Molecular mass determination of low-molecular-mass heparins

Application of wide collection angle measurements of light scattering using a high-performance gel permeation chromatographic system equipped with a low-angle laser light-scattering photometer

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ABSTRACT

A high-performance gel permeation chromatographic system with on-line low-angle laser light-scattering detection (HPGPC–LALLS) was used to determine molecular masses of low-molecular-mass heparins (LMMHs). Measurements at wide and narrow collection angles were compared and the application of the HPGPC–LALLS method to small molecules, with molecular masses in the range 1000–10 000, was assessed. The molecular mass averages of fractionated heparins and commercially available LMMHs were also determined by ordinary HPGPC analysis using an LMMH molecular-mass calibrant, supplied by the National Institute for Biological Standards and Control for the calibration of columns. The LALLS intensity at the routinely used narrow collection angle ($\theta_{col} = 1°$) about doubled at the wide collection angle ($\theta_{col} = 2°$) and the signal-to-noise ratio was improved. The present study thus indicates that wide collection angle measurement of light scattering allows the application of the HPGPC–LALLS method to very small biopolymers of molecular mass <10 000.

INTRODUCTION

Recently, a great deal of research on the chemical, biological and therapeutic properties of low-molecular-mass heparins (LMMHs) has been carried out [1–9]. LMMHs are generally prepared by the enrichment of low-molecular-mass fractions or by the depolymerization of unfractionated heparins [10,11], which are polysaccharides composed of alternate sequences of differently sulphated residues of uronic acid (usually α-L-iduronic acid) and α-D-glucosamine-linked $\alpha(1 \rightarrow 4)$ bonds [12] with a wide molecular mass range between 3000 and 40 000 [12,13]. LMMHs are claimed to possess anticoagulant properties with reduced bleeding, greater bio-

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availability and longer biological half-lives than unfractionated heparins [7,14].

Commercially available LMMHs from different manufacturers differ in their molecular mass distribution and in end-unit chemical structure according to the preparation. Recent production methods involve chemical depolymerization using nitrous acid or hydrogen peroxide [10] and enzymatic depolymerization [11], the resultant LMMHs varying in structure, molecular mass distribution, anticoagulant activities and pharmacological properties [5,6,10]. The anticoagulant properties in particular are related to the molecular mass distribution [14] and therefore the determination of the molecular mass averages and ranges is important for batch characterization.

Several methods for the measurement of heparin molecular mass have been commonly used [5,8,15–19]. Recently, it was shown that low-angle laser light-scattering (LALLS), incorporating high-performance gel permeation chromatography (HPGPC) coupled to an absolute molecular mass detector, can provide molecular mass profiles of heparins [20], but there are only a few data available on LMMHs. For LMMHs having molecular mass averages of about 6000, reliable evaluation is difficult because of the weak light-scattering intensity inherent with very small molecules.

Takagi and co-workers [21–24] applied HPGPC–LALLS to determine the molecular mass of biopolymers and have published extensively on molecular mass measurements of membrane proteins. Krull and co-workers have demonstrated that these detectors can be used to determine biopolymer molecular masses when coupled with reversed-phase [25] and hydrophobic interaction [26] chromatography. Recently, Dollinger et al. [27] demonstrated that a simple fluorimeter for high-performance liquid chromatography can be used as a 90° (corresponding to θ_{obs} in Fig. 1) classical light-scattering detector for the on-line molecular mass determination of biopolymers. This detector was shown to be sensitive, relatively immune to dust and column particles and easy to use.

In this study, in an attempt to improve the application of HPGPC–LALLS analysis to
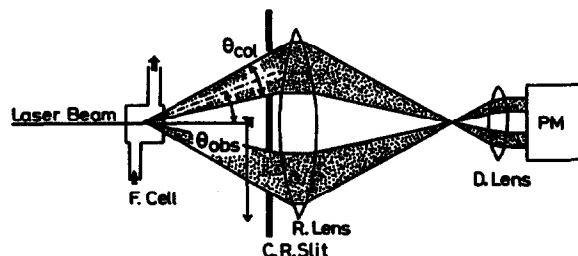

Fig. 1. Schematic illustration of light-scattering collection in a LALLS detector. F. Cell = Flow cell; C.R. Slit = circular ring slit; PM = photomultiplier; R. Lens = relay lens; D. Lens = detector lens.

