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GEL CHROMATOGRAPHY OF HEPARIN

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SUMMARY

It is assumed that heparin is a heterogeneous substance. In order to further investigate the purification of heparin, a column chromatographic technique for the fractionation of heparin is described using various diameters of bead form cross-linked dextran gels and an automated apparatus. It was observed that Sephadex G-50 resulted in the separation of three well formed peaks and provided superior resolution compared to all other gels. One of the peaks, representing 51% of the original material, possessed strong anticoagulant activity as measured by the recalcification time, partial thromboplastin time, thrombin time and the anti-X_a test. This peak also possessed strong metachromasia after electrophoresis as well as having a very potent anticoagulant effect in vivo. This technique may have a significant role in the purification of this agent from tissue sources.

INTRODUCTION

Heparin, whose main use as a drug is for the prevention and control of thromboembolism, is a poly-dispersed mixture of highly electronegative chains with molecular weights ranging from 3,000 to 40,000 daltons; the isolation of the component that possesses the anticoagulant activity has been sought after for many years.

A recent study has shown that thirteen fractions could be obtained by partition fractionation and may provide a tool for isolating and characterizing heparins [1]. Yet, the use of electrofocusing [2] has fractionated heparin into 21 components of different molecular weights; however, this has been disputed [3, 4]. Gel chromatography was and still is being used in attempts to purify this agent [5—19]. In general, these investigations using the Sephadex type gels have shown that heparin gave rise to one peak or to a broad peak that had multiple shoulders.

The main purpose of this study was to systematically examine and compare the chromatography of heparin by employing gel chromatography using various diameters of bead form cross-linked dextran gels. Coagulation tests

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and electrophoresis on agarose gel were used to analyze the isolated fractions. It was shown that column chromatography, employing Sephadex G-50, resulted in the resolution of several well formed peaks; one of the peaks possessed strong anticoagulant activity.

EXPERIMENTAL

Materials

Sodium heparin (Abbott, North Chicago, IL, U.S.A.), was used in these experiments. It was a white amorphous powder with a nitrogen content of 2.1%. The potency was measured at 169 units per mg. Reagents for the various clotting tests were: human thrombin (Ortho Diagnostics, Raritan, NJ. U.S.A.); 0.02 *M* calcium chloride (Difco Labs., Detroit, MI, U.S.A.); activated cephaloplastin reagent (Actin™, Dade, Miami, FL, U.S.A.); factor X_a (Sigma, St. Louis, MO, U.S.A.); agarose (Indubiose A-45, L'Industrie Biologique Française, Gennevilliers, France); toluidine blue (Fisher Scientific, Pittsburgh, PA, U.S.A.); barbital buffer, pH 8.6; Tris · HCl buffer, pH 8.0. The plasma for the in vitro clotting tests was obtained from rats; blood was collected into 1/10 volume of 0.1 *M* sodium citrate and centrifuged immediately to obtain the plasma.

Gel types

Sephadex (Pharmacia, Uppsala, Sweden) G-type for gel chromatography was employed throughout the experiments. These were G-200, G-150, G-100, G-75, G-50 and G-25. The first four types had a dry bead diameter of 40—120 μm; the last two had a dry bead diameter of 50—150 μm. The dry gel was always mixed with 200 ml of 0.086 *M* Tris·HCl buffer, pH 8.0. The amounts of dry gel employed were: G-200 and 150, 2.5 g; G-100, 3.5 g; G-75, 4.5 g; G-50, 6.5 g and G-25, 11 g. After swelling and degassing, the wet gels were then used for the preparation of the columns. The size of the columns was 1 × 60 cm.

Column development

Spectrum analysis was done in a Unicam SP 800B spectrophotometer in order to determine the optimum wavelength for following the elution of peaks from the columns; maxima were found at 218, 255 and 275 nm. A Spectrochrom 130 (Beckman) analyzer was used for almost all of the chromatographic runs [20, 21]; the effluents from the columns were alternatively measured at the 218, 255 and 275 nm wavelengths employing both 1 and 0.25 cm pathlengths and recorded on a logarithmic scale. Heparin (200 mg) was dissolved in distilled water and charged to the column. The washing solution for the columns was 0.086 *M* Tris·HCl, pH 8.0 containing 1 *M* sodium chloride; the columns were jacketed to maintain the temperature at 25°C. The flow-rate of the washing solution was approximately 7.5 ml/h and 2.5-ml fractions were collected. It was not possible to develop the G-200 columns in the Spectrochrom 130. Instead, the fractions from these gel columns were obtained using a Buchler fraction collector; absorption of each fraction was then determined in the Unicam SP 800B spectrophotometer. The quantita-

tion of the peaks was carried out by two methods, the first by determining the area under the curve and the second by cutting out the individual peaks from the chromatogram and then weighing them; the total was then equal to 100% and each peak was a percentage of this. There was very little difference between the two methods and the averages of both methods were used to quantitate the peaks of the chromatograms.

