Gradient Polyacrylamide Gel Electrophoresis for Determination of Molecular Weights of Heparin Preparations and Low-Molecular-Weight Heparin Derivatives

R. E. EDENS*, A. AL-HAKIM*, J. M. WEILER‡, D. G. RETHWISCH¶, J. FAREED§, AND R. J. LINHARDT*x

Received March 12, 1991, from the *Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52242, the ‡Department of Internal Medicine, College of Medicine, University of Iowa, and Iowa City Veterans Administration Medical Center, Iowa City, IA 52242, the ¶Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, IA 52242, and the §Departments of Pathology and Pharmacology, Loyola University Medical Center, Maywood, IL, 60153. Accepted for publication September 26, 1991.

Abstract □ The M_r values of pharmaceutical heparins and low-molecular-weight (LMW) heparin derivatives were examined as part of a collaborative study to develop methods for their characterization. Standard methods of M_r determination rely on gel permeation high-performance liquid chromatography (HPLC). We report the use of gradient polyacrylamide gel electrophoresis (PAGE) to determine the M_r values of pharmaceutical heparins and LMW heparin derivatives. This approach offers certain advantages over the HPLC method. Gradient PAGE analysis was performed in parallel, on multiple samples, with the same standard curve. HPLC was performed serially. Gradient PAGE gave higher resolution than HPLC, and thus, a mixture of easily obtained standards was used in place of individual standards for the construction of a standard curve. Heparin and various LMW heparin samples were analyzed by both gradient PAGE and conventional gel permeation HPLC methods. The number-average M_r, weight-average M_r, and polydispersity were examined by both techniques and found to be similar. This study demonstrates that gradient PAGE analysis is a sensitive method for the determination of the M_r values of heparin and LMW heparin.

Heparin is a polydisperse [M_r, 5000–40 000; average M_r, 14 000], highly sulfated, linear copolymer of 1→4 linked glucosamine and hexuronic acid and has been used clinically over the last half-century as an anticoagulant.[1] The undesirable side effects of heparin include hemorrhagic complications[2]; thus, whereas heparin is in widespread clinical use, it has been cited as the drug most responsible for death in otherwise healthy patients.[3] Low-molecular-weight (LMW) heparins (average M_r, 3000–8000[4]) have recently been introduced as heparin substitutes and are claimed to have reduced side effects (a higher safety:efficacy ratio), more predictable pharmacological action, sustained antithrombotic activity, and better bioavailability.[5] These potential advantages have led to the development of several commercial LMW heparin preparations.

LMW heparin is prepared, in low yield, by size fractionation of heparin, whereas LMW heparin derivatives are prepared in higher yield by the partial, controlled depolymerization of heparin. An LMW heparin standard, prepared by partial nitrous acid depolymerization,[6] has recently been introduced by the National Institute of Biological Standards and Control (NIBSC; London, England) under the auspices of the World Health Organization (WHO; Geneva, Switzerland).[7] A physiochemical characterization of this WHO standard has recently been published.[8]

The M_r values of LMW heparin derivatives affect their biological activity and bioavailability.[9] The M_r value of heparin has been estimated by using several physical methods. Low-pressure gel permeation chromatography relies on soft gels such as Sephadex G-100[10] and Ultrogel AcA44.[11] Although these methods still have importance for the preparation of heparin fractions, they have largely been replaced by the faster and more reproducible method of gel permeation high-performance liquid chromatography (HPLC). The M_r value of heparin has been estimated by viscometric studies from the Mark–Houwink relationship.[12,13] Ultracentrifugation, in conjunction with the Svedberg equation, also gives the M_r value of heparin,[12,14] but its dependence on the partial specific volume of heparin (a term that is difficult to measure) can lead to substantial error in the calculated M_r.[15] Light-scattering methods,[14] including quasielastic light scattering[16] and low-angle X-ray scattering,[17] have also been used with some success in the determination of the M_r value of heparin. The relatively small size of heparin complicates the use of light scattering, and the method is even more difficult to apply to the study of LMW heparins. However, light scattering can be used to measure the particle size range of pharmaceutical heparin and LMW heparin derivatives.[18]

