HEPARIN FRACTIONATION BY ELECTROFOCUSING: PRESENCE OF 21 COMPONENTS OF DIFFERENT MOLECULAR WEIGHTS

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SUMMARY: Electrofocalization of heparin with ampholyte mixtures pH 3.0 to 5.0 has shown the presence of at least 21 components in several commercial heparin preparations. The difference between these 21 components resides exclusively in their molecular weights which range from 3 000 to 37 500.

INTRODUCTION: Several reports have shown that heparin is a polydisperse mucopolysaccharide with molecular weight ranging from 5 000 to 20 000. Fractions with different molecular weights obtained by ultracentrifugation, ion exchange chromatography and molecular sieving of commercial heparin preparations have shown differences in biological activities but no striking differences in chemical composition. For a review see (1).

Total degradation of heparin by a purified heparinase from Flavobacterium heparinum has demonstrated that heparin is composed of tri- and disulfated disaccharide repeating units in a proportion of approximately 2 to 1 (2, 3). Hence, the polydispersity of heparin could be due to the differences in the number of these disaccharide repeating units in the various molecules, and about 25 molecular species should be present in heparin preparations with molecular weights ranging from 5 000 to 20 000.

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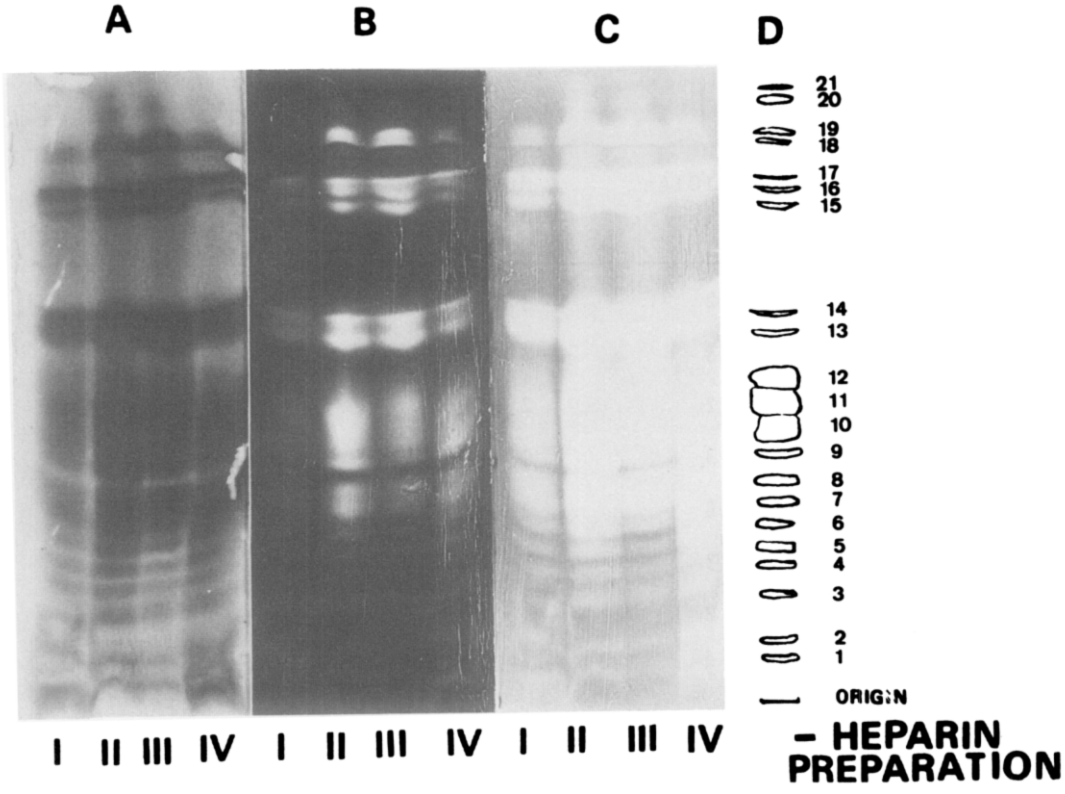

Figure 1. Heparin fractionation by electrofocusing.

I, II, III, IV - commercial heparin preparations.
A - polyacrylamide-ampholyte gel stained with toluidine blue.
B, C - contact photography of A in two different exposures.
D - line drawing composition of A, B, and C.

The present paper reports the fractionation of commercial heparin preparations into 21 components by electrofocusing.