LMMHs, light scattering was measured at a wider collection angle ($\theta_{col} = 2°$; see Fig. 1) than that routinely used ($\theta_{col} = 1°$) for the determination of the absolute molecular masses of polymers and proteins with values above 10 000 [21–24,26].

EXPERIMENTAL

Materials

Commercially available LMMHs, Enoxaparin (lot R573), Fraxiparine (9NDE104), Sandoparin (80005), Fluxum (B72315), Fragmin A (84920-51) and B (84921-51), Logiparin (9005A) and the Japanese Pharmacopoeia Standard for LMMH (JRS-LMMH) (lot JP911) were purchased from Pharmuka Laboratories (Gennevilliers, France), Sanofi Chimie (N.D.-de-Bondeville, France), Sandoz-Wander Pharma (Berne, Switzerland), Opocrin (Corlo, Italy), Kabi-Vitrum (Stockholm, Sweden), Novo-Nordisk Pharma (Gentofte, Denmark) and the National Institute of Hygienic Sciences (Tokyo, Japan), respectively. Fractionated heparins, N-17 (lot KBJ6056-1), N-10 (KBJ6104-1), N-6 (KBJ6076-4), N-3 (KBJ890315) were kindly provided by Novo-Nordisk Pharma, K-6 (DxN89) and K-4 (DxN49) were gifts from Kabi-Vitrum and S-5 (97F0454) and S-3 (97F0455) were obtained from Sigma (St. Louis, MO, USA). An LMMH molecular mass calibrant (Code 90/686) was supplied by the National Institute for Biological Standards and Control (NIBSC), (Potter's Bar, Hertfordshire, UK). All heparins were obtained in powdered form except for Sandoparin, which was provided as a physiological salt solution.

Pullulan P-400 (average molecular mass 380 000 and dispersivity 1.12) was obtained from Showa Denko (Tokyo, Japan). Water for injection (Ohtsuka Pharmaceuticals, Tokushima, Japan) was used for the preparation of eluent and sample solutions. All reagents were of analytical-reagent grade.

Chromatographic conditions

The eluent used was 0.2 M aqueous sodium sulphate and 50 mM Tris–HCl (pH 7.0), filtered using a 0.2-μm pore-size filter (Japan Millipore, Tokyo, Japan) and degassed before use. Elution was performed at a flow-rate of 0.6 ml/min.

HPGPC analysis. A system consisting of an on-line degasser (Model SD-8012, Tosoh, Tokyo, Japan), a pump (Tosoh Model CCPD), an injector (Rheodyne Model 7125) equipped with a 100-μl loop, a guard column for gel permeation (Tosoh TSK guard column SWxl, 40 mm × 6.0 mm I.D.), gel permeation columns (Tosoh TSK gel G2000 SWxl and G3000 SWxl, both 300 mm × 7.8 mm I.D.) and a precision differential refractometer (Tosoh Model RI-8011; light source, photodiode with an effective wavelength of 660 nm) kept at 35°C were used. The columns were maintained at 30°C using a column oven (Tosoh CO-8010). For the calibration of the columns, especially using the NIBSC LMMH molecular mass calibrant as a standard, the absorbance at 235 nm was also monitored at the same time as the differential refractive index (RI) at room temperature (about 20°C) with a Model SPD-6A UV spectrophotometer (Shimadzu, Kyoto, Japan). The detector outputs were fed into a computer (PC-9801DA; NEC, Tokyo, Japan) and used to calculate the molecular mass profiles of the various LMMHs using the program Chromato Data Processor Ver. 2.03, supplied by Tosoh, in a gel permeation chromatographic mode.