The number-average M_r (M_N), weight-average M_r (M_W), and polydispersity (P) of heparin and LMW heparin are typically obtained by gel permeation HPLC.[19] Because of the microheterogeneity of heparin, its P value refers both to variation in degree of polymerization (dp) and variations in its chemical structure (i.e., degree of sulfation). As part of a collaborative study of the M_r distributions of heparin, participants from the pharmaceutical industry and academia have examined various HPLC-based methods. This paper describes the analysis of the M_r distribution of heparin and LMW heparin derivatives by gradient polyacrylamide gel electrophoresis (PAGE).[4,20] Previously, PAGE had been successfully applied to determine the M_r values of other related glycosaminoglycans, including chondroitin sulfates, dermatan sulfates, and hyaluronic acid.[21–23] The application of PAGE to LMW heparin derivatives has recently been proposed.[24] This paper reports the determination of the M_N, M_W, and P values of various heparins and LMW heparin derivatives by gradient PAGE. The values obtained were compared with those obtained by conventional gel permeation HPLC.

Experimental Section

Materials—Six heparin and LMW heparin samples were obtained as part of a collaborative study from Dr. G. van Dedem, Diosynth BV, The Netherlands: United States Pharmacopeia (USP) heparin standard K (1), European Pharmacopeia heparin standard (2), Novo F913B (9), Novo F913C (10), LMW heparin M 85/571 (11), and LMW heparin standard 85/600 (12). (Samples 9–12 are available from NIBSC.) Six heparin and LMW heparin samples were a gift from Dr. E. Coyne, Loyola University Medical Center, Maywood, IL: internal standard (15 MP-16VC) (3), Abbott fraction 17300 (4), Abbott fraction

0022-3549/92/0800-0823$02.50/0

12700 (5), Abbott fraction 6200 (6), S. Lasker (prepared by Dr. Lasker, New York Medical College, Valhalla, NY) LMW heparin 5317 (7), and S. Lasker LMW heparin 4300 (8). Bovine lung heparin (145.7 USP units/mg; sodium salt) was obtained from Sigma Chemical Company (St. Louis, MO). Heparin lyase (heparinase, EC 4.2.2.7) was either purified from *Flavobacterium heparinum* [5 units (μmol/min)/mg][25] or obtained from Sigma. Acrylamide (ultrapure), tris(hydroxymethyl)amino methane (Tris), alcian blue dye, bromophenol blue dye, and ammonium persulfate were obtained from Boehringer Mannheim (Indianapolis, IN). Glycine hydrochloride, disodium ethylenediaminetetraacetic acid, azure A dye, boric acid, sucrose, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were from Fisher Chemical Company (Fair Lawn, NJ). All other chemicals were reagent grade. Desalting was performed with a Spectropore dialysis tubing (M_r cutoff, 1000) from Spectrum Medical (Los Angeles, CA) or with a P-2 gel desalting column from Bio-Rad (Richmond, CA).

Instrumentation—Electrophoresis was performed on a Hoefer (San Francisco, CA) SE600 vertical-slab-gel unit equipped with a Bio-Rad model 1420B power source. The Ultro Scan XL laser densitometer for gel scanning was from LKB Produkter (Bromma, Sweden), and the Jandel Scientific digitizing tablet (model 2210.0.30 TL.F) was from Numonics Corporation (Montgomeryville, PA). The densitometer was connected to an IBM-compatible personal computer, and Sigma-Scan software (version 3.90; Jandel Scientific, Corte Madera, CA) was used. Gel permeation HPLC was performed with a TSK 2000 column (30 × 0.75 cm) with a M_r fractionation range of 500–20 000 determined with dextrans. A Waters 840 liquid-chromatographic system (Waters, Milford, MA) equipped with a model 490 multiwavelength UV detector and a Digital 300 series minicomputer was used. Waters 840 software specifically designed for applications in the determination of M_r values for polymers was used.

Determination of M_r Values of Heparin and LMW Heparin Derivatives by Gel Permeation HPLC—Prior to use, each gel permeation column was calibrated with a collection of heparins and LMW heparin derivatives as M_r standards.[26] These standards were prepared by Sanofi (Paris) from porcine mucosal heparin by partial nitrous acid depolymerization. The peak M_r values determined by ultracentrifugation were 2, 2.5, 3.1, 3.6, 4.3, 5, 5.5, 5.9, 6.4, 6.8, 7.9, 8.8, and 10 kilodaltons. The LMW derivative (20 μL, 10 mg/mL) to be determined was then injected into the column and eluted with 0.5 M sodium sulfate at a flow rate of 1 mL/min. The eluant was monitored by absorbance at 205 nm or by refractometry. Values for retention time, P, and M_r were determined and used with the known M_r values of the standards to prepare a calibration curve by third-order polynomial regression on a computerized Waters gel permeation chromatographic system (GPC 840). The computer also divided the elution curve into 100 equal time slices to yield 100 M_r and retention time values, which were used to determine P.

