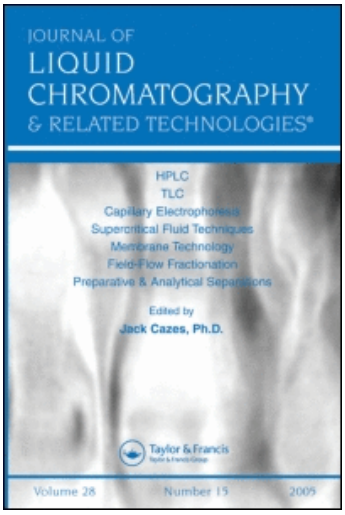

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THE MOLECULAR WEIGHT DISTRIBUTION OF HEPARIN DETERMINED WITH A HPLC-LALLS COUPLING TECHNIQUE

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ABSTRACT

Molecular weight distribution and the polydispersity of Heparins are important parameters for the characterisation and the standardisation of Heparin samples isolated from various raw materials as well as for correlations and ultimately predictions of biological activity toward thrombin. The combination of high pressure liquid chromatography (HPLC) with a low angle laser light scattering photometer has been applied to three standard heparins and a sodium and calcium salt of a particular heparin sample and is shown to be a rapid and accurate method for the determination of these parameters. The molecular weights of the standards thus determined are in good agreement with the results from classical light scattering and analytical ultracentrifuge measurements, while the data from various laboratories using conventional HPLC differ within a wide range.

INTRODUCTION

Heparin is an acidic mucopolysaccharid consisting of repeating units of hexuronic acid and glucosamin.

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The former residues consist mainly of iduronic acid with a minor content of glucuronic acid.[1] The amino groups and the hydroxyl groups are partially sulfonated. The degree of sulfonation of heparins isolated from various mammalian species varies[2]. Depending on the raw material and on the manufacturing procedure heparins differ in molecular weight distribution, in degree of dissociation and in biological activitiy.[3-5]

During the past years numerous papers have been published, dealing with the correlation of the molecular weight of heparins and anticoagulant activity[6-8]. The biological activity is correlated with the probability, to find in a heparin sample with a random distribution of molecular weight a dodecasaccharid with a sequence capable of binding to antithrombin. The probability to find such a sequence is raising with an increasing molecular weight and an increasing width of molecular weight distribution.[9]

In the past molecular weight distributions have been determined by the classical size exclusion chromatography (GPC). In the recent time high pressure liquid chromatography (HPLC) on high performance porous silica gel columns was used.[10-12] Both methods depend on calibration with standards of high monodispersity, which are not readily available. Calibration with standards having different molecular structures causes errors due to different interactions with the gel matrix. Thus a difference of approx 60 % in molecular weight resulted from chromatography using columns calibrated with dextrans and narrow molecular weight heparin fractions, respectively.[13]

As the elution volume of a GPC chromatogram is used for the evaluation, the results are influenced by changes of flow rate, pressure, temperature and by a diffusion controlled peak broadening. The latter must

be corrected for by using standards with narrow molecular weight distributions.

The combination of high performance liquid chromatography with a low angle laser light scattering photometer (LALLS) has the advandage to overcome the limitations of conventional GPC mentioned above. The LALLS photometer measures directly the molecular weight without the necessity for calibration and is independend on peak broadening and experimental flow parameters.[14)]

Herein we report on the application of this method with heparin standards and on the comparison with results from classical light scattering measurements.

METHOD

Theoretical Background

The direct determination of the molecular weight distribution with HPLC in combination with a LALLS-photometer was performed using the following equipment. The column of a high pressure liquid chromatography system was attached in series with a flow-through cell of the LALLS photometer and a differential refractometer detector (DRI) using low dead volume fittings.

The signals of the detectors were recorded with a two channel recorder and directly used for the calculation of the excess Rayleigh coefficients, the concentration of heparin and the molecular weights.

The concentration of heparin is obtained from the DRI-chromatogram using the following equation,[14)]

$$c_i = \frac{m\, S_i}{\Delta V_i \Sigma S_i}$$

where m is the mass of sample in the injected volume, S_i is the DRI recorder signal and ΔV_i the eluent volume passing through the cell during the i-th elution volume interval.

