SEPARATION AND IDENTIFICATION OF ACID POLYSACCHARIDES BY THIN-LAYER CHROMATOGRAPHY*

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Thin-layer chromatography has previously been used for the successful separation of lipids and other relatively small molecules. In the present study, thin-layer chromatography (TLC) has been successfully applied to the difficult problem of separating sulfated acid polysaccharide molecules. The advantages of TLC, in contrast to many conventional paper and electrophoretic methods, include both an increased reliability of identification and a sensitivity to less than 1 microgram of sulfated acid polysaccharides.

MATERIALS AND METHODS

Preparation of media

Both cellulose powder and Sephadex have been used as media for TLC. The necessary equipment used, as well as details of the method of preparation of thin-layer plates, are outlined in a recent review[1]. Ten (10) grams of Cellulose powder** (C.P.) are mixed with 5–10 ml aliquots of water with constant mixing until approximately 60 ml of water have been added. The resultant slurry is spread as a 250 μ thick layer on glass plates and allowed to dry for at least two hours. Sephadex*** thin-layer chromatograms are prepared by the gradual addition of aliquots of water to G-50 Sephadex (6 grams). The mixture is constantly stirred until a total of approximately 50 ml of water has been added. The slurry is then spread on the plates as above and is allowed to air dry at least three hours.

Solvent systems

Separation of acid polysaccharides (APS) on cellulose powder plates is achieved with a freshly prepared mixture of *n*-propanol–iso-propanol–0.037 *M* citric acid (v/v). The citric acid is previously buffered to a pH of 3.1 with sodium hydroxide. The solvent system is equilibrated in a glass chamber at 6° for approximately three hours. The plate is then inserted into the solvent.

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** MN-Cellulose Powder 300, Brinkmann Instruments, Inc., 115 Cutter Mill Road, Great Neck, N.Y.

*** Pharmacia Fine Chemicals, Inc., Box 1010, Rochester, Minn.

Separation of APS on Sephadex plates is achieved with *n*-propanol–isopropanol–absolute ethanol–0.037 *M* citrate buffer, pH 3.1 (1:4:2:13, v/v). Sephadex plates are inserted into the solvent after the chamber has been equilibrating for three hours at room temperature.

Developing time is approximately 18–36 h for Sephadex and 13 h for cellulose powder plates. After development in either system, plates are allowed to air dry in a vertical position.

Spraying solution

Sulfated acid polysaccharides are detected by their metachromatic (red) color reaction to toluidine blue. The spraying solution is prepared by dissolving 200 mg of toluidine blue O* in a mixture of 400 ml acetone, 1800 ml methanol and 50 ml of 2 % acetic acid[2]. The pH is approximately 4.5. This stain will detect sulfated APS down to a level of 0.1 μg after resolution of the spots on cellulose powder plates. Non-sulfated hyaluronic acid does not stain.

Standards

All of the acid polysaccharides (APS) used were prepared from animal tissues and purified in this laboratory[3] as the sodium salt. The exceptions were chondroitin sulfate C and hyaluronic acid (obtained through the kindness of Dr. KARL MEYER), and hyaluronic acid sulfate (obtained through the kindness of G.D. Searle and Co.). Reference standards of other polysaccharides (including chondroitin sulfates A and B, heparitin sulfate, keratosulfate, and hyaluronic acid) were also made available to us through the kindness of DR. KARL MEYER. Chondroitin sulfate B and heparitin sulfate reference standards were also obtained through the kindness of DR. ALBERT DORFMAN.

Degradation of APS by hyaluronidase

In a typical experiment, 1 mg of acid polysaccharide is added to 600 units of hyaluronidase** in 1.5 ml of 0.1 *M* acetate buffer containing 0.15 *M* NaCl at pH 6.0. After incubation for six hours at 37°, the mixture is immediately dialyzed for 24–48 h at 4° with constant stirring[4,5]. Thirty microliters of the dialyzed solution are spotted on cellulose powder plates and chromatographed. Control solutions are treated in the same manner, except that hyaluronidase is not included in the mixture. The two solutions are placed on adjacent spots for comparison.