Preparation of Polyacrylamide Gels—The resolving gel buffer and lower chamber buffer contained 0.1 M boric acid, 0.1 M Tris, and 0.01 M disodium ethylenediaminetetraacetic acid at pH 8.3 and were prepared as previously described.[27] Gradient polyacrylamide resolving gel was prepared from two resolving gel buffer solutions, one containing 11.52% (w/v) acrylamide and the other containing 20% (w/v) acrylamide, with 0.48 and 2% (w/v) *N,N'*-methylenebisacrylamide and 1 and 15% (w/v) sucrose, respectively. Gels were poured vertically between glass plates (16 × 32 cm) separated by 1.5-mm spacers.[27] Gradients were poured by adding 12% solution into the reservoir and 22% solution into the mixing chamber of the linear gradient maker. Ammonium persulfate and TEMED were added to both the reservoir and the mixing chamber. Polyacrylamide solution from the mixing chamber, passing by gravity into two channels leading to the top of the glass plates, formed a linear gradient from the bottom to the top. The unpolymerized gel was overlaid with water. After polymerization, the water layer was removed. A solution of stacking gel made of 4.75% (w/v) acrylamide and 0.25% (w/v) *N,N'*-methylenebisacrylamide in stacking gel buffer (identical to the resolving gel buffer but adjusted to pH 6.3 with hydrochloric acid), containing TEMED and ammonium persulfate, was added to the top of the resolving gel. A comb (well formers) was inserted and removed after polymerization. Each well was rinsed with stacking buffer and then with the upper chamber buffer composed of 0.2 M Tris and 1.25 M glycine at pH 8.3.

Electrophoresis of Oligosaccharide Samples—Samples (20 μg/mL in 40 μL of distilled water) were combined with an equal volume of 50% (w/v) sucrose in distilled water containing trace quantities of bromophenol blue (10 μg/mL) and loaded into the bottom of each well with a microsyringe. Electrophoresis was performed for 16 h at 400 V while cooling with circulating tap water (10–15 °C). The gel was removed from the glass plates and stained with alcian blue (0.5%, w/v) in 2% (v/v) aqueous acetic acid for 30 min. Destaining was achieved with several washings with 200-mL volumes of 5% (v/v) aqueous acetic acid.

Determination of M_r Values of Heparin Preparations and LMW Heparin Derivatives by Gradient PAGE—M_r and P values of intact LMW heparin derivatives and heparin samples were also determined by gradient PAGE.[20,27] The M_r calibration curve was constructed with an oligosaccharide ladder of bands (dp, 2–20) of partially (80% completion) depolymerized bovine heparin.[4,20] These bands were identified by their comigration with authentic standards.[20]

Photography and Scanning of Gels—Black and white negatives were obtained for each gel, with care taken not to overexpose the film. The negatives were printed as glossy photographs (Figure 1). Full-size films (Translite, Kodak) of the negatives were prepared for densitometry scanning. These films were scanned along each lane at a rate of 2.5 cm/min, and the absorbance was plotted with a Hewlett-Packard Thinkjet printer. The scans produced by this method were digitized with a digitizing tablet driven by Sigma-Scan software. The x value obtained corresponded to distance (cm) from the origin, and the y value corresponded to the absorbance of light by Translite film. Between 400 and 1100 x,y pairs (of approximately equal spacing of x points) were assembled as an ASCII file, imported into Lotus 123, and processed to obtain values of M_r and P.

Preparation of a Standard Curve and Calculation of M_r Values—The M_r values of the peaks obtained on scanning the track corresponding to the heparinase-depolymerized bovine heparin ladder (lanes a in Figure 1) were assigned on the basis of comigration with oligosaccharide standards (dp, 2–20; see *Results and Discussion*) and, tentatively, by pattern recognition (dp, 22–46). The logarithms of the M_r values were plotted as a function of migration distance to prepare two standard curves (Figure 2). Only the linear portions of these curves were used to obtain eqs 1 and 2 for gels A and B, respectively:

$$\ln M_i = -0.006954x + 4.759 \quad (1)$$

$$\ln M_i = -0.006784x + 4.588 \quad (2)$$

The M_r values of heparin and LMW heparin derivatives were obtained with these standard curves. First, a collection of x,y pairs corresponding to a scanned gel track was selected. Each x point (i.e., x_i) was converted from distance (cm) to M_r (M_i) by using the standard curve obtained on the same gel. Each y point was used directly as