Molecular weight determination by the LALLS-method depends on the knowledge of the excess Rayleigh coefficient: $\bar{R}_{\theta i} = R_{\theta i} - R_{\theta solvent}$. $R_{\theta i}$ is obtained from the ratio of the intensity of scattered light $I_{\theta i}$ at an angle θ and the illuminating beam of irradiance I_o in the scattering volume V.

$$R_{\theta i} = \frac{I_{\theta i}}{I_o V}$$

Since the scattering intensity of the solvent is electronically compensated, the excess Rayleigh coefficient is directly obtained from the LALLS-recorder signal. The measurements were carried out at a very small scattering angle, so that the molecular weight M_i could be calculated without a Zimm-extrapolation using the relationship,[15]

$$\frac{k\, c_i}{\bar{R}_{\theta i}} = \frac{1}{M_i} + 2A_{2i}c_i$$

were k is the optical constant of the photometer, c_i the concentration, $\bar{R}_{\theta i}$ the excess Rayleigh coefficient and M_i the molecular weight of a component i in the i-th interval of the chromatogram. The optical constant k is equal to

$$k = 4{,}08 \cdot 10^{-6} n_o^2 \left(\frac{dn}{dc} \right)^2$$

were n_o is the refractive index of the solvent and (dn/dc) the refractive index increment of the solution. The second virial coefficient A_{2i} of a component i depends also on the molecular weight. For very exact results it must be calculated for all components using an iterative method calibrated with narrowly distributed standards. However, we have chosen a system of measurement near theta-conditions ($A_{2i} = 0$) and have used a second virial coefficient averaged from the data of three fractions. Because of this simplification the iteration step was unnecessary. The resulting error was about 1 %[16].

In order to obtain the percent distribution of molecular weight the concentration and the molecular weight were calculated from the recorder signals for each volume interval of the chromatogram. The weight and the numerical averages of the molecular weight were calculated using:

$$M_w = \frac{\Sigma c_i M_i}{\Sigma c_i} \qquad M_n = \frac{\Sigma c_i}{\Sigma c_i / M_i}$$

In the classical light scattering measurements the Rayleigh coefficients were determined at different concentrations and the weight averages of the molecular weights were calculated from the intercept of a straight regressions line using a plot of $kc/\bar{R}_\theta$ versus c.

Instrumentation

HPLC-system

Column: combination of two 250 x 4 mm columns, 100 and 500 Diol (10 µm), Lichrosphere (Merck AG)

Eluens:	$NaClO_4$, 1 mol/l
Flow rate:	0,71 ml/min
Pump:	HPLC-pump-52 (Dr. Knauer GmbH),
Pressure:	1.10^6 Pa
Detector:	DRI-detector (Dr. Knauer GmbH)
LALLS-detector:	Chromatix KMX-6 LALLS photometer (Chromatix Inc. Sunnyvale, Calif.)
Light source:	1,8 mW He/Ne laser
Wave length:	632,8 nm
Angle of scattered radiation:	4,85°
Field stop:	0,15 mm
Flow through cell:	5 mm
Scattering volume:	0,1 µl
Second virial coefficient:	$2,23.10^{-3}$ ml mol/g^2
Volume interval:	2 mm $\hat{=}$ 0,0355 ml
Refractive index of the solvent:	n_o = 1,3398

The refractive index increment of the solution (dn/dc) was measured with a Brice Phoenix differential refractometer at 633 nm and 23° C. Prior to the determination of the refractive index all solutions were dialyzed against $NaClO_4$ solutions (1 mol/l).

MATERIALS

Prof. G. Barlow (Michael Reese Research Foundation Chicago) kindly provided a small amount of the heparin

standard fractions 2, 3, 5 at our disposal. The sodium salts of this reference standards which were fractionated by preparative gel chromatography were analysed at different institutes. The results were published by G. Barlow[17] at 1982.

In addition to this experiments, we have measured the molecular weight distributions of a sodium and a calcium salt of a new potent heparin isolated from mucosa salt- brine[18]. This heparin was shown to have a higher degree of purity and an increased content of high molecular weight units. The salts were prepared by treatment of the free heparin with an anion-exchanger bed (Cl^- - form) and a subsequent elution with NaCl or $CaCl_2$ solution. The corresponding salts were precipitated with methanol from the eluate, filtered and dried.

RESULTS AND DISCUSSION

The molecular weight determination of heparins is influenced by the amount of ionic groups, present in the moleculs which in turn depends on the raw material used. They produce exclusion effects and a electrostatic attraction and thus influence the separation behaviour of a GPC system using water as eluent. All this effects are strongly influenced by the ionic strength of the eluent as well.[19]

Molecular weight measurements of heparin with a light scattering photometer are also dependent on the solvent system. This dependence is demonstrated by Debye plots of a sodium heparin measured by classical light scattering experiments in solvents with different contents of NaCl (figure 1 and table 1). The weight averages of the molecular weight decrease with increasing ionic strength of the solvent and come to be

