STANDARDIZATION OF HEPARINS BY MEANS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
EQUIPPED WITH A LOW ANGLE LASER LIGHT SCATTERING DETECTOR

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ABSTRACT

This study shows that HPLC-LALLS (high performance liquid chromatography with a light-scattering detector) is a convenient and reliable method for the characterization of standard heparin samples, provided that polyelectrolyte artefacts are suppressed by a suitable dialysis procedure. The method has been successfully applied to the assessment of calibrated relations like the Mark-Houwink relation and the calibration curve of gel permeation chromatography. Furthermore, this study confirms that gel permeation chromatography for heparins cannot be calibrated with dextrans or polyethyleneglycols, but in addition to this well known fact it is shown that even the universal (hydrodynamic volume) calibration procedure does not work. Moreover, in addition to the familiar conclusion that heparins should be calibrated with heparins, it is shown that calibrated relations are valid only when the calibration has been made with heparin samples of the same origin (i.e. same tissue source, same animal source, and same purification scheme).

INTRODUCTION

Commercially available heparins from different manufacturers differ in molecular mass distribution and in chemical composition, according to animal source, tissue source and purification scheme (1). In this respect tissue source seems to be more important than animal source (2). This has drawbacks for the determination of average molecular masses from calibrated relations like the Mark-Houwink relation between intrinsic viscosity and mass average molecular mass $\bar{M}_w$ (3,4). Once established by means of well-characterized heparin samples, it is valid only for samples consisting of polymer

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molecules of the same chemical composition. That is, for heparins stemming from the same origin (i.e. same animal source, same tissue source and same purification scheme). Thus, even the calibration of a Mark-Houwink relation for heparins with heparins is not enough: in principle, a systematic error is introduced when applying the Mark-Houwink relation established for heparin-samples of one origin (characterized by the same tissue and animal sources and the same purification scheme (2)) to samples of another origin. In this paper we will show that there is such an error indeed.

The same problem will arise with the assessment of other calibrated relations. Generally speaking, those are (usually) relations between some easily measurable transport property of a polymer and some not so easily accessible average molecular weight of this polymer. Thus, the crucial relation in gel permeation chromatography between top-of-peak elution volume and molecular mass (the so-called calibration curve), once established for known heparin samples of a certain origin, can only be applied to unknown samples of the same origin. This will also hold true for still another calibrated relation: that between sedimentation coefficient and $\bar{M}_w$ (3).

Thus, the assessment of calibrated relations for heparins from a certain origin requires (i) a reliable method for the determination of molecular weight distributions of heparin samples of the same origin that will serve as standard samples, and (ii) a reliable method for the construction of a calibration curve from the molecular mass distributions of these standards. In this paper we will show that HPLC-LALLS (high performance gel permeation chromatography coupled to an absolute molecular mass detector, LALLS: low angle laser light scattering (5)) provides molecular mass distributions of standard heparin samples in a quick and reliable way.

The application of HPLC-LALLS analysis to heparin samples has also been referred to by Van Dedem (6) in a comparative study on the determination of heparin molecular mass distributions with HPLC. Our study, however, will present some proviso's as to the reliability and the accuracy of the HPLC-LALLS method. Moreover, we will discuss the applicability of the light scattering method as such to aqueous solutions of a copolymeric polyelectrolyte like heparin.

In addition, we will show that gel permeation chromatography of heparins using other polymers (like dextrans (7) or polyethyleneglycols) as standards leads to wrong results, even when processed by the "universal calibration" procedure (where the calibration curve consists of hydrodynamic polymer volume rather than molecular mass plotted against elution volume (8)).

MATERIALS AND METHODS

Heparins: Two heparins (sodium salt, 164 E/mg) of the same animal and tissue source (porcine intestinal mucosa), and the same purification scheme were obtained as gifts (we regret that we cannot provide batch numbers), with no more specifications than those regarding expected molecular weight (range), and origin and purification scheme. A low molecular mass product was a gift of Kabi Vitrum, Amsterdam, the Netherlands. The other product had a broad molecular mass distribution and was a gift of Diosynth, Oss, the Netherlands. The latter product (10 g) was fractionated over a Biogel column (P200, Biorad, Richmond, CA, USA) as described previously (9). Six fractions with decreasing molecular masses were obtained. The yields, after exhaustive dialysis against

water and lyophilization, were 0.2 g, 1.7 g, 2.0 g, 2.2 g, 1.9 g and 1.5 g for fractions #1 to #6, respectively.