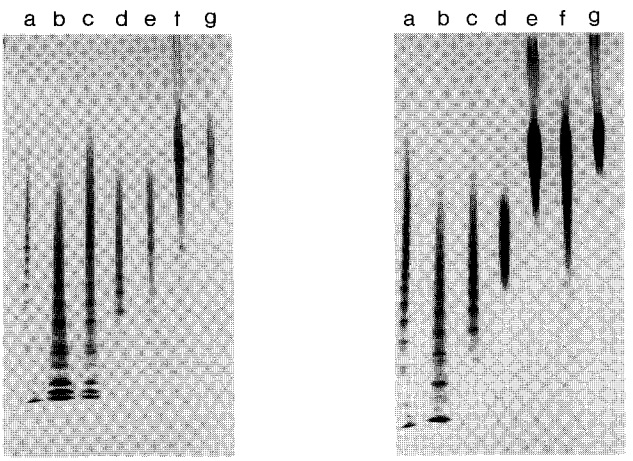

Figure 1—Gradient polyacrylamide gels stained with alcian blue to analyze pharmaceutical heparins and LMW heparin derivatives. (**Left**) Gel A: analysis of (a) molecular weight standards and six samples (b) **8**, (c) **7**, (d) **6**, (e) **5**, (f) **3**, and (g) **2**. (**Right**) Gel B: analysis of (a) molecular weight standards and six samples (b) **10**, (c) **9**, (d) **11**, (e) **12**, (f) **1**, and (g) **4**.

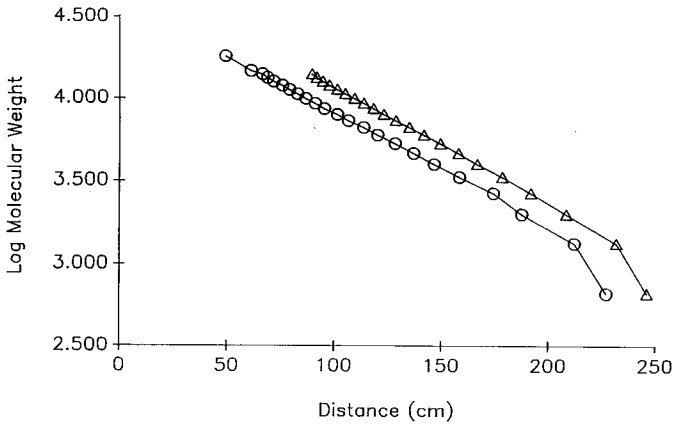

Figure 2—Standard curve showing the logarithm of oligosaccharide M_r as a function of migration distance (cm). These curves were obtained by scanning lane a of gel A (△; Figure 3A, panel a) and by scanning lane a of gel B (○; Figure 3B, panel a).

absorbance (i.e., A_i). The absorbance at any position on the gel is directly proportional to the mass of the sample at that point:

$$A_i = KW_i \tag{3}$$

In eq 3, W_i (equal to N_iM_i) is the mass of sample at point i, K is the proportionality constant, and N_i is the number of molecules at point i:

$$N_iM_i = W_i = A_i/K \tag{4}$$

By definition:

$$M_N = \sum N_iM_i/\sum N_i \tag{5}$$

$$M_W = \sum N_iM_i^2/\sum N_iM_i \tag{6}$$

Substitution of eq 4 into eqs 5 and 6 gives:

$$M_N = \sum(A_i/K)/\sum(A_i/KM_i) = \sum A_i/\sum(A_i/M_i) \tag{7}$$

$$M_W = \sum[(A_i/K)M_i]/\sum(A_i/K) = \sum(A_iM_i)/\sum A_i \tag{8}$$

$$P = M_W/M_N \tag{9}$$

Equations 7–9 were used to analyze the PAGE results.

Results and Discussion

Heparin is a sulfated linear copolymer of 1→4 glycosidically linked glucosamine and hexuronic acid. Disaccharide [→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→)] is its major repeating unit (IdoAp, iduronic acid; GlcNp, glucosamine; S, sulfate), comprising ~71 mol % of the polymer.[1] Heparin is a polydisperse collection of polysaccharide chains, and thus, it is generally described with a peak or average M_r. Although LMW heparin fractions can be prepared by fractionation of heparin, fractionation is not a commercially viable process because the LMW components (M_r, 5000–8000) are <15 wt % of the total material.[19] Instead, LMW heparin derivatives are prepared by partial chemical or enzymatic depolymerization of heparin.[4] The resulting LMW heparin derivatives have average M_r values of approximately one-third that of the parent drug.