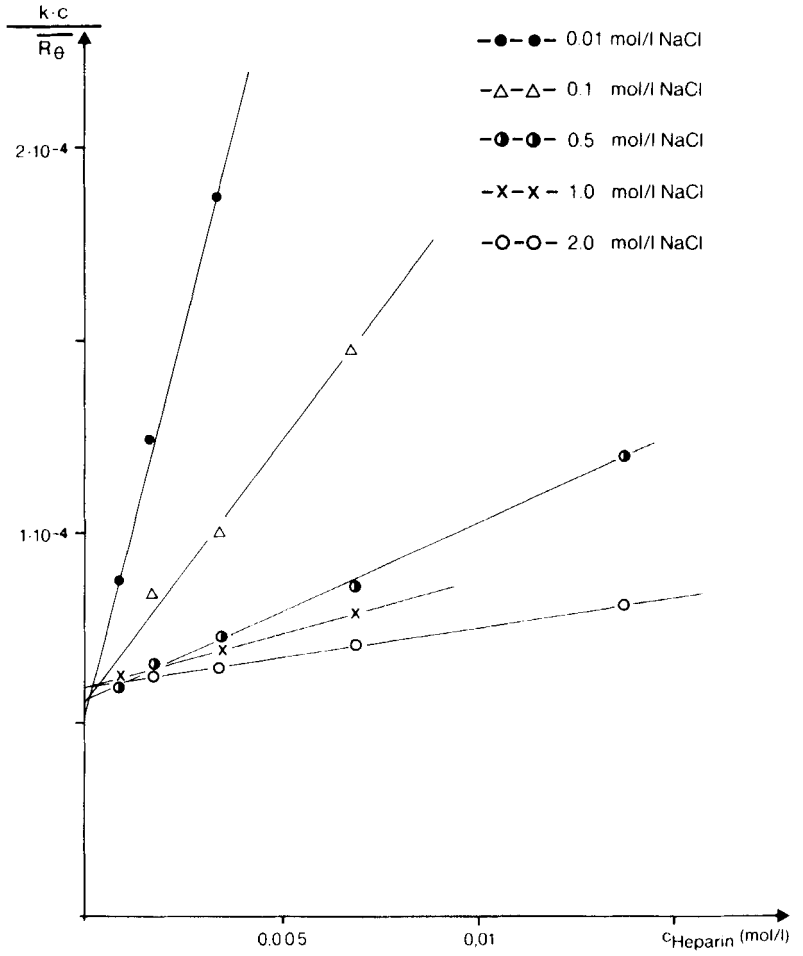

FIGURE 1

Debye Plots of a Na-Heparin in aqueous Solutions differing in the NaCl Concentration.

TABLE 1

Classical Light Scattering Measurements with a Na-Heparin at different Concentrations of NaCl

c_{NaCl} (mol/l)	M_w (g/mol)	A_2 (ml mol/g^2)	$\frac{dn}{dc}$ (ml/g)
0,01	19230	$1,59 \cdot 10^{-2}$	0,142
0,1	17860	$5,84 \cdot 10^{-3}$	0,137
0,5	17240	$2,52 \cdot 10^{-3}$	0,126
1,0	16700	$1,52 \cdot 10^{-3}$	0,121
2,0	16700	$7,8 \cdot 10^{-4}$	0,112

constant at about 1 mol/l NaCl (figure 2). The slopes ($2A_2$) of the Debye plots are reduced and approximate zero (Theta conditions).

The chromatograms of the reference standards, obtained with a HPLC-LALLS coupling technique are shown in figure 3. The results of the analysis of data are listed in table 2 together with the weight average of the molecular weights obtained from the classical light scattering experiments, as well as the range of the results from different laboratories which used conventional HPLC.[17)]

Molecular weights measured with a HPLC-LALLS coupling technique are well correlated with the classical light scattering experiments, whereas the results of the comparative study deviate strongly. However, the molecular weights of the standard heparin fractions measured with an analytical ultracentrifuge are in good agreement with our results (see table 2). Since the ultracentrifuge and the light scattering