HPLC-LALLS analysis: Our HPLC-LALLS equipment was assembled from a pump (Waters, model 6000 A), an injector (Waters U6K), one or two HPLC columns (see below), a LALLS molecular mass detector (Chromatix KMX-6) and a concentration detector (Waters differential refractometer, R 401). Columns used were: Toyosoda/TSK PW 3000 (300 x 7.5 mm; molecular mass exclusion limits for polyethyleneglycol (PEG) in water: 100 and 10000; between these limits the column is not linear, however), Toyosoda/TSK PW 4000 (300 x 7.5 mm; (non-linear) molecular mass range (PEG): 1000-100,000) and Toyosoda SW 3000 (600 x 7.5 mm; linear molecular mass range (PEG) 10,000-100,000).

Elution was performed at room temperature at a flow rate of 1.0 ml/min using 0.1 M NaAc buffer, adjusted with acetic acid to pH 6.0, as the mobile phase. Heparin solutions (50 microliter) with a concentration of 1 % (w/v) were injected.

Heparin samples used were dialysed against the elution buffer solution in order to eliminate (10) Donnan-type artefacts in the molecular mass determination by light scattering. To this end also, the refractive index increments (dn/dc) of the samples (at the LALLS wavelength $\lambda = 633$ nm and at $T = 25^\circ\text{C}$) were measured against the dialysis equilibrium liquid as the blank (10) in a Brice-Phoenix differential refractometer. We found dn/dc values ranging from 0.110 ± 0.002 ml/g (for the lowest molecular mass sample) to 0.118 ± 0.002 ml/g (for the high molecular mass sample). (We have no indications that during this dialysis procedure heparin was lost: all losses must have occurred during the fractionation procedure and the subsequent exhaustive dialysis described above (see "Heparins").)

Calculation procedures for molecular mass distributions and averages are described below (results section). The reproducibility of the top-of-peak elution volumes was within 1% on each set of columns, and that of molecular mass averages calculated from HPLC-LALLS or from HPLC only (based on previous LALLS calibration) was within 8% or 5%, respectively. The LALLS signal becomes vanishingly small for molecular masses lower than about 6000; hence the KABI sample was at the verge of non-detectability.

Membrane osmometry: The $\bar{M}_n$ values derived from HPLC-LALLS measurements were checked against osmometric measurements (11). A Stabin (type M2) membrane osmometer equipped with a dialysis tubing membrane (063-F-09, Tamson, Zoetermeer, the Netherlands) was used. Measurements were carried out for series consisting of at least 3 dilutions (the highest one being 10 mg/ml), at $25 \pm 0.002^\circ\text{C}$, in a 0.1 M NaAc adjusted with acetic acid to pH 6.0 as the solvent. Some NaN_3 (0.03%) was added to the solvent in order to protect the solutions against bacterial growth during the long osmometric experiment.

The hydrostatic pressure method (static osmometer) was applied (11) and a period of 48 hours was required to obtain equilibrium. No correction for the Donnan effect (as is the case with light scattering) is needed for an osmometry experiment (10). Concentration-dependent $\bar{M}_n$ values were derived for each concentration from the equilibrium osmotic pressure. Straight line extrapolation of $1/\bar{M}_n$ to zero concentration was possible, yielding the true $\bar{M}_n$ with a 10-15% reproducibility. A much worse reproducibility was found, however, for the determination (from the same plots) of second virial coefficients. Values ranging from 0.001 to 0.008 mol.ml/g (not systematically varying with $\bar{M}_n$) were found - this kind of measurement usually has no good

reproducibility (11). In all cases, however, significantly positive slopes were found. Hence, our solvent is certainly not a theta-solvent (as is, for instance, water containing 0.5 M NaCl for heparin from another origin (2)).

Viscometry: Viscosity measurements were performed at 25 ± 0.002 °C using an Ubbelohde viscometer (11). Flow times were determined in triplicate for at least 3 dilutions (for solvent and highest concentration see osmometry, above) and did not differ more than 0.2 seconds for each concentration. Intrinsic viscosities $[\eta]$ were calculated by plotting η_{sp}/c and $(\ln \eta_r)/c$ against c , and taking the mean of the intercepts thus obtained (11). The experimental error in each intrinsic viscosity value was ± 0.2 ml/g.