A collection of heparins, fractionated heparins, and LMW heparin derivatives were assembled for this study (Table I). Three heparins in this study are the current USP and European standards (**1** and **2**) and the original international standard (**3**). The average M_r values of the heparin standards had been determined previously by various techniques (Table I). The fractionated heparins **4**, **5**, and **6** were prepared from porcine mucosal heparin by Abbott in the 1960s (by column fractionation) and analyzed by Barlow[28] by ultracentrifugation. Of the LMW heparin derivatives, **7**, **8**, and **11** were prepared by partial nitrous acid depolymerization; **9** and **10** were prepared by heparinase-catalyzed partial depolymerization; and **12** was prepared by fractionation of porcine mucosal heparin (Table I).

Our laboratory uses heparinase in the partial, controlled depolymerization of bovine lung heparin to prepare a set of oligosaccharide standards of known structure and molecular weight. These oligosaccharides have the structure ΔUAp2S(1→[4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→]$_n$4)-α-D-GlcNp2S6S [where n = 0 (disaccharide), 1 (tetrasaccharide), 2 (hexasaccharide), etc., and ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid]. Oligosaccharides having this structure and n = 0–9 (disaccharide to eicosasaccharide) have been prepared and purified by strong-anion-exchange–HPLC[20] and preparative gradient PAGE.[27] The structures of the standards from disaccharide through octasaccharide were established by proton nuclear magnetic resonance spectroscopy and fast-atom-bombardment mass spectral analysis.[27,29,30] The structures of the large oligosaccharides (n = 4–9) were determined by treating the purified oligosaccharide exhaustively with heparin lyase. Treatment afforded only a single disaccharide product, and the number of equivalents formed from each equivalent of oligosaccharide confirmed their size.[31,32] The identity of each major band, from disaccharide through eicosasaccharide (M_r, 665–6650) in the ladder formed on gradient PAGE analysis (lanes a in Figure 1) was established by comigration with the appropriate standard. By using this pattern, the remaining major bands in the gradient PAGE ladder were tentatively identified. The dp ranged from 22 to 44 (n = 10–21), corresponding to M_r values of 7315–14 630.

The M_N, M_W, and P values of each sample were determined by gradient PAGE analysis and gel permeation HPLC (Table II). Both analytical methods gave similar results. The slightly higher M_N and M_W values obtained by PAGE might have resulted from the reduced staining (by cationic dye) of smaller oligosaccharides, particularly those having a low level of sulfation. The small differences observed in M_N and M_W values obtained by gel permeation HPLC with UV and refractometric detection (Table II) may be related to the presence of UV-absorbing contaminants or structural differences in the end groups found in LMW heparin derivatives prepared with different methods.

Table I—Average M_r Values of Heparin Preparations and LMW Heparin Derivatives

Sample	M_r
1	16 000[a]
2	15 000,[a] 16 000[b]
3	15 000,[a] 16 000[b]
4	17 300[b]
5	12 700[b]
6	6200[b]
7	5317[b]
8	4300[b]
9	3600 (M_N), 5900 (M_W),[a] 4100 (M_P)[c]
10	2500 (M_N), 3700 (M_W),[a] 2600 (M_P)[c]

[a] Determined by gel permeation HPLC. [b] Determined by ultracentrifugation. [c] Peak M_r.

Table II—M_N, M_W, and P Values of Heparin Preparations and LMW Heparin Derivatives

Sample	Values Determined by Gradient PAGE			Values Determined by Gel Permeation HPLC		
	M_N	M_W	P	M_N	M_W	P
1	11 900	15 313	1.29	—[a]	—	—
2	11 183	15 788	1.41	—	—	—
3	13 509	16 905	1.25	—	—	—
4	18 013	22 999	1.28	—	—	—
5	16 786	20 638	1.23	—	—	—
6	6265	7985	1.27	—	—	—
7	5044	5945	1.18	—	—	—
8	2136	3363	1.58	—	—	—
9	3608	6501	1.80	3446[b]	4816[b]	1.40[b]
10	2335	3621	1.55	2481[b]	3372[b]	1.36[b]
11	4724	6475	1.37	5995[c]	6609[c]	1.10[c]
				5061[b]	6203[b]	1.23[b]
12	5837	8420	1.44	5540[c]	6377[c]	1.15[c]
				5016[b]	6868[b]	1.37[b]

[a] —, Not determined. [b] Detection by UV absorbance at 205 nm. [c] Detection by refractometry.

