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Note

Fractionation of aorta glycosaminoglycans by high-performance liquid chromatography

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Various analytical techniques have been reported for the fractionation of individual glycosaminoglycans (GAGs). The techniques include fractional precipitation by ethanol [1], the use of quaternary ammonium salts [2], ion-exchange chromatography [3], and cellulose acetate electrophoresis [4]. We [5] developed a sensitive automated ion-exchange chromatographic procedure using a Technicon sugar chromatography system with continuous monitoring of uronic acid by the orcinol–sulfuric acid reaction or by the carbazole–sulfuric acid reaction [6]. In an effort to enhance the sensitivity further and reduce the analysis time of the chromatography we now have developed a high-performance liquid chromatographic (HPLC) procedure using an ion-exchange column for GAG fractionation.

EXPERIMENTAL

Materials

Samples of hyaluronic acid and heparan sulfate were available in the laboratory from other investigations, after extensive preparative and fractionation tech-

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niques. Chondroitin sulfates A and C were purchased from Miles Labs. (Elkhart, IN, U.S.A.). Glucuronolactone was purchased from Corn Products Refineries (New York, NY, U.S.A.). Reagent-grade sulfuric acid was purchased from Dupont de Nemours (Wilmington, DE, U.S.A.). Analytical-grade sodium chloride, HPLC-grade water, and absolute methanol were from J.T. Baker (Phillipsburg, NJ, U.S.A.). Millex-HV 0.45-μm filter unit and 0.45-μm FH type filter for organic solvent were purchased from Millipore (Bedford, MA, U.S.A.). Chondroitin 6-sulfatase and 4-sulfatase and the chondroitinases AB and AC were purchased from Miles Labs.

Isolation of glycosaminoglycans

GAGs were isolated from intima preparations of aortas by methods previously described [7,8]. Briefly, the method of isolation of GAGs consisted of digestion of the tissue with alkali, followed by hydrolysis with papain, precipitation of peptide material in the digest by trichloroacetic acid (TCA), removal of the precipitate by filtration on celite pad, dialysis of the filtrate against distilled water, and lyophilization of the dialysate. GAG material was reconstituted in a known volume of distilled water. An aliquot of the solution was analyzed for uronic acid by the method of Bitter and Muir [9]. The remainder of the solution was filtered through Millex-HV 0.45-μm filter unit and was lyophilized. The GAG material was reconstituted in 150–175 μl of HPLC-grade water and used for chromatography to quantify individual GAGs.

High-performance liquid chromatography

A Perkin-Elmer Series 3 liquid chromatograph with a loop injection of 175 μl and a 150×7.5 mm Ultropac TSK 545 DEAE HPLC ion-exchange column (LKB Instruments, Gaithersburg, MD, U.S.A.) were used for fractionation of GAG mixtures.

A gradient solvent system consisting of solvent A (10% methanol in 1 *M* sodium chloride, v/v) and solvent B (10% methanol in water, v/v) was used for the separation of GAGs. The solvents were separately filtered and degassed through a 0.45-μm FH membrane filter in order to prevent the accumulation of particulate matter in the column and a consequent increase of back-pressure of the column. The solvent program mode and Series 3 microprocessor-controlled pump module were used to program the chromatographic conditions. The maximum pressure for the column was 24.54 bar. A three-segment solvent gradient program starting with 0.05 *M* and ending with 0.75 *M* sodium chloride was used with the following flow program sequence [10]: segment 1, 0.05–0.5 *M* curve No. 5 for 20 min; segment 2, 0.5–0.65 *M* curve No. 1 for 25 min; segment 3, 0.65–0.75 *M* curve No. 4 for 25 min. The flow-rate was 0.6 ml/min. The outlet of the column was connected to a fraction collector and fractions were collected every 4 min. Each fraction was analyzed for uronic acid by the method of Bitter and Muir [9]. Based on uronic acid profiles, fractions were pooled, dialyzed exhaustively against distilled water, lyophilized, and used for various analyses for characterization and quantification of individual GAGs.

Measurement of conductivity

The conductivity measurements were made in a Mili MHO conductivity meter (London Company, West Lake, OH, U.S.A.).

Electrophoresis

Electrophoresis of pooled fractions eluted from the column was performed on cellulose acetate strips (Helena Labs., Beaumont, TX, U.S.A.) in pyridine–formic acid buffer, pH 3.3, and GAGs were located on the strips by alcian blue stain [11].

Analysis of mixtures of chondroitin sulfates

Fractions containing mixtures of chondroitin sulfates and dermatan sulfate were analyzed for chondroitin 4- and 6-sulfates and dermatan sulfate by the procedure of Saito et al. [12] using chondroitinases.