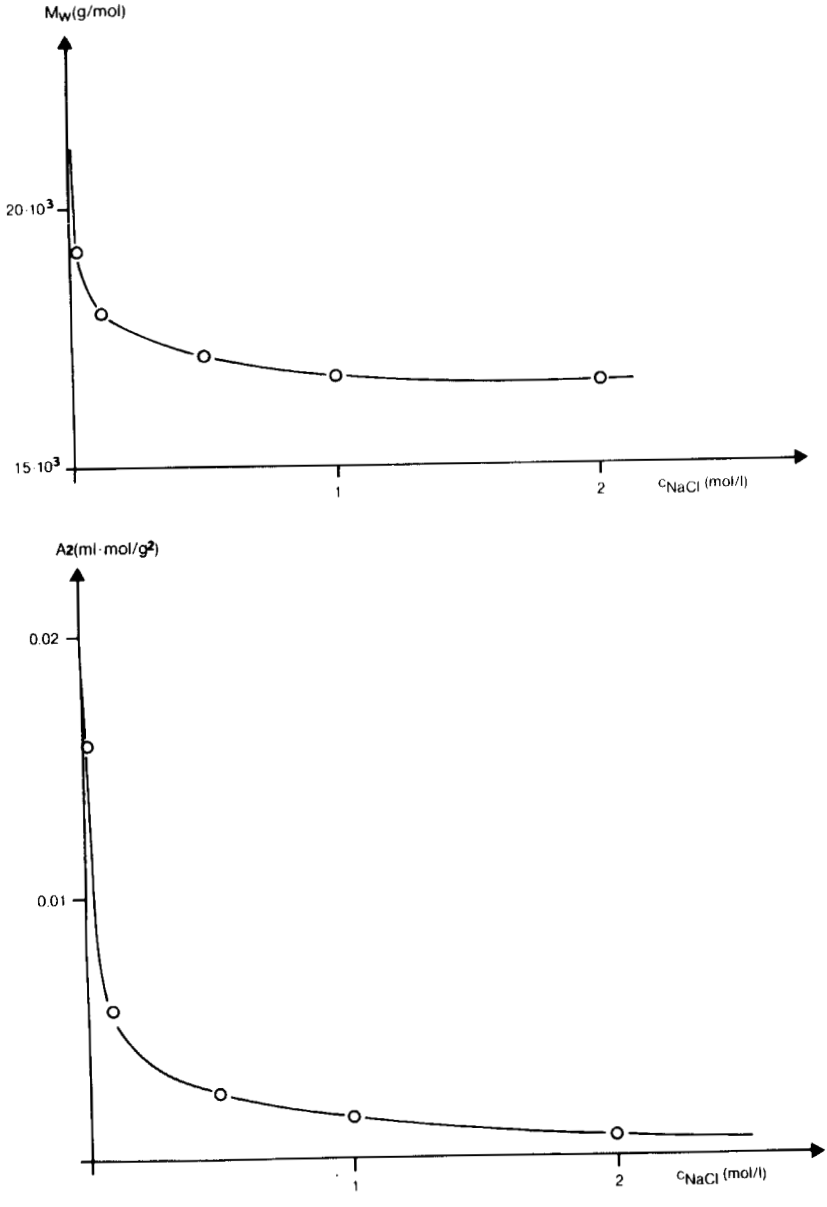

FIGURE 2

Molecular Weight and second Virial Coefficient of Heparin as Function of the Salt Concentration.