Mathews and Decker[21] first used PAGE to examine chondroitin sulfates with various M_r values that had been determined by viscometry. PAGE analysis gave a good correlation between the reported M_r values and the migration distance. The PAGE separation of sulfated oligosaccharides is based on molecular size and is very similar to sodium dodecyl sulfate–PAGE used to separate proteins. Min and Cowman[22] also used PAGE to determine the M_r values of chondroitin sulfates and introduced the use of a partially depolymerized hyaluronic acid to serve as a "counting ladder". This ladder represented a set of internal standards from which the M_r values of unknown samples were calculated. These authors warned about the possibility of misidentification of bands and recommended the use of purified oligosaccharide standards to calibrate gel permeation HPLC chromatograms. Attempts by Hittner and Cowman[23] to fit data (obtained with PAGE) for the polymers chondroitin 4-sulfate, dermatan sulfate, and hyaluronic acid to the same calibration curve were unsuccessful. Their results suggest that, although chondroitin 4-sulfate and dermatan sulfate had similar hydrodynamic radii, hyaluronic acid was somewhat smaller. For this reason, we constructed our standard curve for heparin and LMW heparin with oligosaccharides derived from the same polymer, heparin.

For these samples, the M_r values we obtained by gel permeation HPLC and gradient PAGE (Table II) were also similar to reported M_r values obtained by both gel permeation HPLC and ultracentrifugation methods (Table I). The major strength of gradient PAGE is that it permits rapid, parallel analysis of multiple samples by virtually any laboratory. Although the banding ladders constructed with partially depolymerized chondroitin sulfates,[20] dermatan sulfates,[33] and hyaluronic acid[22] result in sharp, regularly spaced bands, the assignment of M_r values to each band is often complicated by the failure of some of the smaller bands to stain, as a result of their low charge.[34] In heparin analysis, pure oligosaccharide standards are required only to confirm the assignment of bands in the oligosaccharide ladder. The reproducibility of this ladder (Figures 1 and 2) and the intense staining of all the important bands within the ladder suggest that the routine use of pure standards is probably unnecessary. A laboratory wishing to do such an analysis would be required simply to prepare partially depolymerized heparin and not to purify and characterize standards. These standards are then run on each slab gel used to determine M_r values.

The sensitivity of gradient PAGE (1–5 μg of heparin by alcian blue and 50–100 ng of heparin by silver staining) is also an important advantage. Gel permeation HPLC uses refractive index detection and generally requires 0.1–1 mg of sample for each analysis. An equal amount of several specially prepared M_r standards also are required for each determination. Gradient PAGE can simultaneously analyze up to 15 samples on a single gel. Previous studies have demonstrated that the staining intensity resulting from this type of cationic dye is proportional to the sample mass as long as the chain length is above a critical size[35]; for heparin, this chain length is typically larger than an octasaccharide. Although silver staining can be used to increase sensitivity, the staining intensity by this method is proportional to the logarithm of the sample mass[34]; therefore, the accuracy of M_r determinations based on silver staining is severely compromised.

Although the method described here results in the determination of M_r values, several improvements can simplify it. First, it is unnecessary to photograph the gel before scanning;