The "Clorox–benzidine" stain for APS

This technique[6] has been modified for use on cellulose powder TLC. The plate is air dried. The "Clorox" reagent is prepared immediately before use by adding 5 ml Clorox brand bleach*** to 50 ml distilled deionized water. Five milliliters of glacial acetic acid are then added. This reagent is sprayed on a plate placed on a horizontal surface in a ventilated hood. The plate is allowed to dry thoroughly in the air. It is then resprayed with benzidine reagent as described[6].

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* National Aniline, 40 Rector Street, New York 6, N.Y. (73 % Dye Content).
** Alidase, G.D. Searle and Co.
*** The Clorox Company, 850, 42nd Avenue, Oakland 1, Calif.

RESULTS

Cellulose powder TLC

The cellulose powder chromatograms are divided into three visible zones by secondary and tertiary solvent fronts (Fig. 1). The mean R_F of the secondary solvent front is 0.77 (range = 0.73–0.80) and that of the tertiary solvent front is 0.54 (range = 0.50–0.57).

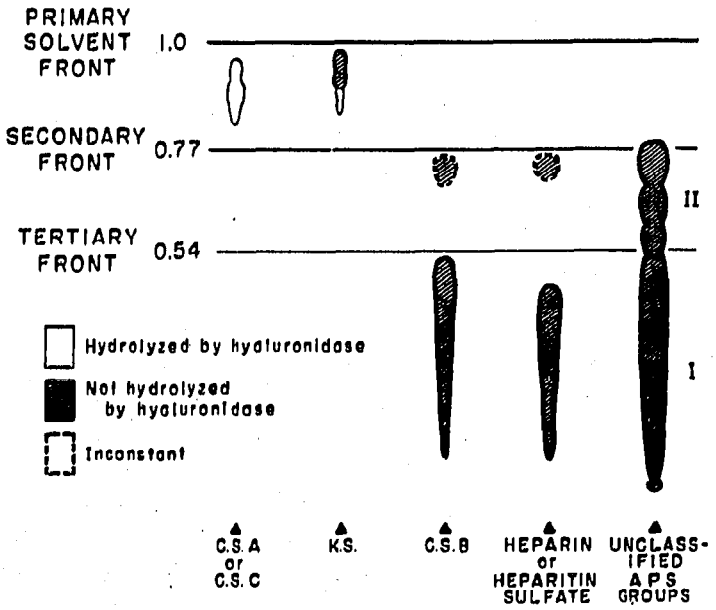

Fig. 1. Schematic representation of the migration of metachromatic acid polysaccharides on cellulose powder TLC. Abbreviations: C.S. A, B and C = chondroitin sulfates A, B and C; K.S. = keratosulfate. Note the position of the chondroitin sulfate B spot here in contrast with its position in Fig. 2.

Chondroitin sulfates A and C migrate in the zone between the primary and the secondary solvent front. They are both hydrolyzed by hyaluronidase. Some keratosulfate preparations may include two components which also migrate in this same zone. In these instances, the top component is located at, or close to, the primary solvent front, and the second is found immediately below this more rapidly moving fraction. Only this second spot is hydrolyzed by hyaluronidase. (Other lines of evidence suggest that the second moiety is either chondroitin sulfate A, or C, which happened to be included in the keratosulfate preparation).

Chondroitin sulfate B, heparitin sulfate, heparin, and hyaluronic acid sulfate (a synthetic APS) migrate as polydisperse molecules. They all extend from just above the application point to the middle half of the plate. These spots end approximately at the level of the tertiary solvent front. These APS are not hydrolyzed by hyaluronidase. Hyaluronic acid also migrates in this area, but it is not metachromatic under these conditions. However, hyaluronic acid can be revealed with the "Clorox–benzidine" method.