Our Mark-Houwink relation (equation (1) below) was calculated by means of an iterative procedure in order to obtain K and a values independent of polydispersity. First, $\bar{M}_v$ (equation (2) below) was estimated for each fractional distribution using preliminary K and a values derived from a plot of $\log[\eta]$ against $\log \bar{M}_w$. Then, the $\log[\eta]$ were plotted against the $\log \bar{M}_v$ thus obtained, and new K and a were calculated from intercept and slope, respectively. These new values were used for the recalculation of new $\bar{M}_v$ values, that were plotted again. After three iterations, K and a were constant and a straight line with regression coefficient 0.97 for the 8 data-points was obtained.

Ultracentrifugation: The $\bar{M}_w$ and $\bar{M}_v$ derived with the HPLC-LALLS method were checked, for the unfractionated Diosynth sample and for fraction #3, by equilibrium ultracentrifugation (in a Beckmann Spinco model E). Our experimental procedure was the same as that of Barlow (2), who processed the experimental data according to a computer program used in Scholte's "variable rotor speed" procedure (12). We could not use this program, however, our solvent being not a theta-solvent. Therefore, we performed our measurements at more than one concentration (see above for the concentration range) and calculated molecular mass averages according to another procedure outlined by Scholte (13) for the variable rotor speed method. The reciprocal values of the molecular masses were extrapolated to zero concentration, and again significantly positive second virial coefficients were found in the range as mentioned above for the osmometric measurements. The reproducibility of the true molecular masses thus obtained is comparable to that of osmometry: 10-15%.

Again, a dialysis procedure as described above for the HPLC-LALLS method prevented the occurrence of Donnan-type artefacts in the results. In this case, not only refractivity index increments were measured against the dialysis equilibrium liquid as the blank, but also the buoyancy factors $(1 - \bar{v}d)$ (10). For the partial volume $\bar{v}$ we found 0.43 ml/g for Diosynth and 0.50 for fraction #3, in accordance with earlier measurements by other workers (2,3). These data were calculated from density measurements in a PAAR Digital Density Meter (DMA 50, Anton Paar K.G., Graz).

RESULTS

HPLC-LALLS measurements: Two series of HPLC measurements were carried out. The first for characterization of heparin standards with the LALLS as a detector, the second for studying the transferability of the standard data for calibration. For the first (characterization) series of measurements we used two columns in series (PW 3000 and 4000, specifications given above), connected to the LALLS and to the refractometer, for the second (calibration)

series the LALLS was disconnected and only one column (the SW 3000) was used. This column has a higher resolution than the two PW columns in series because it is linear over the range of expected molecular masses, and therefore it should be preferred for calibration purposes over the PW columns. The SW 3000 column, however, released small particles during the experiments, which renders it unreliable for use with the subtle LALLS detecting system (too much noise on the LALLS recorder signal).

A microcomputer processed the detector signals and calculated the molecular mass distributions and the mean molecular masses. For the characterization of samples by means of the LALLS system, correction for the second virial coefficient was necessary and performed as indicated by Ouano (14). The second virial coefficient (determined by osmometry and confirmed by ultracentrifugation, see above) was assumed to be 0.0045 mol.ml/g for the whole range of molecular masses. The bad reproducibility of the second virial coefficient determination causes an uncertainty of 15% in the molecular masses, determined by the HPLC-LALLS method, presented in Table 1.

Correction parameters for column dispersion were calculated directly from the HPLC-LALLS measurements by a procedure outlined by Zhi-Duang He e.a. (15)) and were found to be very small for the two column system. For the SW 3000 column the same had been found earlier by Leypoldt e.a. (16). According to these workers, the $\bar{M}_n$ and $\bar{M}_w$ calculated with and without such small dispersion correction parameters hardly differ. Hence, we omitted these parameters from our calculations, the more so, because the errors in the molecular masses are determined almost exclusively by the experimental uncertainty in the second virial coefficient. Molecular mass distributions calculated from our HPLC-LALLS measurements in the same way as the average molecular masses are shown in fig. 1 (next page).