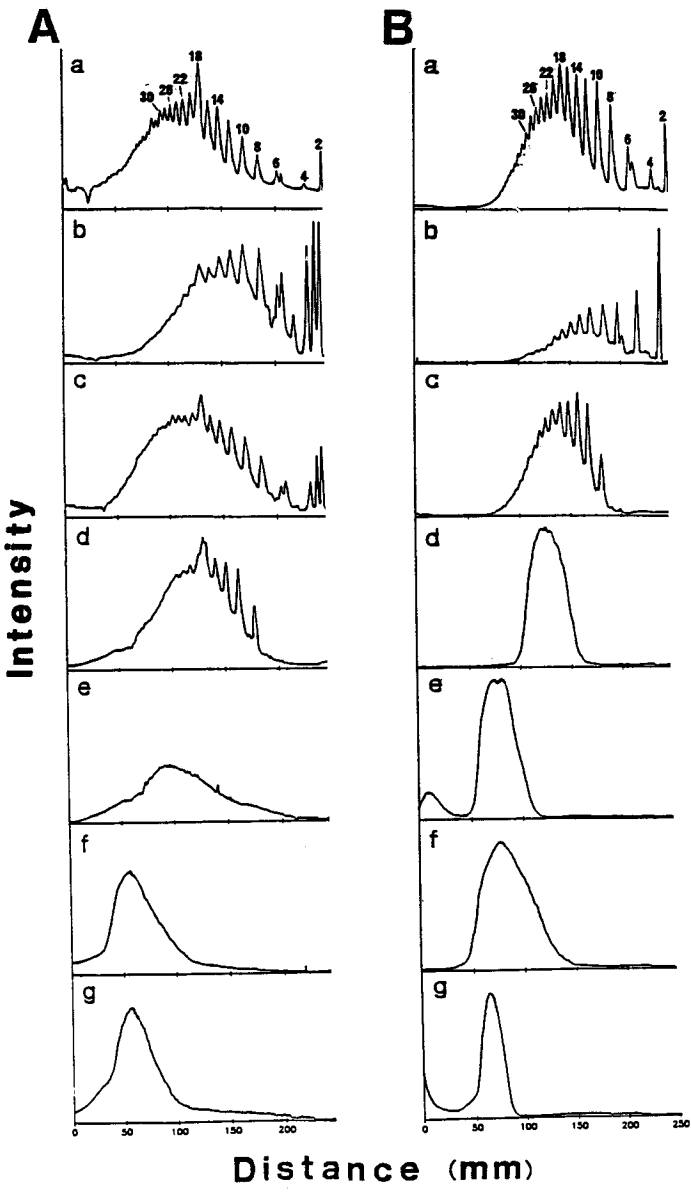

Figure 3—Densitometer scan of polyacrylamide gels shown in Figure 1: (**A**) scans of gel A lanes a–g; (**B**) scans of gel B lanes a–g.

our goal was to prepare a permanent record to facilitate repeated analysis. Photographic reproduction not only adds a step but also can introduce error in the determination of M_r values. For example, if the film is overexposed, the relative intensity of staining may be altered. Ideally, the gel should be scanned directly, with the analogue output from the scanner converted to digital signal and entered directly into a computer programmed to calculate M_r. Such data processing also permits automatic subtraction of background, which is particularly important when the gel has been incompletely destained and the sample size is small. The background of the destained gel was darker at the top than at the bottom of the gels in Figure 1. In Figure 1 (**Right**) lane e, insufficient sample was loaded to produce high-contrast staining of the sample; thus, to obtain a more accurate M_r, it was necessary to subtract manually the background (Figure 3B, panel c). In a completely automated system, background can easily be subtracted by scanning the empty lane on either side of the sample track and subtracting the background data set from the scanned sample lane data set prior to processing.

Conclusions

This study demonstrates that gradient PAGE can be used to determine the M_r values of heparin preparations and LMW heparin derivatives. The M_r values obtained with gradient PAGE are similar to those obtained by other methods, and yet much less material is required for analysis. Furthermore, multiple samples can be run simultaneously and in parallel. The high resolution inherent to gradient PAGE permits the use of multicomponent mixtures of standards. Additional work is required to automate this method for routine use by the pharmaceutical industry.