The concentration of each sample was *ca.* 10 mg/ml, except for Sandoparin (70 mg/ml). Heparins are usually hygroscopic. In this study, therefore, their water contents were determined by thermogravimetric analysis under the assumption that the decrease in sample mass can be ascribed to evaporation of water. The concentrations of heparins were then corrected for the

water content. The sample injection volume was 50 μl, except for Sandoparin (20 μl).

HPGPC–LALLS analysis. In the measurement of LALLS, a guard column for gel permeation (Tosoh TSK guard column PWxl, 40 mm × 6.0 mm I.D.) and a gel permeation column (Tosoh TSK gel G3000 PWxl, 300 mm × 7.8 mm I.D.), were used instead of SW-type columns. Although the latter have a higher resolution they release small particles during experiments, which render them unreliable for use with subtle LALLS detection systems (too much background noise on the LALLS recorder signal in spite of careful experiments), as pointed out by Hennink *et al.* [20]. A LALLS photometer (Tosoh Model LS-8000), whose introduction was described in detail in a recent review [24], and a differential refractometer were used as detectors. The light source of the LALLS photometer is a 5-mW helium–neon laser with a wavelength of 633 nm. A circular light slit with an aperture wider ($\theta_{col} = 2°$) than the conventional one ($\theta_{col} = 1°$) is an option which was obtained from the manufacturer (Tosoh). The columns were kept at 40°C. An ultrafilter with pore size 0.45 μm (Type FP-045; Sumitomo Electric, Tokyo, Japan) was connected between the columns and the LALLS photometer. Other components were the same as for HPGPC analysis. The sample concentrations and injection volumes were determined as the maximum values, at which RI detector saturation did not occur, being *ca.* 10 mg/ml and 80 μl, respectively, except for Sandoparin case (35 mg/ml and 30 μl). The molecular mass profiles of LMMHs were calculated using the analysis program in the LALLS mode.

RESULTS AND DISCUSSION

HPGPC measurements

Calibration of columns using an NIBSC LMMH molecular mass calibrant. The method adopted in this study is based on that of Van Dedem and Nielsen [28]. The principle is as follows: the molecular mass calibrant is a sample of bovine mucosal heparin partially degraded by *endo*-lyse heparinase. Cleavage with this enzyme leaves an unsaturated uronic acid residue at the

non-reducing end of the resulting fragment, which demonstrates UV absorption with a λ_{max} at 235 nm. The action of heparinase is assumed to be random with regard to the substrate size and position of the bonds broken. The UV absorption at 235 nm may, therefore, be assumed to give a relative measure of molar concentration over the whole molecular mass range of the material. It is further assumed that the RI is a relative measure of mass concentration and that, accordingly, the RI/UV ratio is a relative measure of molecular mass. The resolved peaks of the RI and UV outputs on the chromatogram correspond to the low-molecular mass-end and the peak positions (retention times) are assigned to degrees of polymerization. At the top of each peak, the RI/UV ratios correspond to an integral multiple of molecular masses for a disaccharide unit approximated as 600. The resulting data for retention times and molecular masses at the resolved peaks were used to derive a calibration for the chromatographic system by fitting the equation $\log M = at^3 + bt^2 + ct + d$, where t is the retention time, and a, b, c and d are parameters calculated in the fitting process. The values for the parameters were $a = -2.952 \cdot 10^{-4}$, $b = 3.040 \cdot 10^{-2}$, $c = -1.092$ and $d = 17.63$.

Measurements of the molecular mass profiles of heparins. After calibration of the system, RI detection alone was used for LMMHs of unknown molecular mass distributions. One of the molecular mass parameters, the mass-average molecular mass (M_w), was calculated from these data.

Table I gives the evaluated M_w values of various LMMHs together with a variety of fractionated heparins. Wide variations were observed in the M_w values. These findings are compatible with the results of Fareed et al. [8].

