PAPER CHROMATOGRAPHY OF HEPARIN AND RELATED SULFATED MUCOPOLYSACCHARIDES*

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SUMMARY

A paper chromatographic system was developed for the resolution of mixtures of sulfated mucopolysaccharides. The effect of varying the ammonium formate buffer/isopropanol ratio in the developing solution on the R_F values of heparin and chondroitin sulfate is presented.

A complete separation of heparin from chondroitin sulfate was obtained with three solvent mixtures containing different proportions of buffer and isopropanol.

One of these solvent mixtures, ammonium formate buffer–isopropanol (65:35) v/v, resolved commercial beef heparin into two principal components, on "fresh" paper. This system was used to compare beef heparin with mouse mast cell tumor heparin, rat heparin, sheep lung heparin, chondroitin sulfate, and β-heparin. A common metachromatic component with an R_F of approximately 0.5 was found in all of the heparin preparations chromatographed with this solvent mixture.

"Aging" of the filter paper resulted in the loss of the ability of the buffer–isopropanol mixture (65:35) v/v to resolve the beef heparin. Increase of the buffer–isopropanol ratio to (70:30) v/v restored the resolution of the beef heparin on "aged" paper. Heparitin sulfate and α-heparin monosulfate were compared to beef heparin using the latter system.

INTRODUCTION

A procedure for the extraction of heparin from tissues was reported recently from this laboratory[1]. For a study of heparin biosynthesis in a mouse mast cell tumor[2–4], it was necessary further to purify the heparin preparation obtained by this technique. Filter paper chromatography appeared to be the most desirable method for separating the heparin from other sulfated mucopolysaccharides, in particular, chondroitin sulfate.

A search of the literature revealed only two reports[5,6] in which a separation of heparin from chondroitin sulfate was accomplished by paper chromatography. Two solvent systems were used to effect this separation. When the mixture ethanol–phosphate buffer was used, heparin remained essentially on the origin, while chondroitin sulfate moved close to the solvent front. With the other solvent system

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described, *n*-propanol–phosphate buffer, heparin was separated from chondroitin sulfate, but both migrated close to the solvent front, and close to one another. It appears that in order to establish chondroitin sulfate contamination of a heparin preparation, both of these solvent systems must be used. In addition, the patterns obtained, while adequate for a characterization of the chondroitin sulfate, were not considered satisfactory for a characterization of the heparin. Other published reports[7,8] of the filter paper chromatography of heparin do not adequately describe the conditions used, nor, as mentioned above, is the behavior of chondroitin sulfate in these systems indicated.

An attempt was made, therefore, to develop a relatively simple and more convenient paper chromatographic technique for the separation of heparin from closely related compounds. Using a solvent system consisting of buffer at a lower pH than previously employed, and isopropanol, the effects of varying the buffer/alcohol ratio of the developing solution on the chromatographic behavior of heparin and chondroitin sulfate were investigated. The results of these experiments, and a paper chromatographic technique for the separation of heparin from related compounds developed in the course of this study, are presented below.

Procedure

Using an aqueous solution containing 6 mg of a single mucopolysaccharide/ml, a quantity of 30 μg of material* was applied (spot diameter, 7–10 mm) to a strip of Whatman No. 1 filter paper, 4¼ in. × 16 in., at a distance of 3 in. from the end of the strip. Spots containing a mixture of mucopolysaccharides contained 30 μg of each substance.

In general, apparatus standard for descending filter paper chromatography was employed. The atmosphere of a chromatographic jar (6 in. × 18 in.) was equilibrated overnight with the solvent to be used. The spotted paper was then placed in the jar. An anti-siphon rod was in position 1.5 in. from the spots, and 1.5 in. from the end of the paper, which was held in a glass trough. After equilibration of the paper with the atmosphere of the jar for 3–4 h, the solvent was introduced into the trough. The chromatogram was developed for about 12 h at room temperature (26 ± 2°). The paper was then removed from the jar, the solvent front marked with a pencil, and the paper dried at room temperature. The results shown in Fig. 1 were obtained in this manner.