Coagulation assay of heparin

The anticoagulant activity of each fraction was measured by several techniques. Because of the large amounts of heparin used for chromatography, the eluted fractions had to be diluted before the clotting tests were performed. The diluted samples were then mixed with 0.45 ml of rat plasma and the anticoagulant potential determined using the activated partial thromboplastin time, recalcification time, thrombin time [22] and factor X_a inhibition assay [23].

Electrophoresis

This technique consisted of coating microscopic slides (7.5 × 2.5 cm) with 0.9% agarose. Two slits were made at 2 cm from the cathode end of the slide. A 3-μl aliquot of each fraction obtained from the column was then applied to the slits and electrophoresis started immediately using barbital buffer, pH 8.6; the electrophoresis time was 25 min at 130-V using a Gelman apparatus. After electrophoresis, the slides were fixed, stained with toluidine blue and the spots measured [10].

In vivo activity of isolated peaks

After in vitro testing of the fractions of each peak from G-50 columns, they were combined and peaks I, II and III were concentrated in vacuo from the frozen state and tested in triplicate for anticoagulant activity in vivo. A plastic cannula was inserted into the femoral artery of rats; the blood in the cannula was kept from clotting by the use of citrate. Approximately 0.25 mg of each peak was dissolved in 1 ml of saline and administered by tail vein; other rats given similar amounts of non-chromatographic heparin served as controls. Samples of blood were collected over a 5-h period and tested for anticoagulant activity using the thrombin time. Before each blood sample was tested, sufficient amount of blood was discarded from the cannula to eliminate the citrate effect; more blood was taken and mixed with 0.1 volume of 0.1 *M* citrate and the thrombin time determined.

RESULTS AND DISCUSSION

There have been many attempts to purify heparin by gel chromatography and in only a few instances [5, 10, 13, 15, 17—19] have chromatograms from the chromatography on non-fractionated commercial heparin been shown. The present investigation gave us an opportunity to compare the chromatography of commercial heparin by a systematic approach using various types of cross-linked dextran gels.

Only one peak was found when G-200 was used in the column; all the

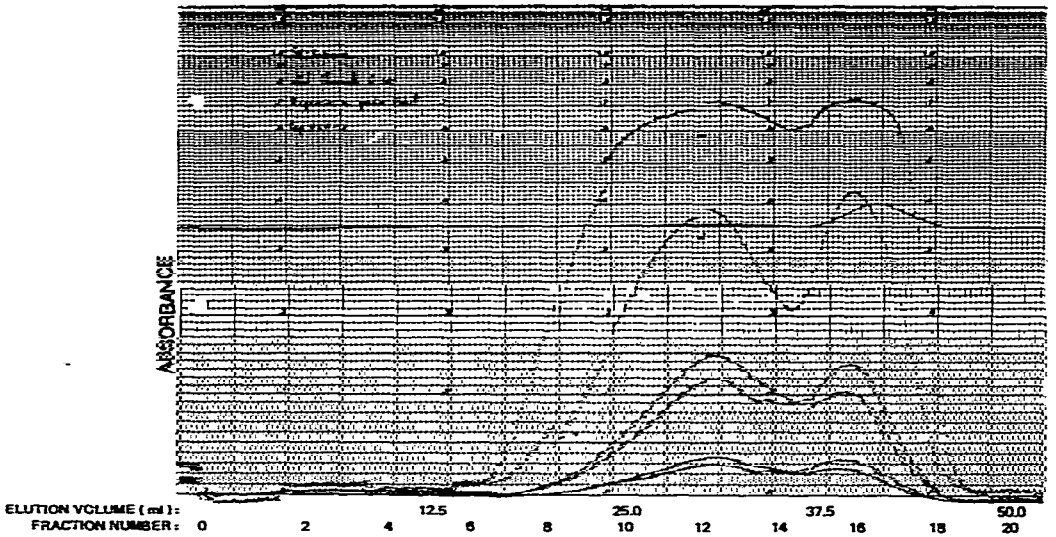

Fig. 1. Chromatogram from a gel G-100 column; 200 mg of heparin were applied to 3.5 g of gel. Column size was 1 x 60 cm. The different tracings on the recording paper represent absorption readings on a logarithmic scale at 218, 255 and 275 nm wavelengths for both the 1.0 and 0.25 cm pathlengths.