MATERIALS AND METHODS: Commercial heparin preparations were kindly supplied by Lederle Laboratories (Pearl River, New York), UpJohn Co. (Kalamazoo, Mich.) and Roche Laboratories (São Paulo, Brazil). Heparin was also purchased from Fisher Scientific Co. (Fairlawn, New Jersey). Polyacrylamide gel slabs (5.0 x 15 cm), 0.2 cm thick containing 2% ampholyte (pH 3.0 to 5.0) from LKB (Sweden) were prepared as described by Vesterberg (4). 100 to 200 µg of heparin and other mucopolysaccharides were applied to the gel in 2 µl volume at 2 cm from the negative end and subjected to a potential of 15 V/cm for 18 hours. The gel slab was then stained with the toluidine blue reagent (5). For semi-large scale preparation up to 2 mg of heparin in 20 µl of water were applied to the gel. After electrofocalization, the gel containing the visible precipitated fractions was cut and eluted with

TABLE I

Molecular weight distribution of the heparin ampholyte fractions.

FRACTION NO.	% OF TOTAL HEPARIN	MOLECULAR WEIGHT	MW INTERVAL BETWEEN FRACTIONS
1	5.2	37,500	
			7,500
2	6.9	30,000	
			500
3	2.2	29,500	
			4,500
4	1.3	25,000	
			1,500
5	3.0	23,500	
			2,500
6	4.3	21,000	
			1,500
7	2.2	19,500	
			1,500
8	3.5	18,000	
			2,000
9	4.4	16,000	
			2,000
10	10.9	14,000	
			2,500
11	4.4	11,500	
			1,000
12	10.9	10,500	
			1,000
13	5.2	9,500	
			2,200
14	1.7	7,300	
			1,300
15	11.3	6,000	
			1,200
16	8.7	4,800	
			200
17	2.2	4,600	
			250
18	4.4	4,350	
			950
19	5.2	3,400	
			200
20	0.8	3,200	
			200
21	1.3	3,000	

0.05 M NH_4OH. Molecular weight determinations in polyacrylamide gel electrophoresis were performed as described by Hilborn and Anastassiadis (6), except that gel slabs instead of tubes were used (5). Enzymatic degradation and chemical determinations of heparin fractions were performed as previously described (3).

RESULTS AND DISCUSSION: Figure 1 shows the electrofocalization of heparin at pH 3.0 to 5.0 in polyacrylamide gel. At least 21 bands are visible after staining with toluidine blue reagent. These bands are also clearly visible as white precipitates before the staining with the reagent. The 21 bands are distributed between pH 4.2 and 3.2. Each one of the bands were prepared in semi-large scale and resubmitted to focalization. Most of the bands precipitate at their own specific pHs. Enzymatic degradation of the 21 bands with a purified heparinase from Flavobacterium heparinum failed to show any difference in degradation products. All the fractions were degraded by the enzyme and produced trisulfated disaccharide and disulfated disaccharide as the main products. This result indicates that the 21 bands are chemically identical as far as the products formed by enzymatic degradation are concerned.

Molecular weight determinations of the individual fractions are shown in Table I. There is an inverse correlation between the distance migrated and the molecular weight of each fraction. For instance, fraction 21 that precipitates near the positive pole (more acidic pH) has the smaller molecular weight and fraction 1 that precipitates near the point of application (less acidic pH) has the higher molecular weight. The relative amount of each fraction and the molecular weight intervals between the fractions are also shown in Table I.

Preliminary determinations of the anticoagulant activities of the fractions have shown that only fractions 1 to 14 have significant anticoagulant activities.

The results shown in this paper confirm the reports on the polydispersity of heparin. It is also shown that heparin is

composed of at least 21 molecular species which differ from each other only by their molecular weight and that only species with molecular weights higher than 7 000 are responsible for the anti coagulant activity of heparin.

The molecular weight intervals between most of the fractions are somewhat constant. Taking into consideration the error of the determinations, the molecular weight intervals are around 1 500 to 2 000 which is about the weight of heparin hexasaccharide (7). If these molecular species represent intermediates in a biosynthetic pathway, it could be suggested that the biosynthesis of "high molecular weight heparin" is made by addition of hexa-saccharide units, and not mono- or disaccharide units as has been proposed (8). If the different molecular species varied by disaccharide units (molecular weight about 600) the number of heparin molecular species between the higher and the lower molecular weights reported in the present paper (from 3 000 to 37 500) would be about 57. It is possible however that the present method does not separate all the heparin molecular species.

The parameters involved in this fractionation of heparin by electrofocalization are not fully understood. Heparin does not have isoelectric point at the pHs where the precipitations occur. Preliminary experiments suggest that the nature of the ampholytes together with the differences in molecular weight of the components of heparin play a strong role in the precipitation. The nature of the support does not seem to be important for the fractionation of the different components since agarose can replace the polyacrylamide in this fractionation.

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