References and Notes

1. Linhardt, R. J.; Loganathan, D. In *Biomimetic Polymers*; Gebelein, G., Ed.; Plenum: New York, 1990, pp 135–175.
2. Gervin, A. S. *Surg. Gynecol. Obstet.* **1975**, *140*, 789.
3. Porter, J.; Hershel, J. *JAMA* **1977**, *237*, 9.
4. Linhardt, R. J.; Loganathan, D.; Al-Hakim, A.; Wang, H. M.; Walenga, J. M.; Hoppensteadt, D.; Fareed, J. *J. Med. Chem.* **1990**, *33*, 1639.
5. Breddin, H. K.; Fareed, J.; Bender, N. *Haemostasis* **1988**, *18*, 1.
6. Shivley, J. E.; Conrad, H. E. *Biochemistry* **1976**, *15*, 3932.
7. Barrowcliffe, T. W.; Curtis, A. D.; Tomilson, T. D.; Hubbard, A. R.; Johnson, E. A.; Thomas, D. P. *Thromb. Haemostasis* **1985**, *54*, 675.
8. Neville, G. A.; Racey, T. J.; Rochon, P.; Rej, R. N.; Perlin, A. S. *J. Pharm. Sci.* **1990**, *79*, 425.
9. Emanuele, R. M.; Fareed, J. *Fed. Proc.* **1987**, *46*, 868.
10. Laurent, T. C.; Tengblad, A.; Thunberg, L.; Hook, M.; Lindahl, U. *Biochem. J.* **1978**, *175*, 691.
11. Johnson, E. A.; Mulloy, B. *Carbohydr. Res.* **1976**, *51*, 119.
12. Lasker, S. E.; Stivala, S. S. *Arch. Biochem. Biophys.* **1966**, *115*, 360.
13. Liberti, P. A.; Stivala, S. S. *Arch. Biochem. Biophys.* **1967**, *119*, 510.
14. Patat, F.; Elias, H. G. *Naturwissenschaften* **1959**, *46*, 322.
15. Nieduszynski, I. In *Heparin, Chemical and Biological Properties Clinical Applications*; Lane, D. A.; Lindahl, U., Eds.; CRC: Boca Raton, FL, 1989; pp 57–63.
16. Tivant, P.; Turq, P.; Drifford, M.; Magdeleat, H.; Menez, R. *Biopolymers* **1983**, *22*, 643.
17. Stivala, S. S.; Herbert, M.; Kratky, O.; Pilz, I. *Arch. Biochem. Biophys.* **1968**, *127*, 795.
18. Neville, G. A.; Mori, F.; Racey, T. J.; Rochon, P.; Holme, K. R.; Perlin, A. S. *J. Pharm. Sci.* **1990**, *79*, 393.
19. Fareed, J.; Walenga, J.; Racanelli, A.; Hoppensteadt, D.; Huan, X.; Messmore, H. L. *Haemostasis* **1988**, *18*, 33.
20. Rice, K. G.; Rottink, M. K.; Linhardt, R. J. *Biochem. J.* **1987**, *244*, 515.
21. Mathews, M. B.; Decker, L. *Biochim. Biophys. Acta* **1971**, *244*, 30.
22. Min, H.; Cowman, M. K. *Anal. Biochem.* **1986**, *155*, 275.
23. Hittner, D. M.; Cowman, M. K. *J. Chromatogr* **1987**, *402*, 149.
24. Barrowcliffe, T. W.; Mulloy, B.; Johnson, E. A.; Thomas, D. P. *J. Pharm. Biomed. Anal.* **1989**, *7*, 217.
25. Yang, V. C.; Linhardt, R. J.; Bernstein, H.; Cooney, C. L.; Langer, R. *J. Biol. Chem.* **1985**, *260*, 1849.
26. Hoppensteadt, D., Masters Thesis; Loyola University Medical Center, Maywood, IL, 1989.
27. Al-Hakim, A.; Linhardt, R. J. *Electrophoresis* **1990**, *11*, 23.
28. Barlow, G. H. *Thromb. Res.* **1983**, *31*, 513.
29. Mallis, L. M.; Wang, H. M.; Loganathan, D.; Linhardt, R. J. *Anal. Chem.* **1989**, 1453.
30. Linhardt, R. J.; Wang, H. M.; Loganathan, D.; Lamb, D.; Mallis, L. M. *Carbohydr. Res.* **1992**, *225*, 137.
31. Linhardt, R. J.; Rice, K. B.; Kim, Y. S.; Engelken, J.; Weiler, J. *J. Biol. Chem.* **1988**, *263*, 13090.
32. Rice, K. G., Doctoral Thesis; The University of Iowa, Iowa City, IA, 1987.
33. Linhardt, R. J.; Al-Hakim, A.; Liu, S. Y.; Kim, Y. S.; Fareed, J. *Sem. Thromb. Hemostas.* **1991**, *17*, 15.
34. Al-Hakim, A.; Linhardt, R. J. *Appl. Theor. Electrophor.* **1991**, *1*, 305.
35. Grant, A. C.; Linhardt, R. J.; Fitzgerald, G. L.; Park, J. J.; Langer, R. *Anal. Biochem.* **1984**, *137*, 32.

Acknowledgments

We thank the National Institutes of Health (GM38060, AI 22350, and HL29797), the Department of Veteran Affairs, and the USP (in the form of a fellowship to Erik Edens) for supporting this research. We also acknowledge the help of Dr. Erwin Coyne of Loyola University Medical Center for the standards and for reviewing the manuscript.