In order to obtain a longer distance from origin to solvent front, a chromatocab (16.5 in. wide, 27 in. long, 25.5 in. deep) was used in some instances. Whatman No. 1 filter paper strips, 22 in. long, were used. The width of these strips depended on the number of individual samples to be chromatographed simultaneously. Materials were applied 4 in. from one end of the paper. An anti-siphon rod was in position 1.5 in. from the applied spots, and 2.5 in. from the end of the paper which was held in the glass trough. The time of development was approx. 16 h. Other details of technique were described above for the jar system. The results represented in Figs. 2–4 were obtained in the chromatocab.

The solvents used to develop the chromatograms consisted of a mixture of 0.04 *M*

* In the case of the mouse mast cell tumor heparin, three units were applied (assay according to U.S.P. XIV).

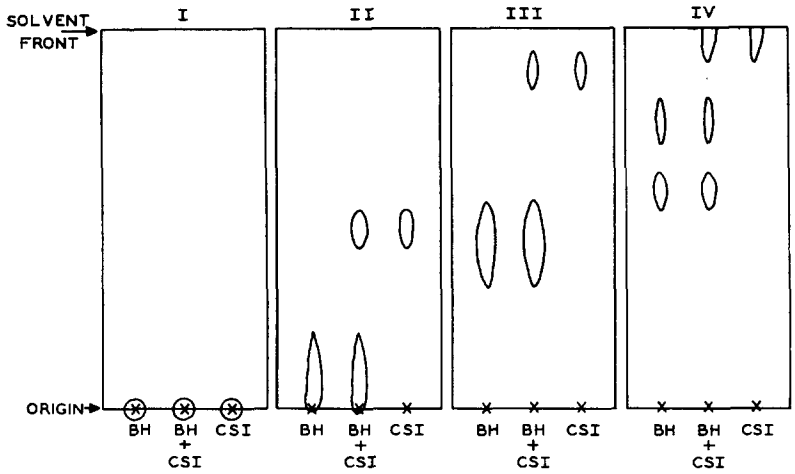

Fig. 1. Paper chromatography of commercial beef heparin (BH) and chondroitin sulfate CSI. Solvent systems consist of 0.04 *M* ammonium formate buffer, pH 4.3, and isopropanol in the following proportions; I: 50:50 (v/v); II: 55:45 (v/v); III: 60:40 (v/v); IV: 65:35 (v/v).

TABLE I

COMPOSITION OF SOLVENT SYSTEMS USED

Buffer: 0.04 *M* ammonium formate (General Chemical Division, Allied Chemical and Dye Corp., Reagent) adjusted to pH 4.3 with formic acid (Mallinckrodt Chemical Works, A. R., 88%). Isopropanol (General Chemical Division, Allied Chemical and Dye Corp., Reagent, A.C.S.).

Solvent system	*Parts buffer (v)*	*Parts isopropanol (v)*
I	50	50
II	55	45
III	60	40
IV	65	35
V	70	30

ammonium formate buffer, pH 4.3, and isopropanol, in varying proportions, as shown in Table I.

Dried chromatograms were stained by immersion in an Azure A solution for a period of 4 to 5 min. This solution was prepared by dissolving 200 mg of Azure A (National Aniline Division, Allied Chemical and Dye Corp., certified, 93% dye) in 50 ml distilled water, and then adding 400 ml acetone (General Chemical Division, Allied Chemical and Dye Corp., Reagent, A.C.S.), and 1800 ml methanol (General Chemical Division, Allied Chemical and Dye Corp., Reagent, A.C.S.) to this solution. Upon removal from the staining solution, the paper was dried in air at room temperature.

Some of the Whatman No. 1 filter paper strips were used relatively soon after having been purchased; this paper was designated "fresh". In other instances, the paper strips were stored in a closed, but not airtight, container for approximately one year; these strips were designated "aged".

The mouse mast cell tumor heparin was prepared from Dunn-Potter tumor[9]

tissue grown and transplanted in Dba/2 mice. The material was extracted and purified essentially according to a procedure reported recently[1]. The other mucopolysaccharides were obtained from the sources indicated; they were chromatographed without further treatment.