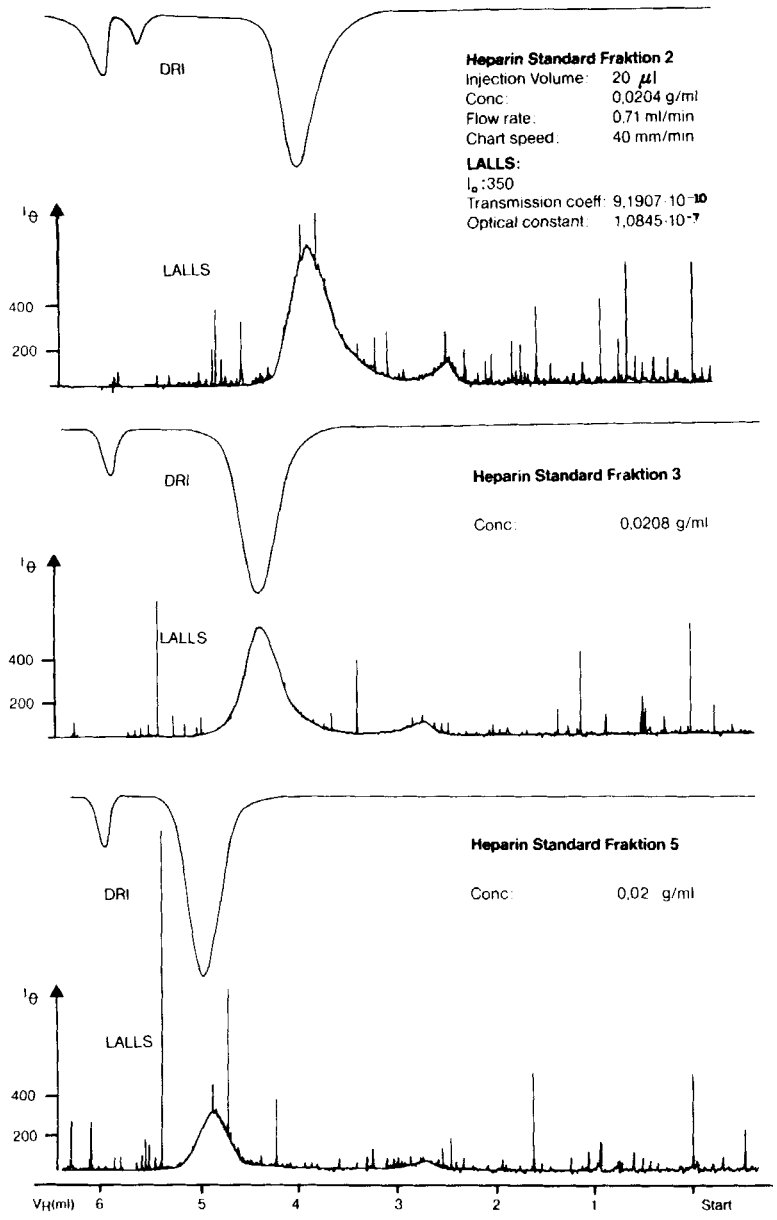

FIGURE 3

HPLC-LALLS Coupling of Heparin Standard Fractions in $NaClO_4$ Solutions (1 mol/l).

TABLE 2

Experimental Results of the Heparin Reference Standards and Comparison with Literatur Data

	HPLC-LALLS coupling techn.					classical LS		intercomparison study HPLC		UC
Fr.	M_w	M_n	M_w/M_n	V_R	k_{av}	M_w	A_2	M_{max}	- M_{min}	M_w
2	18025	13470	1,34	4,08	0,44	17700	$1,4.10^{-3}$	32300	- 15200	17100
3	12560	9480	1,32	4,51	0,54	13300	$2,5.10^{-3}$	18000	- 12000	12800
5	6500	3993	1,38	5,05	0,69	6450	$2,8.10^{-3}$	7700	- 6300	6000

M_w = weight average (g/mol), M_n = numerical average (g/mol)

V_R = retention volume (ml) - HPLC

A_2 = sec. virial coeffizient (ml mol/g^2) - classical LS

K_{av} = average partition coefficient - HPLC

UC = ultracentrifuge

technique are absolute methods for a molecular weight determination, the good agreement of both results demonstrates the qualification of the HPLC-LALLS coupling technique for the characterisation of heparin-standards.

Figure 4 shows the calculated molecular weight distributions of the three fractions in a distributiv and a cumulative manner. Comparison of the separations of samples of heparins differing in molecular weight shows the rather good separating power of the HPLC-system used (figure 3). According to the molecular weight, different elution volume are observed. Since in all 3 chromatograms the amount of heparin introduced onto the columns was the same, the peak areas of the DRI signals are equal while the areas of the LALLS peaks increase with increasing molecular weight, giving a linear relationship between the ratio A_{LALLS}/A_{DRI} and the molecular weight averages (figure 5). Also the values of a sodium and a calcium salt fall on this line.

In figure 3 there is an additional peak observable only with the LALLS photometer eluting prior to the heparin peak. This peak is tentatively assigned to result from a high molecular weight heparin present in a concentration which is to low for detection by DRI. The elution volume of this peak was taken as the void volume of the gel matrix. The elution volume of the salt peaks eluting after the heparin and only detected by DRI were taken as the total volume V_T of the column[20]. Using the following equation the available volume partition coefficient k_{av} was calculated.

