Journal of Chromatography, 622 (1993) 13–20
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7123

Characterization of heparins with different relative molecular masses (from 11 600 to 1600) by various analytical techniques

Nicola Volpi

Department of Animal Biology, Chair of Biological Chemistry, University of Modena, Via Berengario 14, 41100 Modena (Italy)

(First received June 8th, 1993; revised manuscript received September 7th, 1993)

ABSTRACT

Heparin was extracted and purified from beef intestinal mucosa, and its structure and physico-chemical properties, *e.g.* disaccharide pattern (by specific enzymatic cleavage), relative molecular mass and sulfate-to-carboxyl ratio, were evaluated by different techniques. Heparin fractions with different relative molecular mass (from $M_r = 7560$ to $M_r = 1600$) were prepared by chemical degradation and gel-permeation chromatography. The fractions were characterized with respect to relative molecular mass, disaccharide pattern, sulfate-to-carboxyl ratio and percentage of slow moving and fast moving components by agarose-gel electrophoresis. The percentage of the two heparin species was calculated by densitometric analysis and specific calibration curves. The amount of the slow moving component decreases with relative molecular mass. The disaccharide pattern is different for the two heparin fractions. The percentage of trisulfated disaccharide decreases and the amount of mono- and disulfated disaccharides increases with a decrease of the relative molecular mass. The charge density, evaluated as the sulfate-to-carboxyl ratio, also decreases with the molecular mass of the fractions. This study confirms the heterogeneity of the structure, as evaluated by the constituent disaccharides, of the physico-chemical properties, such as relative molecular mass and charge density, and of the relative amount of the two heparin components also for low molecular mass heparins particularly produced by chemical depolymerization in the presence of free radicals.

INTRODUCTION

Heparin is a very complex glycosaminoglycan composed of alternate sequences of differently sulfated residues of uronic acid (usually α-L-iduronic acid) and α-D-glucosamine linked by α(1→4) bonds [1]. The backbone of heparin contains O-sulfate groups in different amounts and O-linkages at different positions. The prevailing heparin monosaccharides are α-L-iduronic and β-D-glucuronic acids, N-acetyl-α-D-glucosamine and N-sulpho-α-D-glucosamine. Sulfate groups can be O-linked at position 2 of the uronic acids, at position 6 of N-acetyl-α-D-glucosamine and at positions 3 and 6 of N-sulpho-α-D-glucosamine [2–4]. The primary heparin structure is commonly represented by its prevalent disaccharide sequences obtained by different approaches, such as enzymatic cleavage [5–7] and chemical depolymerization [8,9]. Trisulfated disaccharide [(1→4)-(O-α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-(2-deoxy-2 sulfamino-α-D-glucopyranosyl-6-sulfate)] is the main sequence in commercially available heparin disaccharides [1], the percentage varying with the type of heparin [5]. Due to the complexity of its primary structure, heparin is a polydisperse heteropolysaccharide strongly heterogeneous in terms of M_r, physico-chemical properties and biological activities [10].

Heparin is an anticoagulant and antithrombotic agent [11] already used for more than 50 years in the treatment and prevention of venous thrombosis [12]. However, it exhibits significant

0378-4347/93/$06.00

side effects, such as hemorrhagic complications [13,14] and thrombocytopenia [15], sometimes causing the patients' death [16].

Over the last years, non-heparin glycosaminoglycans, heparinoids and heparin derivatives [17] have been developed as anticoagulant, antithrombotic [11], thrombolytic, fat-clearing [18], and antiatherosclerotic agents with less serious side effects.

A group of heparin derivatives, the low-molecular-mass heparins (LMW heparins) have become a topic of great clinical interest [12]. These derivatives differ from native heparin in their anticoagulant and antithrombotic activities [14,19,20], their pharmacokinetics [11,21], and their interactions with several proteins [20,22]; they are used for the treatment of deep venous thrombosis [23], prevention of post-operative thrombosis and treatment of manifest thrombosis [20].

Commercial heparin preparations can be fractionated in two main components by selective barium precipitation [24–26] characterized as the slow moving and the fast moving component in discontinuous agarose-gel electrophoresis [25,27]. These two components have a different M_r, different charge density, different disaccharide pattern [25], and specific antithrombotic, antilipemic and anticoagulant activities [24].