HPGPC–LALLS measurements

Data handling. The outputs of the LALLS and RI detectors on chromatograms are related to the molecular mass (M_t) of the sample at the retention time t according to the following equa-

TABLE I

MASS-AVERAGE MOLECULAR MASSES (M_w) OF VARIOUS LMMHS AND FRACTIONATED HEPARINS

LMMH	M_w		
	HPGPC	HPGPC–LALLS	
		Wide angle	Narrow angle
Fraxiparine	4519 ± 31[a]	–[b]	–[b]
Enoxaparin	4206 ± 35	4230 ± 57	5035 ± 85
Logiparin	6742 ± 30	6713 ± 45	7301 ± 43
Fragmin A	5946 ± 19	5779 ± 56	6050 ± 54
Fragmin B	6071 ± 22	5910 ± 48	6042 ± 63
Fluxum	5819 ± 29	6077 ± 50	6395 ± 72
Sandoparin	5087 ± 40	5094 ± 42	5652 ± 70
LMMH-JRS	6216 ± 27	5964 ± 51	–[b]
N-6	8291 ± 20	8272 ± 38	7806 ± 55
N-3	3682 ± 10	3637 ± 71	3986 ± 109
K-6	6983 ± 25	–[b]	6246 ± 51
K-4	5067 ± 25	5030 ± 45	–[b]
S-5	6136 ± 28	6023 ± 46	–[b]
S-3	3042 ± 9	2932 ± 85	–[b]

[a] Mean ± S.D. (values for data from three determinations).
[b] Not measured.

tion, which is derived from the classical Rayleigh equation [29]:

$$\frac{(LS)_t}{(RI)_t} = k_1(dn/dc)_t M_t \qquad (1)$$

where $(LS)_t$ and $(RI)_t$ are the LALLS and RI detector outputs, respectively, k_1 is a constant and $(dn/dc)_t$ is the specific refractive index increment at the retention time t. This equation is valid when both the heparin concentration and light-scattering angle (θ_{obs}; see Fig. 1) are small enough to allow the concentration-dependent terms in the Rayleigh light-scattering equation to be ignored [29]. Using the present system, these requirements were satisfied in most instances.

If k_1 and $(dn/dc)_t$ are determined using a standard substance, for which the M_w is already known, the molecular mass distribution can be calculated from the LALLS to RI output ratio on the chromatogram. However, even if heparin is adopted as a standard of known molecular mass, the correctness of the following two assumptions should be ascertained: (1) the $(dn/dc)_t$ of the samples is equal to that of the standard, and (2) $(dn/dc)_t$ is independent of the molecular mass and is constant in the molecular mass range studied.

The specific refractive increment, (dn/dc), can be assumed to be proportional to RI_{area}/C, where RI_{area} and C are the area on the RI chromatogram and the molar concentration, respectively. Fig. 2 depicts the dependence of RI_{area}/C values for the various fractionated heparins on the molecular masses. Open circles show the RI_{area}/C values for a variety of fractionated heparins prepared by Kabi-Vitrum, these being almost constant and independent of molecular mass. Such a small molecular mass effect is uncommon for the molecular mass range studied [30]. For small molecules of molecular mass below 20 000, usually the dn/dc slightly decreases with increase in molecular mass [30]. On the other hand, it is almost constant and independent of the molecular mass for large molecules with molecular mass above about 20 000 [30]. This behaviour was found with polystyrenes and also with pullulans and has been ascribed to changes in specific volume
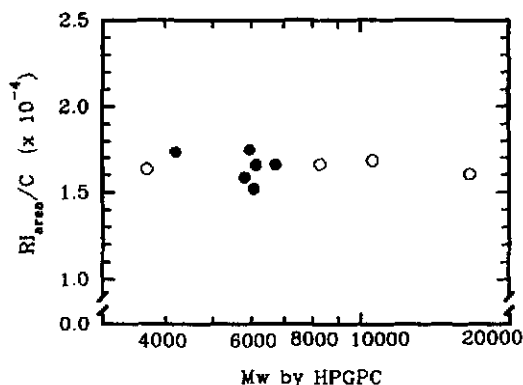

Fig. 2. Dependence of the ratio of area on the RI chromatogram to molar concentration (RI_{area}/C) of various fractionated heparins on the molecular mass. $\bigcirc = RI_{area}/C$ values for a variety of fractionated heparins prepared by Kabi-Vitrum; $\bullet = RI_{area}/C$ values for various LMMHs. Standard deviations evaluated from at least three determinations are within the size of the circles.

caused by the end-groups of molecules [30]. The fractionated heparins in this study were all prepared by the same depolymerization and enrichment procedures but although they seem to have the same end-groups, the behaviour of dn/dc differed. The results indicate that $(dn/dc)_t$ is almost independent of the molecular mass of LMMHs and may be assumed to be constant in the molecular mass range studied.