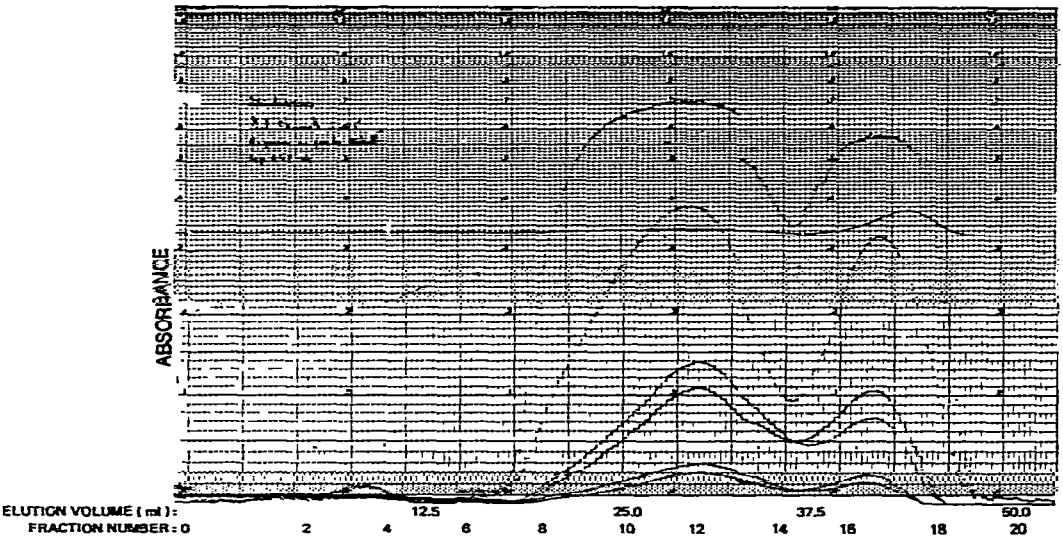

Fig. 2. Chromatogram from a gel G-75 column; 200 mg of heparin were applied to 4.5 g of gel. Column size was 1 x 60 cm. The different tracings on the recording paper represent absorption readings on a logarithmic scale at 218, 255 and 275 nm wavelengths for both the 1.0 and 0.25 cm pathlengths

fractions from this peak possessed anticoagulant and metachromatic activity and did not show any difference to the original material. When G-150 was substituted for G-200 in the columns, one peak was again obtained and gave similar results in clotting and electrophoretic analysis as to those found with G-200. However, as can be seen in Fig. 1, chromatography employing G-100

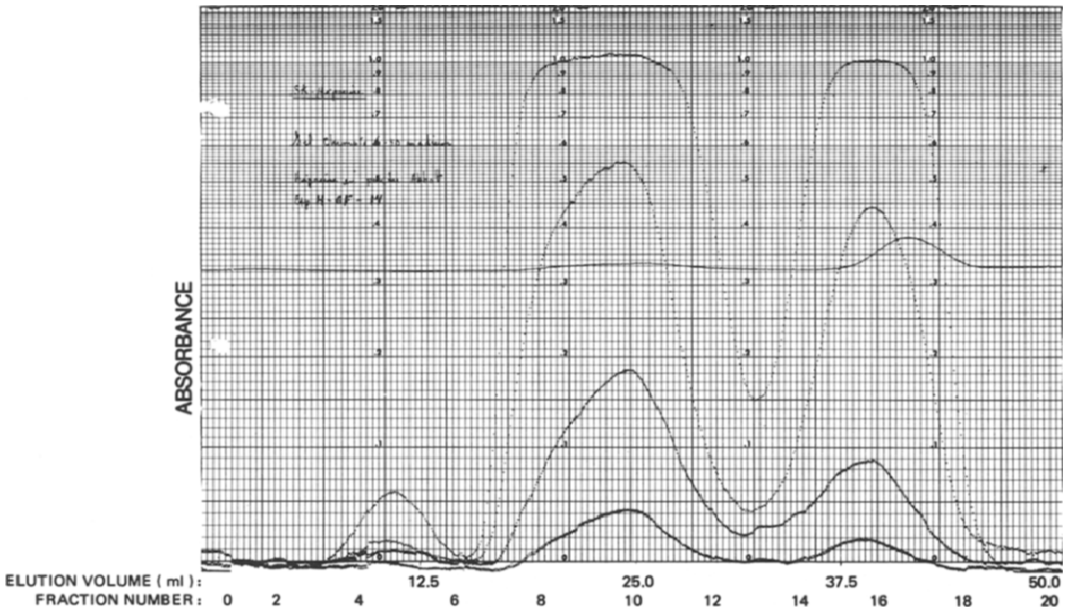

Fig. 3. Chromatogram from a gel G-50 column; 200 mg of heparin were applied to 6.5 g of gel. Column size was 1 × 60 cm. The different tracings on the recording paper represent absorption readings on a logarithmic scale at 218, 255 and 275 nm wavelengths for both the 1.0 and 0.25 cm pathlengths.

began to resolve the heparin material; two peaks were found which is similar to the observations of Walton et al. [5] and Laurent et al. [13]. The fractions from both these peaks possessed anticoagulant and metachromatic activity. However, the second peak was much less active in both of these parameters.