Heparin was purified from beef intestinal mucosa and depolymerized in LMW fractions by a chemical process in the presence of free radicals. These fractions were characterized with respect to M_r, disaccharide pattern and the relative percentage of slow moving and fast moving components by agarose-gel electrophoresis.

EXPERIMENTAL

Extraction and purification of heparin

Beef intestinal mucosa was ground and treated with proteolytic enzymes at 65°C for 12 h in a reaction vessel fitted with a thermostat, stirrer and thermometer. After heating at 100°C for 30 min, the product was filtered on a diatomite filter (High Performance Filter Aids from Dicalite, Los Angeles, CA, USA), and the solution containing the polysaccharides was percolated through a strong anion-exchange column (Purolite A860, batch 1/88 from Purolite, Pontyclun, Wales, UK). Peptides, nucleic acids and glycosaminoglycans with a low charge density (chondroitin sulfate, dermatan sulfate, heparan sulfate) were eluted with 0.7–1.8 *M* NaCl. Heparin was eluted with 2.0–3.0 *M* NaCl. The heparin containing fractions were diluted with 1.0–1.5 volumes of acetone [6]. Residual dermatan sulfate was removed by selective precipitation as its copper salt, as reported elsewhere [28].

The slow moving and fast moving components of heparin were purified as their barium salt at different temperatures, as previously reported [25].

Evaluation of heparin purity

The presence of glycosaminoglycans (dermatan sulfate and chondroitin sulfate) as contaminants in the preparations of heparin was determined by agarose-gel electrophoresis [25] and cellulose polyacetate (Titan III from Helena Laboratories, Beaumont, TX, USA) [29] electrophoresis.

Specific optical rotation was determined with a polarimeter at 25°C and at a concentration of 5% in bidistilled water.

Preparation of LMW heparin fractions

Different LMW fractions were obtained from purified heparin by a controlled chemical depolymerization process induced by free radicals in the presence of copper salt, as previously reported [6]. Five grams of heparin and 0.2 g of copper acetate monohydrate (0.02 *M*) were dissolved in 50 ml of water in a reaction vessel. The temperature was kept at 60°C and the pH was adjusted to 7.5 with 1 *M* NaOH. A 9% (v/v) hydrogen peroxide solution was added at a rate of 10 ml/h. The reaction with heparin was stopped at different times, and the Chelex 100 chelating resin (Cod. 142-2832 from Bio-Rad, Richmond, CA, USA) was used to remove pollutant copper from the product. A strong anion-exchange resin (Amberlite IRA-400, Cod. 1-0326 from Supelco, Bellefonte, USA) in the OH^- form was used to remove acidic pollutants. The pH of the percolate was adjusted to 6.0 by adding excess acetic acid and then two volumes

of acetone were added. The precipitate was collected by filtration, washed with acetone and dissolved again in 100 ml of water. One gram of sodium acetate was added to this solution, and then the different sodium salt M_r fractions were precipitated by addition of two volumes of acetone. The precipitate was collected and dried. Heparins with different M_r were prepared by stopping the chemical depolymerization process at different times.

The different molecular mass fractions were further fractionated on a column packed with BioGel P6 (BioRad, USA; particle size, 45–90 μm; molecular mass range 1000–6000) and eluted with 1 *M* NaCl. The fractions (determined by high-performance size-exclusion chromatography, HPSEC [30]) were desalted on BioGel P2 (BioRad, USA; particle size, 45–90 μm; molecular mass range 100–1800) and lyophilized.

Determination of M_r by HPSEC

Equipment: HPLC by Jasco (pump, Model 880 PU from Jasco, Tokyo, Japan). Columns Protein Pak 125 (300 × 7.8 mm I.D.; particle size 10 μm; molecular mass ranges: native-form globular M_r from 2000 to 80 000 and random coil M_r from 1000 to 30 000; cod. 84601 from Waters, Milford, MA, USA) and Protein Pak 300 (300 × 7.5 mm I.D.; particle size 10 μm; molecular mass ranges: native-form globular M_r from 10 000 to 400 000 and random-coil M_r from 2000 to 150 000; cod. T72711 from Waters) were assembled in series. The mobile phase was 125 m*M* Na_2SO_4–2 m*M* NaH_2PO_4 adjusted to pH 6.0. The flow-rate was 0.9 ml/min. The UV detector (Model 875 from Jasco, Tokyo, Japan) was set at 214 nm. Peak M_r was determined by a third grade polynomial calibration curve plotted against glycosaminoglycan standards [30].