The same sulfated APS prepared in different laboratories showed no appreciable difference in the R_F values of their major component[7]. Calcium salts of chondroitin sulfate B and of heparitin sulfate had essentially the same R_F values as did

their sodium salts. R_F values were not altered when individual acid polysaccharides were spotted in mixtures.

It should be noted that the present method reveals a group of sulfated APS which have heretofore not been categorized (Fig. 1). For example, distinct subfractions are found not only in APS fractions purified in our own laboratory from various tissues, but also in each of the several APS reference standards obtained from the other investigators. Different isolation techniques have been used to obtain these standards. These "unclassified" APS moieties may be divided provisionally into groups I and II for present purposes of discussion. For example, the migration of the group I moieties is similar to that of heparin or heparitin sulfate. However, they tend to stain more of a blue-purple metachromatic color rather than an intense red. The majority of the APS in group I are hydrolyzed by hyaluronidase. Included in the group II category are the metachromatic spots which migrate between the secondary and tertiary solvent fronts. None of these spots has been hydrolyzed by hyaluronidase.

Sephadex TLC

The only major difference from the above observations on cellulose powder is that chondroitin sulfate B migrates near the primary solvent front together with chondroitin sulfates A and C (Fig. 2).

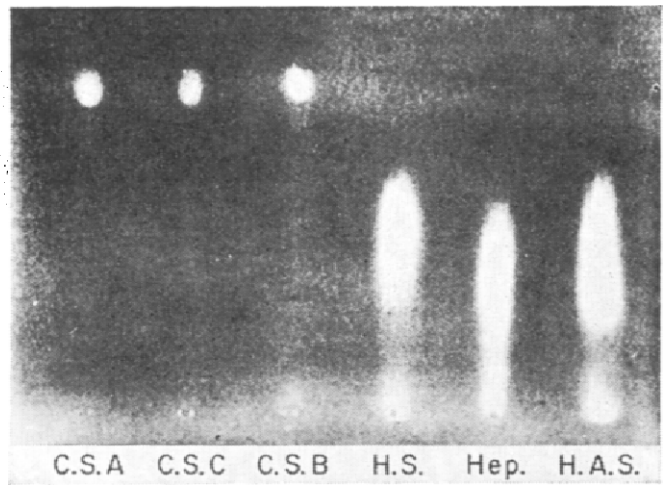

Fig. 2. Toluidine blue stain of a Sephadex G-50 thin layer chromatogram. Left to right: chondroitin sulfates A, C, B, heparitin sulfate, heparin and hyaluronic acid sulfate. (Photographed with Wratten F dark red filter to show the metachromatic spots as light against a contrasting dark background.)

Staining of APS with "Clorox–benzidine"

On cellulose powder TLC, heparitin sulfate and heparin stain an intense dark blue, whereas hyaluronic acid stains a light blue. Chondroitin sulfate B, as well as sulfated hyaluronic acid, stain an even lighter blue. Chondroitin sulfate A and keratosulfate do not stain on TLC plates after they have been separated chromatographically.

There is a non-metachromatic keratosulfate contaminant which stains lightly on TLC cellulose powder plates (R_F 0.50). There is also a non-metachromatic contaminant in chondroitin sulfate A preparations which again stains lightly (R_F 0.23).

The "Clorox–benzidine" staining method is not applicable for TLC on Sephadex because the chromatographed spots are poorly resolved.