TABLE 1

Molecular Mass Averages (in g/mol) from HPLC-LALLS Measurements

HPLC-LALLS (HL) compared to some results of measurements with osmometer (os) and ultracentrifuge (uc). V_e is the elution volume belonging to the top of the distribution curve.

Sample (Name or # of fraction)	V_e ml	$\bar{M}_n$ HL	$\bar{M}_n$ os	$\bar{M}_w$ HL	$\bar{M}_w$ uc	$\bar{M}_z$ HL	$\bar{M}_z$ uc
Kabi	23.25	6200	6700	7400		9300	
6	22.60	6700	8800	8200		10600	
5	22.05	10500	11200	11800		13300	
4	21.80	9800	12500	12000		14900	
3	21.40	11700	13900	15500	13000	18900	25000
Diosynth	21.50	12300	12000	17700	18000	22500	30000
2	20.85	16500	20500	20800		24300	
1	20.00	22200		30300		38900	

Table 1 also compares the HPLC-LALLS results with those of osmometry and ultracentrifugation. Within the experimental errors inherent to each method (see above), the results are reasonably well in agreement. This means that light scattering (and, therefore, HPLC-LALLS) is a valid method for the

determination of molecular masses of heparin - provided that its polyelectrolyte behavior is properly corrected for by the dialysis procedure described above.

FIG.1 Molecular mass distribution (mass fraction frequency) of heparin samples. Not corrected for column spreading (see text).
(●) Diosynth, (□) fraction #1, (○) fraction #6.

Still one remark has to be made concerning Table 1. Usually, top-of-peak elution volumes decrease with increasing average molecular mass. This, however, does not seem to be the case for the unfractionated Diosynth sample. The solution to this paradox is, that top-of-peak elution volumes for polydisperse samples (with broad and skewed elution peaks, like the Diosynth sample) not necessarily coincide with the calibration curve (17). For this curve the $\log \bar{M}_w$ of low polydispersity samples (with $\bar{M}_w/\bar{M}_n < 1.3$, say, and with a narrow unskewed elution peak, like the fractions #1-#6) are plotted against the corresponding top-of-peak elution volumes.

Mark-Houwink relation: Table 2 shows that calculation of molecular mass averages according to the Mark-Houwink equation proposed by Lasker and Stivala (3) leads to results that are very different from all the results of Table 1. In the Discussion Section it is explained that this has to do with the other origin of the samples studied by these workers, and not with the fact that Lasker and Stivala (3) used an aqueous salt-solution (0.1 M NaCl) different from ours (0.1 M Na-acetate). For our heparin samples we obtained the Mark-Houwink relation:

$$(1) \quad [\eta] = K \cdot \bar{M}_w^a, \quad \text{with: } K = (6.97 \pm 0.20) \cdot 10^{-4} \text{ (ml/g)}, \text{ and} \\ a = 1.03 \pm 0.02$$

The $\bar{M}_w$ of the fourth column of Table 2 are derived from the $\bar{M}_w$ as given in the last column. Hence, the rather large experimental uncertainties in K and a of equation (1) are consequences of the experimental uncertainty in the osmotic second virial coefficient correction used in the calculation of molecular masses from the LALLS signals, and of the reproducibility of the LALLS

signals. The $\bar{M}_v$ calculated according to equation (1) when the experimental uncertainties in K and a are taken into account, however, differ significantly from those derived from Lasker and Stivala's Mark-Houwink relation (3) (third column of Table 2).

TABLE 2

Mass- and Viscosity -average Molecular Masses

Molecular masses calculated from Mark-Houwink relations, as determined by Lasker and Stivala (LS) (3) and by us (equation (1)), and compared to the HPLC-LALLS results (HL).

Sample (Name or # of fraction)	ml/g	$\bar{M}_w$	$\bar{M}_v$	$\bar{M}_w$
		LS	(1)	HL
Kabi	5.7	3842	6233	7400
6	8.7	5914	9393	8200
5	10.9	7444	11688	11800
4	12.9	8841	13763	12000
3	14.9	10241	15828	15500
Diosynth	16.9	11646	17884	17700
2	18.9	13054	19934	20800
1	29.1	20277	30295	30300

As a is close to 1, $\bar{M}_v$ is close to $\bar{M}_w$ (w_i in (2) is the mass fraction of species i):

$$(2) \bar{M}_v = (\sum w_i M_i^a)^{1/a} \approx \sum w_i M_i = \bar{M}_w$$

Thus, M_i 's may be calculated directly from our Mark-Houwink relation. Its application to heparin samples of another origin than ours is not directly possible, however.