As represented by the filled circles in Fig. 2, the RI_{area}/C values varied from heparin to heparin, being sensitive to the depolymerization process and/or organ of origin. Therefore, differences in dn/dc between standards and samples should be take into account even if heparin is used as the standard.

As dn/dc is proportional to RI_{area}/C, eqn. 1 can be accordingly modified to give the following equation:

$$\frac{(LS)_t}{(RI)_t} = k_2 \cdot \frac{RI_{areaSTD}/C_{STD}}{RI_{areaSMP}/C_{SMP}} \cdot M_t \qquad (2)$$

where $RI_{areaSTD}$ and C_{STD} are the RI_{area} and C of standard heparin and $RI_{areaSMP}$ and C_{SMP} are those of sample heparins, respectively, and k_2 is a constant dependent on the apparatus. In the present study, N-6 ($M_w = 8291$, determined by HPGPC) was used as a standard for the determination of k_2.

Measurements at wide and narrow collection angles. With our instrument, measurements by the LALLS method were usually carried out at the narrow collection angle ($\theta_{col} = 1°$, $\theta_{obs} = 4.5$–$5.5°$). For large molecules with molecular mass above 10 000, the signal-to-noise ratio is high enough to evaluate the molecular mass distributions at this regular angle because of the strong light scattering by the molecules. In most instances with small molecules, however, the light-scattering intensity is very weak and it is difficult to calculate the molecular masses. In this study, this problem was overcome by making the collection angle wider. Fig. 3 shows the elution profiles with RI and LALLS at both wide ($\theta_{col} = 2°$, $\theta_{obs} = 5.0$–$7.0°$) and narrow collection angles. The LALLS intensity became about twice as strong when the angle was widened and the signal-to-noise ratio was improved.

The LALLS signal-to-noise ratio is not only a factor of the collection angle for a fixed scatter angle but also, and most importantly, of the concentrations of samples injected. Higher concentrations at a narrow collection angle could have resulted in molecular masses as accurate and precise as those reported for lower concentrations injected with a wider collection angle. In our case, however, saturation of the RI detector

occurred using higher concentrations because of the very low molecular masses of LMMHs and fractionated heparins. Therefore, with such small molecules the use of a wide collection angle appears to be the most useful approach to improving the signal-to-noise ratio. This method is also effective in the case of measurements where the amount of sample available is limited.

The lower part of Fig. 4 illustrates the relationships between the k_2 values of various LMMHs and fractionated heparins and their molecular mass averages. The k_2 values are theoretically constant and independent of the molecular mass. The scatter of k_2 in Fig. 4 accordingly corresponds to the error of measurement. In order to clarify this scatter, the ratios of k_2 to average values are depicted in the upper part of Fig. 4. Observation at the wide collection angle was associated with a smaller scatter and an increment in the signal-to-noise ratio. This suggests that observation at the wide angle results in an increase in the accuracy of measurement.

Table I gives the molecular masses of various LMMHs and fractionated heparins measured by HPGPC–LALLS at wide or narrow collection angles. At the wide angle, the molecular mass values obtained by the HPGPC–LALLS method were near to those obtained by the HPGPC
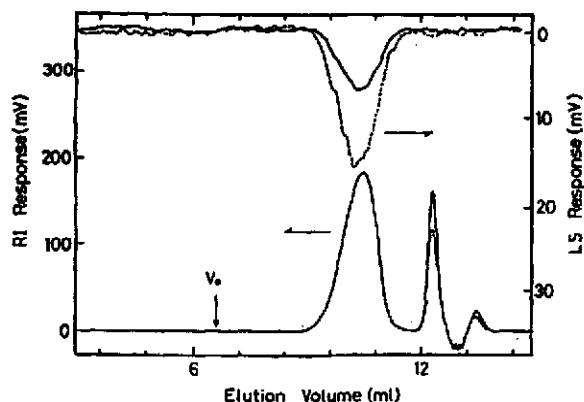