RESULTS

The effects of changing the ratio of ammonium formate buffer to isopropanol in the developing solvent on the relative migration of heparin and chondroitin sulfate are presented in Fig. 1. Heparin spots were bright pink, chondroitin sulfate spots purple, all against a light blue background. With a solvent consisting of equal parts of buffer and isopropanol (system I), neither heparin nor chondroitin sulfate was moved from the point of application. Solvent system II (ratio buffer to isopropanol, 55:45) streaked the heparin without moving it from the origin. The chondroitin sulfate was shifted, however. When the ratio buffer/isopropanol was increased further to 60:40

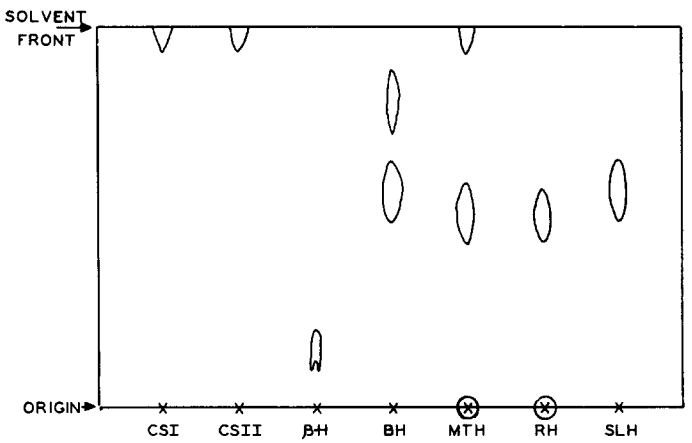

Fig. 2. Paper chromatography of chondroitin sulfate of different source (CS I, CS II), of β-heparin (β-H), and of heparin of different origin (beef, BH; mouse tumor, MTH; rat, RH; sheep lung, SLH). Solvent system IV.

(system III), both heparin and chondroitin sulfate migrated from the origin, and a complete separation of these compounds was accomplished. System IV, with a ratio of 65:35, resolved heparin into two metachromatic spots, a slower moving major component, and a faster moving minor component. Chondroitin sulfate moved with the solvent front in this system.

Solvent system IV was used to compare commercial beef heparin with mouse mast cell tumor heparin, rat heparin, sheep lung heparin, β-heparin*, and chondroitin sulfate** (Fig. 2). The spots represent pink or purple metachromasia with one exception: the point of application of the mouse mast cell tumor heparin stained a deep blue. The major metachromatic component of the mouse mast cell tumor heparin

* The term β-heparin was used, since this preparation was so designated by Dr. WINTERSTEIN, who furnished it. This compound is identical with chondroitin sulfate B [10].

** Two preparations of chondroitin sulfate (CS) were chromatographed: CS I purchased from Aldrich Chemical Co.; CS II supplied by Dr. WINTERSTEIN.

migrated in this system with an R_F slightly less than that of the major component of the commercial beef heparin; a smaller amount of metachromatic material moved with the solvent front. The rat heparin traveled with an R_F essentially equal to that of the major metachromatic component of the mouse mast cell tumor heparin. Some metachromatic material in the rat heparin did not leave the origin. Sheep lung heparin migrated with essentially the same R_F as the major component of commercial beef heparin. β-heparin traveled with a smaller R_F than any of the heparin preparations which were run in this system. Chondroitin sulfate moved with the solvent front. The components of the mouse mast cell tumor heparin and rat heparin which remained at the origin may have been responsible for a slight reduction of the R_F of the major metachromatic components of these preparations.

The prolonged storage of Whatman No. 1 filter paper strips, "aging", resulted in a change in the pattern obtained when beef heparin was chromatographed with solvent system IV: only a single spot with an R_F of about 0.4 was observed. However, when the ratio of buffer to isopropanol was increased to 70:30 (system V), the "aged" filter paper yielded essentially the same pattern for beef heparin as did "fresh" paper with solvent system IV (Fig. 3). "Aging" of the paper did not affect the R_F of chondroitin sulfate or of β-heparin when solvent system IV was used.

Using solvent system V, and "aged" paper, beef heparin was compared with α-heparin monosulfate and with heparitin sulfate (Fig. 4). The latter two substances stained red-violet. The spots were, however, much less intense than those which were obtained with an equivalent amount of beef heparin. α-heparin monosulfate migrated with an R_F essentially equal to that of the faster moving component of beef heparin. Heparitin sulfate yielded two spots corresponding to those of the commercial beef heparin used.