HPLC calibration: As was stated by the inter-laboratory survey report mentioned above (6), the degree of the polynomial describing a HPLC calibration curve based on standard heparins should be of great importance (this is supported by the model calculations on HPLC data treatment by Ogawa and Inaba (17)). Hence, two calibration curves for the second series of HPLC measurements (SW 3000 column, no LALLS) were derived from the first series of measurements, a cubic one and a linear one,

The calibration procedure for the SW column was as follows. The eight heparin samples were injected on the SW 3000 column. For each sample, at elution volumes V_i , separated from each other by 0.5 ml, the weight fraction of heparin having a retention volume $> V_i$ was determined from the chromatogram. The weight fractions, in turn, were correlated to molecular masses using the cumulative mass distributions obtained from the HPLC-LALLS calibration measurements. Due to overlap of the different fractions, 1 to 3 molecular weights were obtained per elution volume. Then least squares cubic and linear fits were calculated through the elution volumes (as the independent variables) and the natural logarithms of the corresponding molecular masses (as the dependent variables).

From the calibration curves, molecular mass distributions were calculated by a procedure outlined by Yau et al. (18). As a check to the reliability of this procedure for the transfer of calibration data obtained on the two PW column set to the one SW column, it was also applied to the calculation of cubic and linear calibration curves for the two-column calibrating system itself (TSK PW 3000 and 4000), from which the molecular mass distributions of the calibrating samples were recalculated.

The results are presented in Tables 3 - 5. The cubic calibration curve (columns "cal 1") for the two column set certainly covers the whole range of original (i.e. HPLC-LALLS) molecular mass averages best, generally speaking, and apart from the results for the Kabi and Diosynth samples (an explanation is given below).

The molecular masses as determined by HPLC-LALLS of the Kabi and Diosynth samples are not very well reproduced by the calibration curve calculation, neither with the cubic curve based on all the samples (including the outliers Kabi and Diosynth), nor with the straight line based on the 3 narrow-peak samples. In our opinion, this has to do with an experimental error inherent to the HPLC-LALLS method. At the high molecular mass tail (low elution volume) the LALLS signal is reasonably high while the dn/dc signal is low, whereas the reverse is true for the high elution volume tail. The - ambiguous (17) - choice of the positioning of the baselines of the two recorder signals, therefore, especially biases the calculation of the molecular mass distributions of the Kabi and Diosynth samples. The Kabi sample has a very small LALLS signal, whereas the elution peak of the Diosynth sample is extended from the lowest to the highest elution volumes (cf. figure 1).

TABLE 3

Number average Molecular Masses from calibrated HPLC Measurements

The results are compared to the same averages calculated from HPLC-LALLS measurements (HL). Explanation of column headings:

cal 1 : two column system, cubic calibration curve based on fractional distribution (see text). cal 2 : same, linear calibration .

cal 3 : one column system, cubic calibration curve based on fractional distribution. cal 4 : same, linear calibration.

cal 5 : one column system, linear calibration with 3 widely different narrow distribution samples only (see the Discussion Section).

Sample (Name or # of fraction)	$\bar{M}_n$ cal 1	$\bar{M}_n$ cal 2	$\bar{M}_n$ cal 3	$\bar{M}_n$ cal 4	$\bar{M}_n$ cal 5	$\bar{M}_n$ HL
Kabi	5000	6300	5300	4900	5900	6200
6	6600	7600	6900	6700	7600	6700
5	8800	9000	9000	8800	9800	10500
4	11000	10700	11000	10900	11800	9800
3	14000	13900	14000	14000	14800	11700
Diosynth	11400	11400	11600	11100	12400	12300
2	18800	19700	18900	19300	19900	16500
1	27900	26200	28700	27900	29700	22300

TABLE 4

Mass average Molecular Masses from calibrated HPLC Measurements

For column headings cf. Table 3.