$$k_{av} = \frac{V_R - V_o}{V_T - V_o}$$

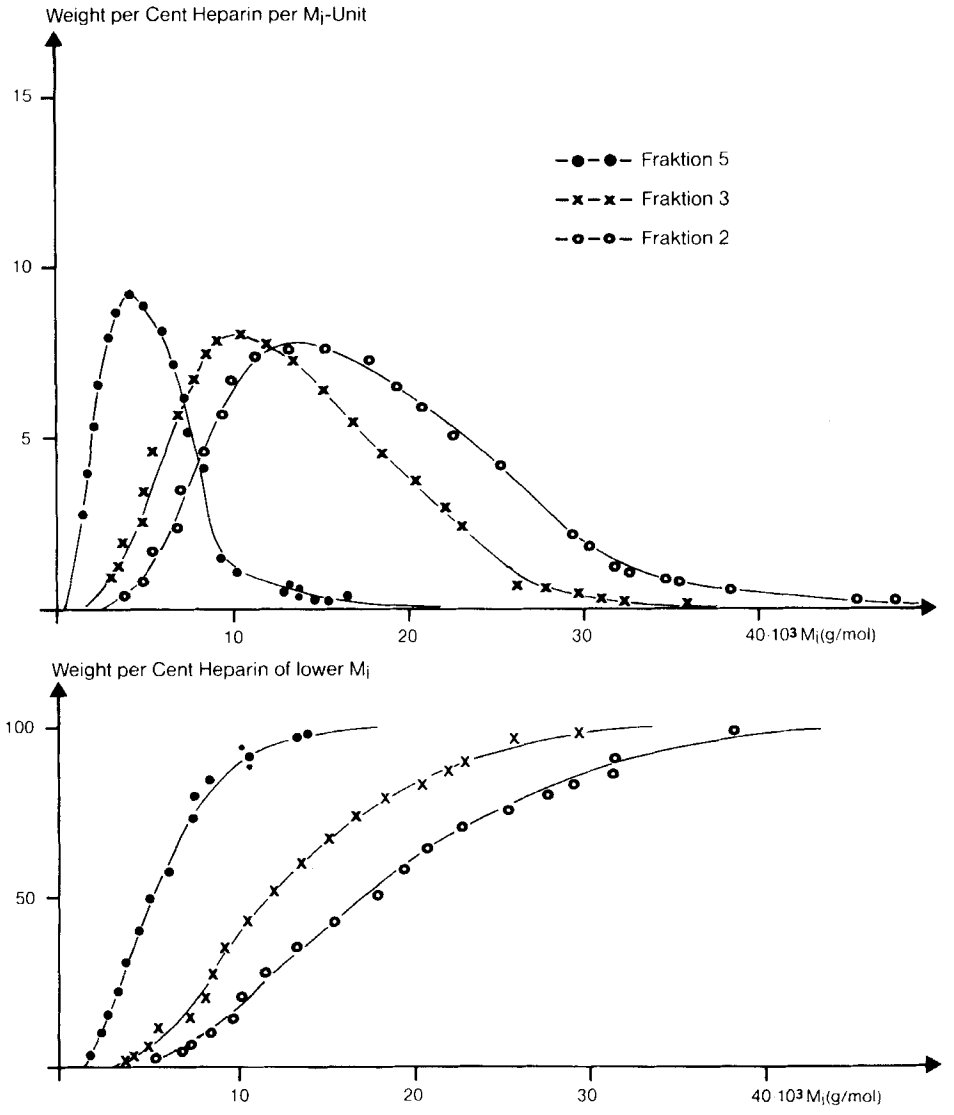

FIGURE 4

Molecular Weight Distribution of Heparin Standard Fractions.

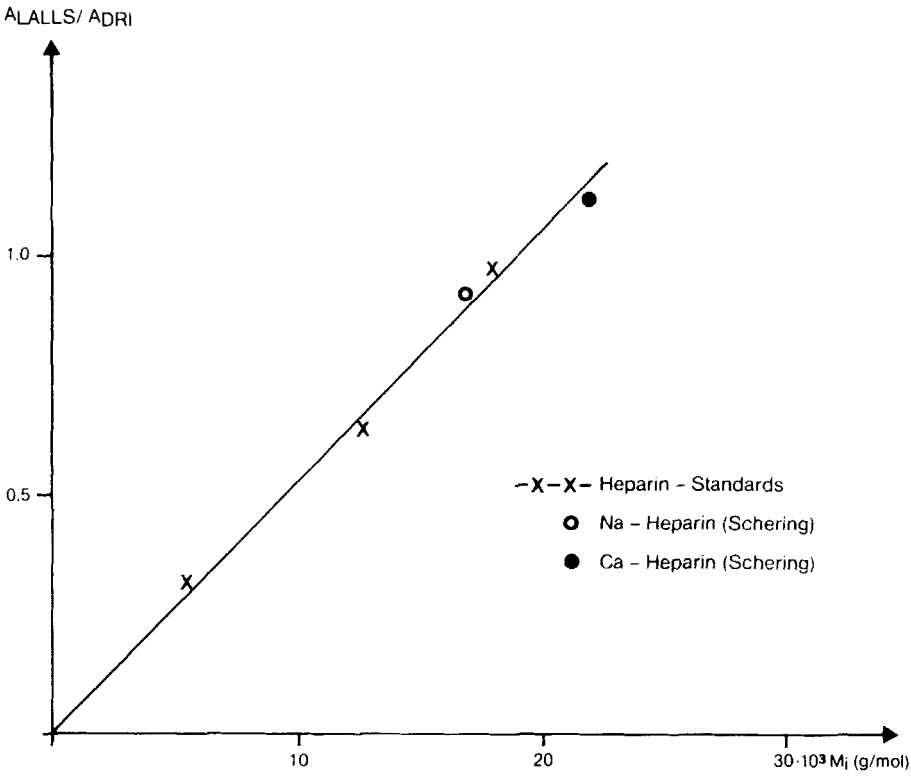

FIGURE 5

Dependence of the Peak Area Ratio A_{LALLS}/A_{DRI} on the Molecular Weight Averages.

