried. The best overall separation of aortic AMPS is achieved using the buffer system reported in the experimental part. The following two buffers were also useful: (a) zinc acetate·H_2O (20 g), glacial acetic acid (100 ml) and distilled water (900 ml), pH 3.5, 0.5 mA, 90 min; and (b) cadmium acetate·$2H_2O$ (20 g), glacial acetic acid (100 ml) and distilled water (900 ml), pH 3.5, 0.5 mA, 90 min.

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF AMPS BY ELECTROPHORESIS AND DENSITOMETRY

AMPS (2.6 μg in 2 μl) were applied on acetylated paper and submitted to electrophoresis in copper acetate buffer and to densitometrical evaluation as described in "Methods".

Sample No.	*Total AMPS (μg)*	*HA (%)*	*HS (%)*	*CS-B (%)*	*CS-C (%)*
1	2.2	19.6	30.3	23.2	26.9
2	2.9	19.3	27.7	25.2	27.8
3	2.7	20.2	29.9	22.4	27.5
4	2.7	19.6	30.4	23.3	26.7
5	2.4	18.4	28.4	26.1	27.1
Mean ± S.E.	2.6 ± 0.1	19.4 ± 0.3	29.3 ± 0.5	24.0 ± 0.7	27.2 ± 0.2

The zinc buffer permitted sharp separation of ChS-B and ChS-A/C* fractions, while the cadmium salt proved advantageous in delineating HS and HA from the chondroitin sulfates.

Concerning staining of AMPS with alcian blue, MOWRY[16] had considered that nonsulfated and sulfated AMPS had different affinities for the dye, but this was denied by QUINTARELLI and associates[17]. In our experiments also, as shown in Fig. 2, the alcian blue stainability of all standard AMPS was virtually identical. It should be noted, however, that only HA was available as an analytical pure compound for standardization. All other AMPS which we have employed as standards were not analytical reagents but were homogenous when examined electrophoretically. Identification of the individual AMPS from rabbit aorta fractionated by the method of TANAKA AND GORE[6] was achieved by electrophoresis on acetylated paper simultaneously with standard AMPS. Extensive study concerning characterization of AMPS from rabbit aorta is presently in progress in our laboratory.

Every determination of the compounds which are not ideally separated involves some uncertainty. As a practical expedient, it was found that separation at the lowest point between adjacent "peak areas" and actual measurement of the weight of the graph paper gave reproducible results[19]. This method, therefore, was applied in our procedure.

Our micromethod was employed for the determination of AMPS in the aortic tissue (intima and media) of the group of 15 healthy New Zealand albino rabbits (females). Data obtained and presented in Table II could be considered as standard quantities of AMPS in the aortic tissue of this particular animal species.

TABLE II

AMPS CONTENT OF THE AORTAE OF HEALTHY NEW ZEALAND ALBINO RABBITS (FEMALES)
Values expressed as mean ± S.E.

No. of rabbits	*AMPS (uronic acid) (mg/g)*	*HA (%)*	*HS (%)*	*ChS-B (%)*	*ChS-C (%)*
15	3.94 ± 0.11	14.6 ± 0.4	31.0 ± 0.7	26.6 ± 0.4	27.8 ± 0.7

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* It was not possible to separate ChS-A from ChS-C, electrophoretically; it is indicated, however, that rabbit aortas contain only ChS-C[20].

SUMMARY

A micromethod for the quantitative estimation of acid mucopolysaccharides (AMPS) in human and animal vascular tissue is described. The procedure involves proteolysis of approximately 10 mg of dry, defatted tissue followed by separation and purification of AMPS by chromatography on an Ecteola cellulose column. Subsequent to electrophoresis, fractions were quantitated (after alcian blue staining) by densitometry.

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