Figs. 2 and 3 show the results of G-75 and G-50 respectively. It can be seen that resolution could still be improved; with the G-50 columns, it was possible to isolate three distinct peaks (identified as peaks I, II and III). The results of the various clotting tests and metachromatic activity of the different fractions of a G-50 column are shown in Table I. The first peak (peak I) which represented 3% of the charged material and consists of the larger molecular weight species was devoid of anticoagulant and metachromatic activity. The last peak (peak III) which consisted of 46% of the applied sample is due to the smaller molecular weight material; it has weak metachromatic and anticoagulant activities. The weak activities observed at the beginning of peak III may be due to the slight lag between the spectrophotometer readings and the collection of the samples and could be part of the second peak. It has been shown that heparin with a molecular weight of less than 6000 daltons has little effect in thrombin neutralization but has high anti-factor X_a activity; just the opposite was found when the molecular weight is greater than 25,000 daltons [15, 24, 25]. The fractions which appeared at the beginning of peak III possessed more anticoagulant potential as measured by the $\dot{X}_a^f$ assay than by the activated partial thromboplastin time.

Peak II proved to be the most interesting; this peak contained 51% of the

TABLE I

COAGULATION AND METACHROMATIC ANALYSIS OF FRACTIONS FROM A G-50 COLUMN

Peak	Fraction No.	Activated partial thromboplastin time (sec)	Recalcification time (sec)	Thrombin time (sec)	Factor X_a inhibition assay (sec)	Metachromasia*
I	1	20.1	50.4	17.3	22	—
	2	22.3	50.7	19.4	21	—
	3	21.2	49.2	18.7	23	—
	4	20.5	48.1	17.2	20.4	—
	5	22.1	50.2	16.0	20.4	—
	6	21.8	50.5	17.8	21	—
II	7	23.7	52.6	18.3	23.1	—
	8	23.7	70.3	19.5	24	+/—
	9	25.6	100.8	30.9	28.9	+
	10	100.8	250.9	55.5	200	4+
	11	72.1	200.8	45.8	200	4+
	12	31.4	140.4	40.1	200	3+
	13	28.3	150.8	42.5	200	2+
III	14	24.2	80.5	33.4	103	+
	15	23.8	60.4	28.3	48	+
	16	21.1	50.1	25.7	30	+
	17	22.1	48.3	20.6	25.5	+
	18	20.2	50.4	22.2	25.0	+
	19	21.2	52.5	19.4	25.2	+/—
	20	20.3	48.1	20	23.3	—
Control		18—21	50—57	17—19	20—23	

*Intensity of metachromasia was measured semiquantitatively after electrophoresis; — = no reaction and 4+ = a strong reaction.

applied heparin. It possessed strong anticoagulant activity as measured by the activated partial thromboplastin time, recalcification time, thrombin time and the anti-factor X_a assay. All these tests were used to measure the heparin anticoagulant activity because of the persistent controversy as to which is the best test that relates the true heparin concentration in patients who are receiving heparin therapy [26, 27]. However, it is generally accepted that heparin treatment should be monitored by clotting tests in order to prevent complications of bleeding on one hand and thrombosis on the other. What difference or advantages that the material from peak II may have in patients receiving antithrombotic therapy will require further investigations. However, preliminary studies in rats have shown that peak II does have a very potent anticoagulant effect. After intravenous injection, it was found that the thrombin time was markedly prolonged (more than 300 sec) at 2 h. This was not observed if we used similar amounts of unfractioned heparin; peaks I and III also failed to show any anticoagulant activity under the same conditions. Chromatographic runs using G-25 were inferior to that of G-50.

Our chromatography results are different from those obtained by Graham and Pomeroy [18] or Mulloy and Johnson [19] who used distilled water to develop their columns. It is the opinion of the latter investigators that fractionation on suitable gels using sodium salts at sufficient concentration gives a separation essentially on a molecular weight basis but this might not be the case if just water is used [19].

It is concluded that chromatography on Sephadex G-50 in the present system results in the optimum isolation of a heparin component that is highly active as measured by several assay methods and therefore should be of use in the preparation of this agent from tissue sources.

ACKNOWLEDGEMENT

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