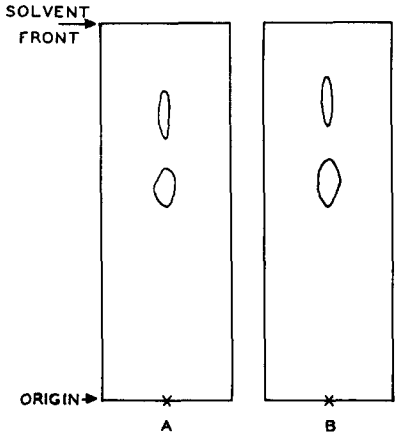

Fig. 3. Chromatography of commercial beef heparin. Comparison of "fresh" and "aged" paper. A: "Fresh" paper, solvent system IV; B: "Aged" paper, solvent system V.

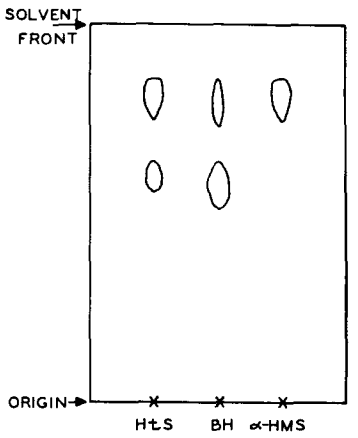

Fig. 4. Paper chromatography of heparitin sulfate (HtS), commercial beef heparin (BH), and α-heparin monosulfate (α-HMS); "aged" paper, solvent system V.

DISCUSSION

The purpose of this study was to develop a convenient chromatographic procedure which would permit the separation of heparin from chondroitin sulfate, and a

characterization of the mouse mast cell tumor heparin. Solvent system IV, with "fresh" paper, appears to satisfy these requirements.

A complete separation of heparin from chondroitin sulfate was obtained with this system, with a greater difference in R_F values than was reported prior to this study. This large difference makes it much easier to evaluate the relative quantities of heparin and chondroitin sulfate in a mixture.

Furthermore, a much more satisfactory characterization of a heparin preparation is possible with this system, since the major metachromatic component of commercial beef heparin migrates with an R_F of 0.57. Sulfated mucopolysaccharides which conceivably could contaminate heparin preparations, such as chondroitin sulfate and β-heparin, migrate with R_F values markedly different from that of heparin (Fig. 2). The characterization of mouse mast cell tumor heparin was based, in part, on its chromatographic behavior with this solvent system[2-4].

The effects of the isopropanol concentration of the solvent system on the R_F values of heparin and chondroitin sulfate appear to be related to the relative non-polarity of the isopropanol. Both heparin and chondroitin sulfate, being highly polar molecules, remained at the origin when the isopropanol content of the developing solvent was too high (system I, Fig. 1). Heparin is more polar than chondroitin sulfate and was not moved from the origin by a solvent mixture (containing less isopropanol) which did move chondroitin sulfate (system II, Fig. 1)*. The polarity difference between heparin and chondroitin sulfate is again apparent from the differences in patterns obtained with solvent systems III and IV (Fig. 1).

The relatively low pH of the buffer reduces the ionization of the carboxyl groups of both the heparin and the chondroitin sulfate. Though this decreases the net negative charge on these molecules, it increases the relative contribution of the sulfate to this charge, and emphasizes the difference in sulfate content between the heparin and chondroitin sulfate.

The patterns obtained upon chromatography of α-heparin monosulfate and heparitin sulfate (Fig. 4) are for the most part consistent with the above-discussed relationship between sulfate content and R_F. The R_F obtained for β-heparin is apparently not related primarily to the sulfate content (Fig. 2). On the basis of sulfate content, one would expect this compound to migrate faster than heparin. The observed smaller R_F may perhaps be related to the reported insolubility of some of the metal salts of β-heparin in dilute alcohol[13-15].

The effect of "aging" of the filter paper on the observed R_F of heparin, which was described above, has not been explained. An increase of the buffer/alcohol ratio, *e.g.* system V, essentially restored to "aged" paper the R_F picture seen with "fresh" paper using solvent system IV. Attempts to circumvent this difficulty, through the use of acid-washed paper, resulted in binding of the mucopolysaccharides on the paper, giving a chromatogram with an R_F of essentially 0.

When this study was initiated, we were not aware of a report by AWE AND STÜDEMANN[16] which presented three solvent systems for the paper chromatography of heparin, heparinoids, and "heparin-like" substances. However, separation of heparin and chondroitin sulfate was not obtained with any of the three solvent systems described by these authors.

* Heparin has either 2.5 or 3 sulfate groups per disaccharide unit[11,12], as compared to only one [13] in the case of chondroitin sulfate.

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