In a semilogarithmic plot of M_i against k_{av} a straight line was obtained for the relationship between M_i and k_{av} (figure 6) which indicates the accuracy of the HPLC-LALLS coupling method.

Figure 7 shows the HPLC-LALLS chromatograms of a Na-heparin and a special Ca-heparin. For the latter a higher molecular weight average and a higher polydispersity was determined (table 3).

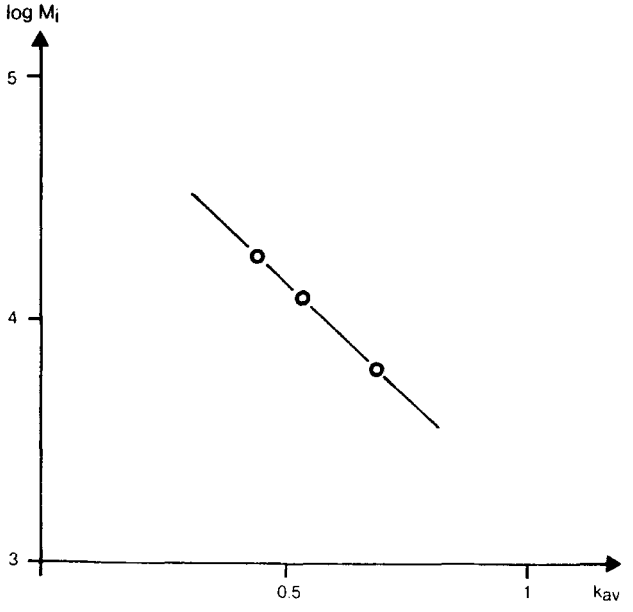

FIGURE 6

Semilogarithmic Plot of Molecular Weight against the Average GPC Partition Coefficient (K_{av}) of the Heparin Standards.

TABLE 3

Comparison of Results of a Sodium and a Calcium Heparin

	classical LS	HPLC-LALLS coupling technique		
	M_w	M_w	M_n	M_w/M_n
Na-Heparin	17860	16980	11770	1,44
Ca-Heparin	20410	22150	13996	1,58

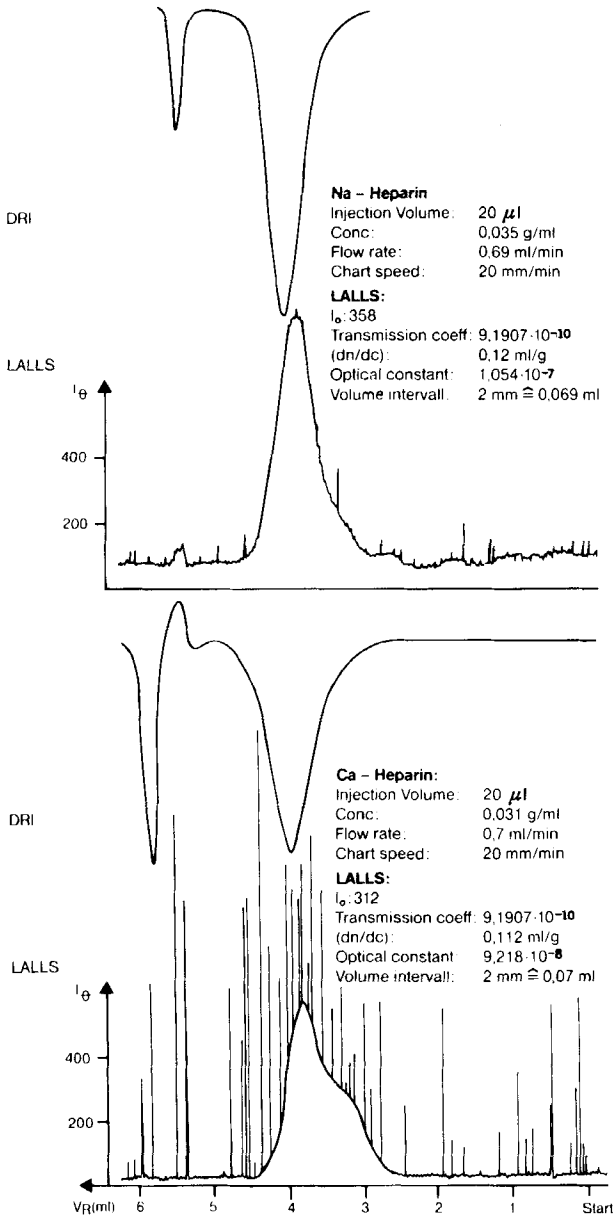

FIGURE 7

HPLC-LALLS Coupling of a Na-Heparin and a Ca-Heparin (Schering AG).