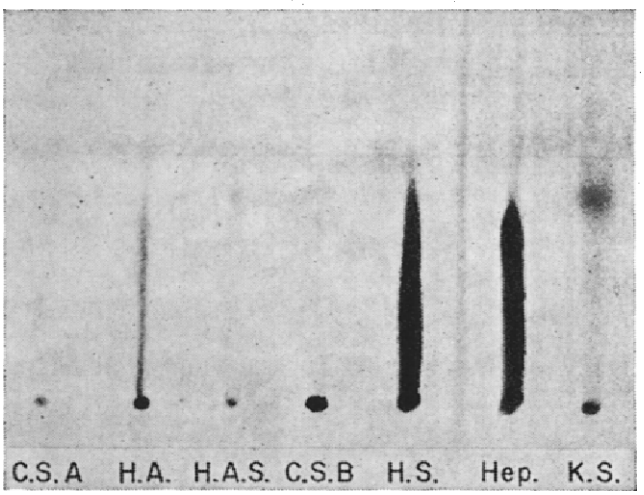

Fig. 3. Clorox–benzidine stain of acid polysaccharides on a cellulose powder thin-layer chromatogram. (Compare with Fig. 1.) Left to right: chondroitin sulfate A, hyaluronic acid, hyaluronic acid sulfate, chondroitin sulfate B, heparitin sulfate, heparin and keratosulfate. Note the intense staining of heparitin sulfate and heparin. Note also that the high R_F spots of C.S. A and K.S. (which would be characteristically metachromatic with toluidine blue), are not revealed with "Clorox–benzidine". The minor spots which are visible in the C.S.A and K.S. preparations represent moieties which are non-metachromatic in parallel studies. One hundred μg of material were spotted at each application point.

DISCUSSION

If a mixture of sulfated APS does *not* contain chondroitin sulfates A or C, then chondroitin sulfate B may be distinguished from the other sulfated APS by comparing its low R_F spot on cellulose with its high R_F spot on Sephadex. If chondroitin sulfates A or C *are* present in the mixture they both can be eliminated with hyaluronidase. Thus, chondroitin sulfate B will remain as a single spot near the solvent front of the Sephadex plate. Other APS including heparitin sulfate, heparin, and hyaluronic acid sulfate are not hydrolyzed under the conditions employed. They migrate with R_F values of less than 0.50 in both chromatographic systems.

Existing methods do not separate the isomeric chondroitin sulfates A and C as intact molecules, but require acid hydrolysis and separation of the products[8]. Keratosulfate migrates at the primary solvent front. Of its two metachromatic components, only the more slowly migrating fraction is hydrolyzed by hyaluronidase. This hydrolysable fraction has been eliminated in the course of recent extraction procedures for keratosulfate in our laboratory. Therefore it may represent a contamination of keratosulfate preparations by chondroitin sulfate A or C, or by other unclassified sulfated APS which also migrate in this region on TLC[3].

The substrate specificities of testicular hyaluronidase preparations are not sharply defined at present[5]. Therefore, it would not be possible to reach precise conclusions about chemical structure from information based solely on hyaluronidase degradation per se. Chondroitinase activity has been shown to parallel hyaluronidase activity in one preparation[9]. It has been noted that hyaluronidase may be inhibited in the presence of substantial quantities of sulfated APS[5]. However, in the present

test system, there is a relatively large amount of enzyme per unit quantity of APS. This excess of enzyme suffices to overcome an inhibitory effect.

The present methods have been successfully used to separate the purified APS fractions which were obtained from a variety of human, bovine, and rat tissues (both normal and abnormal)[10]. In the course of these studies, the present methods have revealed some sulfated APS which, for the most part, are not found in the reference standard APS preparations available to us. To judge from the literature, such moieties have yet to be satisfactorily defined chemically. Hyaluronidase digested the majority of the APS in group I from a normal human kidney. These moieties had R_F values less than 0.50. Also included within most APS extracts from human brain, kidney and urine were some unidentified polysaccharides belonging to group II[3]. These polysaccharides are present as one or more spots at intermediate R_F values between the tertiary and secondary solvent fronts. The finding of these moieties emphasizes how much more basic information is required about the chemistry of acid polysaccharides before APS identifications can be precise.

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SUMMARY

Thin-layer chromatographic procedures are described for the separation and identification of a number of acid polysaccharides. With the present methods, chondroitin sulfates A and/or C, chondroitin sulfate B, keratosulfate, and heparitin sulfate and/or heparin may be distinguished among the normally occurring known sulfated APS. A number of polysaccharides, as yet unclassified, are also revealed by these procedures.

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