RESULTS AND DISCUSSION

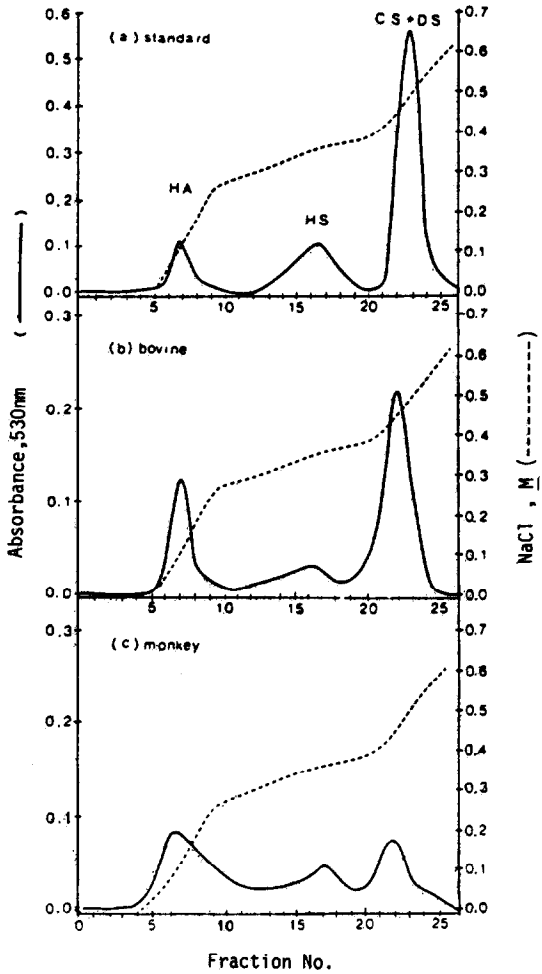
Fig. 1 illustrates the chromatographic profile of a synthetic mixture of hyaluronic acid, heparan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate mixed in equal proportions. Hyaluronic acid eluted from the column at a sodium chloride concentration between 0.05 and 0.30 *M*, heparan sulfate between 0.35 and 0.45 *M*, and chondroitin sulfates and dermatan sulfate coeluted between 0.45 and 0.64 *M*. The nature of GAG in each peak was confirmed by cellulose acetate electrophoresis (Fig. 2).

The reproducibility of the quantitation of individual GAGs by the procedure was checked a total of eighteen times using the same mixture of GAGs on ten consecutive days. Both within-day and day-to-day variations were noted. Within-day variation of individual GAGs ranged from 0 to 9% (one outlier of 16% was not included). Day-to-day variation ranged from 2.7 to 9.9%. The results indicate good reproducibility of the procedure.

The sensitivity of the method was studied using different amounts of the GAG mixture. We observed that under the conditions of chromatography 50–170 μg uronate produced well resolved peaks and the fractions could be easily monitored by the carbazole–sulfuric acid reaction of Bitter and Muir [9].

To study the suitability of the procedure for analysis of GAG mixtures from tissues, we isolated GAGs from bovine and monkey aortas and analyzed them for individual GAGs. Chromatographic profiles of aorta GAGs are shown in Fig. 1. Each of the mixtures resolved into three peaks. Analyses of the fractions under the peaks gave the following results: bovine aorta, hyaluronic acid/heparan sulfate/chondroitin sulfates and dermatan sulfate = 5:3:12; monkey aorta, hyaluronic acid/heparan sulfate/chondroitin sulfates and dermatan sulfate = 4:2:3. The results are in agreement with our earlier observations [13].

The HPLC procedure described above affords a rapid and reproducible method for fractionation of GAGs. The advantage of this technique over the previously reported technique [5] is its shorter analysis time. The chromatogram can be developed and the system can be purged and equilibrated within 2 h as opposed



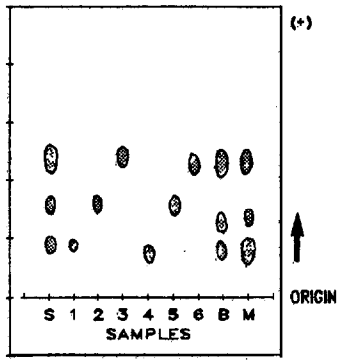


Fig. 1. HPLC resolution of GAG mixtures. Details of the procedure are given in the text. (a) A synthetic mixture of 25 μg each of hyaluronic acid (HA), heparan sulfate (HS), chondroitin 4- and 6-sulfates (CS), and dermatan sulfate (DS); (b) GAGs (150 μg uronate) isolated from bovine aorta; (c) GAGs (100 μg uronate) isolated from monkey aorta. Note that CS and DS coelute from the column. These isomeric GAGs were determined in the mixtures by an enzymatic procedure described in the text. We did not include heparin in the synthetic mixture of GAGs because heparin is not a constituent of aorta GAGs. Had heparin been included in the mixture it would have eluted after CS+DS from the column.

Fig. 2. Illustration of cellulose acetate electrophoresis of GAG fractions from HPLC. Electrophoresis was performed in pyridine-formic acid [11] and the GAGs were localized on the strips by alcian blue. S = Standard mixture of chondroitin 6-sulfate (C6-S), heparan sulfate (HS), and hyaluronic acid (HA); the numbers 1, 2, and 3 refer to standard sample of HA, HS, and CS, respectively; 4, 5, and 6 refer to HPLC peaks labelled as HA, HS, CS+DS, respectively (Fig. 1); B = bovine GAGs; M = monkey aorta GAGs.

to 5 h for development of chromatogram and 3 h for washing and regenerating the column in the previous procedure.

Application of this HPLC procedure to GAG fractionation and quantification is a significant aid to methods currently in use.

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