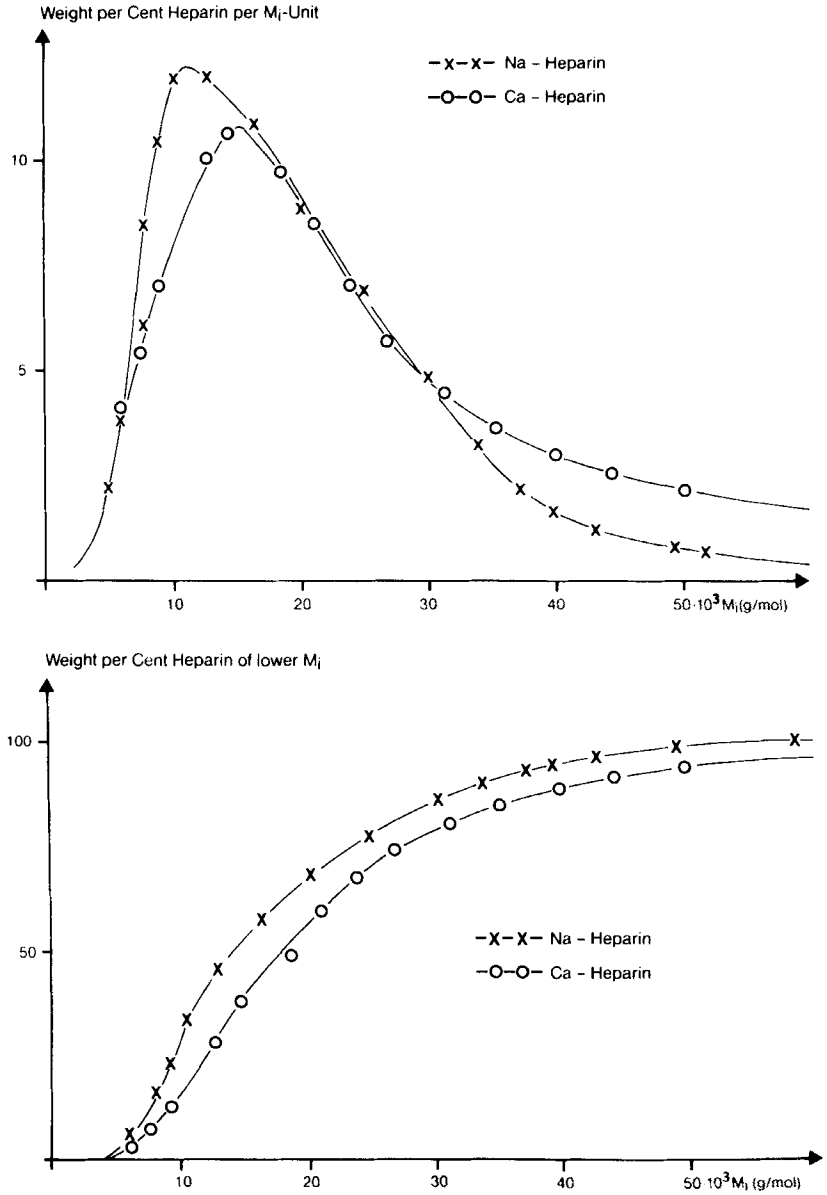

FIGURE 8

Molecular Weight Distribution of a Na-Heparin and a Ca-Heparin (Schering AG).

The LALLS peak of the calcium salt has a well distinct shoulder at its front indicating the presence of high molecular weight components. The relative molecular weight distributions of both salts (figure 8) show no difference in the medium molecular range, but a higher content of molecular weights over 30 000 g/mol for the calcium heparin. It is known that calcium ions are complexed by heparin and that the bound calcium is forming a bridge to connect heparin chains[22,23], so that the high molecular content of calcium heparin could result from this association.

After subcutanous application of the salts to beagle dogs the calcium salt was shown to be slightly more effective than the sodium salt in increasing blood clotting time.[24] Therefore, the interpretation of the results of biological tests should give due consideration to the molecular weight distribution of the sample.

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