Sample (Name or # of fraction)	$\overline{M}_w$ cal 1	$\overline{M}_w$ cal 2	$\overline{M}_w$ cal 3	$\overline{M}_w$ cal 4	$\overline{M}_w$ cal 5	$\overline{M}_w$ HL
Kabi	6000	6600	6200	5700	6800	7400
6	8600	8900	8700	8600	9300	8200
5	11200	11100	11200	11000	11900	11800
4	13200	12900	13200	12900	14000	12000
3	15900	16100	15900	15800	16800	15500
Diosynth	15700	15000	15900	15000	16700	17700
2	21300	21500	21700	21100	22600	20800
1	32800	28300	34600	31400	35300	30300

TABLE 5

Z-average Molecular Masses from calibrated HPLC Measurements

For column headings cf. Table 3.

Sample (Name/# of fraction)	$\overline{M}_z$ cal 1	$\overline{M}_z$ cal 2	$\overline{M}_z$ cal 3	$\overline{M}_z$ cal 4	$\overline{M}_z$ cal 5	$\overline{M}_z$ HL
Kabi	7400	7100	7400	6900	8000	9300
6	11100	11100	11100	11000	11700	10600
5	13700	13700	13700	13400	14400	13300
4	15700	15500	15800	15100	16600	14900
3	18000	18200	18200	17500	19000	18900
Diosynth	21100	18900	22000	19100	22500	22500
2	24200	23100	25100	23000	25800	24300
1	38100	29300	41400	34000	41700	38900

Universal calibration: Figure 2 (next page) presents the results of the universal calibration procedure (8). The calibration curve is a plot of the (logarithm of the) hydrodynamic volumes, μ , of different polymers in the same solvent against the corresponding (top-)elution volumes (the hydrodynamic volume μ is represented by the product of intrinsic viscosity and molecular mass: $\mu = [\eta] \cdot \overline{M}_v$).

In figure 2 the μ -values (determined from measurements in the same solvent as that used for our heparin samples) for three dextrane fractions and four polyethylene glycol fractions are plotted. Within experimental error, these can be described by one and the same curve.

Surprisingly, however, the μ -values of our heparin samples are not covered by this curve and heparin molecular masses derived from this curve are greatly overestimated in the low molecular mass region. Maybe this has to do with the fact that heparin molecules are not randomly coiled (as indicated by the value of the Mark-Houwink exponent $a > 0.8$), while dextrane and polyethylene glycol

are. This explanation has been given by Lambert e.a. (19) for the deviation from the universal calibration curve they found for the xanthan gum. This deviation is precisely the reason why these workers advocate the use of LALLS as a HPLC detector for their polymer.

FIG.2 Universal calibration in aqueous 0.1 M NaAc solution adjusted to pH 6.0: logarithm of hydrodynamic volume (see text) plotted against top-of-peak elution volume. Upper points: polyethyleneglycol and dextrane. Lower points: heparin fractions.

DISCUSSION

The problem of the determination of average molecular masses of heparin, so important for establishing relations between molecular mass and biological activity (9), is solved in principle when one can calibrate relations like that of Mark-Houwink and HPLC calibration curves by well-characterized heparin samples of the same origin. As we have shown, HPLC-LALLS (gel permeation chromatography coupled to a light scattering detector) characterizes such samples conveniently and reliably (i.e. comparison with osmometry and equilibrium ultracentrifugation does not reveal any systematic error).

An objection to light scattering as a method could be that heparin, in a sense, is a random copolymer (1). It is well-known that random copolymers are very difficult to characterize by light scattering of solutions (20). Unless at least three different solvents are used, apparent molecular masses are derived allways from the measurements, sometimes far too high. This does not seem to be the case for our series of heparins: the molecular masses are reasonably well in agreement with those determined by osmometry and ultracentrifugation. Thus, we infer that heparins from the same origin are rather constant in chemical composition (i.e. with respect to sequence length and sequence composition along the chain backbones).

Moreover, a straight line Mark-Houwink relationship between $\log[\eta]$ and $\log \bar{M}_v$ has been obtained for our samples. This would not have been so when they had consisted of molecules greatly differing in sequence distribution. Thorough

chemical analysis of several heparins has revealed (1) that differences in sequence lengths and compositions do occur, but that they are much greater between molecules of heparins from different origin than between heparin molecules belonging to samples from the same origin. Hence, the latter are more like proteins: those are also random copolymers, but of such constancy in chemical composition that no problems arise in the interpretation of light scattering measurements and true molecular masses can always be derived (20).