Fig. 3. Elution profiles of Fragmin B observed by RI and LALLS detectors with wide- and narrow-angle measurements. Solid line, with the narrow collection angle ($\theta_{col} = 1°$, $\theta_{obs} = 4.5$–$5.5°$); dotted line, with the wide collection angle ($\theta_{col} = 2°$, $\theta_{obs} = 5.0$–$7.0°$). The sample size of Fragmin B was 70 μl of a 8.5 mg/ml solution. V_0 indicates the void volume of the column, detected by the RI detector using pullulan P-400 as a marker.
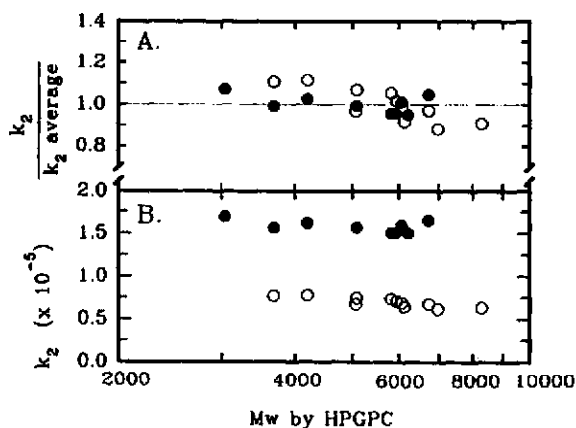

Fig. 4. The k_2 values of various LMMHs and fractionated heparins plotted against their mass-average molecular masses with wide- and narrow-angle measurements. (A) ratios of k_2 to average values; (B) k_2 values. ● = With the wide collection angle ($\theta_{col} = 2°$, $\theta_{obs} = 5.0$–$7.0°$); ○ = with the narrow collection angle ($\theta_{col} = 1°$, $\theta_{obs} = 4.5$–$5.5°$).

method, whereas the conformity was not so good with the narrow angle.

Methodological comparison of HPGPC–LALLS with HPGPC. Several methods have commonly been used to assess the molecular masses of heparins [5,8,15–19]. The HPGPC mode in HPLC with RI detection has been developed, and heparins with poorly defined molecular masses and wide molecular mass ranges have been used for calibration. It has already been shown that the application of HPGPC using other polymers such as dextrans [17], polyethylene glycols or pullulans [31] for the calibration of columns leads to wrong results because of the high negative charge of heparins. HPGPC is not the ideal way to determine accurate molecular masses, even though defined standards are available, and usually the HPGPC calibration graph, as shown in this study, is not linear. Therefore, a reliable method for the determination of molecular mass distributions of standard heparins is still required.

It has been demonstrated that HPGPC–LALLS provides molecular mass distributions of standard heparin samples in a rapid and reliable manner [20]. Light-scattering measurement has for some time been an important method for determining the molecular masses, molecular sizes and second virial coefficients of a variety of particles. Nevertheless, it has hitherto not become popular owing to the long time required for measurement in some instances and the poor precision achieved with a low angle. Owing to the development of laser light sources and improvements in LALLS detectors, the measurement can now be carried out easily. The extra cost of the relatively expensive LALLS detector may be warranted by the merits of the HPGPC–LALLS method: (1) a calibration graph is not necessary; and (2) only a standard for the determination of k_2 is needed, and its molecular mass need not necessarily be in the molecular mass range of the samples being studied. Also, it does not always have to be the same kind of compound.

In conclusion, although HPGPC–LALLS has distinct advantages, it has only rarely been utilized for the determination of the molecular masses of small molecules because of their weak light-scattering intensity. However, this study suggests that this difficulty can be overcome by measurement of light scattering at a wider collection angle. This should lead to the increased application of HPGPC–LALLS to very small molecules with M_w values in the range 1000–10 000.

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