Our finding that dn/dc seems to increase with molecular mass (see above) does not contradict this conclusion about the constant chemical composition of the heparin molecules of our samples. Such a (small) molecular mass effect is not uncommon for the molecular mass range we studied (21).

The exponent a of equation (1) is within experimental error equal to that found by Lasker and Stivala (3) and by Liberti and Stivala (4), namely 0.98 and 1.00, respectively, for fractions of beef-lung heparin in aqueous solutions of an ionic strength equal to that of our solutions. Their K , however, are very different: $1.75 \cdot 10^{-3}$ and $1.54 \cdot 10^{-3}$ (ml/g), respectively. This difference in K should be attributed to differences in sequence distribution between heparins of different origin. The exponent a stems from the long range interactions: those between the polymer molecules and those between them and the salt-ions and the solvent molecules (22). In contrast, K is a complicated function (22) not only of these long range interactions but also of the short range interactions (determined by the mutual positioning of adjacent monomeric units, different for heparins of differing origin). Thus, heparins from different origins but dissolved in the same solvent (see next paragraph) should have similar values for the exponent a and different values for the coefficient K .

Aqueous solutions of 1-1 salts (like NaCl and Na-acetate) are considered by us as the same solvent, provided that (i) the ionic strengths are equal, and (ii) the positively charged ions are the same. We are justified to do so because the oppositely charged (i.e. positively) small ions, and not the co-ions, determine the net charge of the negatively charged heparin molecules and thus the short range and the long range interactions. We would not consider aqueous solutions of $AgNO_3$ and NaCl to be the same solvents, however, even if their ionic strengths were equal, because silver-ions may exhibit "site-binding" to charged groups on heparin molecules whereas sodium-ions do not (10).

The universal calibration method does not seem to work for aqueous solutions of dextrans, polyethyleneglycols and heparins. (Maybe it does within the class of heparins as such, regardless of their origin - this will be the subject of coming work). We also tried to derive heparin molecular masses from the non-universal calibration curves (i.e. $\log \bar{M}_w$ plotted directly against top-of-peak elution volume) of dextrane or polyethylene glycol in the same solvent as that used for heparin. The results were not presented above, as they add nothing new to our conclusion from figure 2: the non-universal calibration curve also overestimates the molecular masses of the heparin samples, but now over the whole molecular mass range. Thus, we have confirmed the familiar conclusion that heparins should be calibrated with heparins and that heparin molecular masses should never be determined from HPLC measurements calibrated by other polymers than heparin. Our conclusion reaches further, however: one should calibrate heparins with heparins from the same origin.

The transfer of the HPLC-LALLS results to the other column does not lead to appreciable deviations from the original $\bar{M}_n$, $\bar{M}_w$ and $\bar{M}_z$ values, as Tables 3-5

show. These are reasonably well reproduced for the whole range of molecular masses, even if a linear calibration line (columns "cal 2" and "cal 4") is applied. Cubic calibration curves (columns "cal 1" and "cal 3" in Tables 3 - 6) seem to be superior in covering the whole range of molecular masses, however, in accordance with the findings of other authors (8), (17).

Surprisingly, even a linear calibration line based on only 3 narrow-peak samples gives acceptable results: see the column "calibration # 5" in Tables 3 - 5. The top-of-peak elution volumes and molecular masses used for the calculation of this line were those belonging to the elution peaks of 3 narrow distribution samples of widely differing mean molecular masses, though equidistantly spread over the $\log(M_w)$ range (fractions #6, #4 and #2). This has an obvious advantage: only 3 well-characterized samples and a linear calibration line are needed for the calibration of a (set of) column(s) when not the utmost accuracy is required.

The determination of complete molecular mass distributions, if desired, is also possible by means of properly calibrated HPLC. For the determination of such distributions, however, one should not omit (as we did) the column dispersion correction: a small correction has almost no influence on the average molecular masses, but it can influence greatly the shape of the distribution at the low and high molecular mass tails (16).

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