Determination of sulfate-to-carboxyl ratio

Sulfate and carboxyl groups were determined by potentiometric titration with 0.1 *M* NaOH in water–dimethylformamide (60:40, v/v) of the acid form of the heparin fraction obtained by removing possible pollutant anions and the salt cations through strong exchange resins (the strongly basic polystyrene gel-type resin IRA-400 and the strongly acidic polystyrene gel-type resin IR-120 PLUS; Supelco, Bellefonte, USA).

The sulfate-to-carboxyl ratio was also determined by enzymatic degradation after HPLC separation of the constituent disaccharides. The ratio was calculated by considering the percentage of carboxyl and sulfate groups for each disaccharide.

Quantitation of the constituent disaccharides by cleavage with heparinases

The enzymatic cleavage of the different heparin fractions was performed as previously reported [6], using heparinase I (EC 4.2.2.7.), heparinase II (no assigned EC number) and heparinase III (EC 4.2.2.8.) (enzymes were from Sigma, St. Louis, MO, USA).

The constituent disaccharides were separated and determined by strong anion-exchange (SAX)-HPLC [6]. Equipment: HPLC by Jasco. Column Spherisorb 5 SAX (250 × 4.6 mm I.D. packed with 5-μm resin with trimethylammoniopropyl $Si–CH_2–CH_2–CH_2–N^+(CH_3)_3$ active groups in the Cl^- form, from Phase Separations, Deeside, UK). Isocratic separation from 0 to 5 min with 0.2 *M* NaCl pH 4.0; linear gradient separation from 5 to 60 min with 100% 0.2 *M* NaCl pH 4.0 to 100% 1.2 *M* NaCl pH 4.0. Flow-rate, 1.4 ml/min. UV wavelength, 232 nm.

The identification and the separation of the heparin disaccharides were performed according to the retention times of standards.

Agarose-gel electrophoresis

The different M_r heparin fractions were analyzed by agarose-gel electrophoresis in barium acetate–1,2-diaminopropane as reported [25] with slight differences. A Pharmacia Multiphor II (from Pharmacia LKB Biotechnology, Uppsala, Sweden) electrophoretic cell equipment was used. The first run was performed in 0.04 *M* barium acetate buffer pH 5.8 for 60 min at 60 mA; the second run was in 0.05 *M* 1,2-diaminopropane (Cod. 821041 from Merck, Darmstadt, Germany) buffered at pH 9.0 with acetic acid for 120 min at 60 mA. After migration, the plate was soaked in 0.1% cetyltrimethylammonium bromide (Cod. 8130 from Merck, Darmstadt, Germany) for *ca.* 3 h, dried and stained with

toluidine blue (0.2% in ethanol–water–acetic acid, 50:49:1, v/v/v) for 30 min. After decoloration with ethanol–water–acetic acid (50:49:1, v/v/v), quantitative analysis of glycosaminoglycans was performed with a densitometer (Macintosh IIsi computer interfaced with a Microtek Color Scanner from Microtek, Hsinchu, Taiwan; an image processing and analysis program, Ver. 1.41 from Jet Propulsion Lab., NASA, FL, USA, was used to perform densitometric analysis of agarose-gel electrophoretic bands), and the slow moving and fast moving components were evaluated by specific calibration curves as reported [25].

RESULTS

The purified beef intestinal mucosa heparin has an M_r of 11 600, a sulfate-to-carboxyl ratio of *ca.* 2.37 as evaluated by enzymatic cleavage, and 65% of the fast moving and 35% of the slow moving species (Table I). In comparison, a commercial bovine heparin of 155 U.I. has an M_r of 12 350, a sulfate-to-carboxyl ratio of *ca.* 2.43, and 58% of the fast moving and 42% of the slow moving species. Purified heparin (specific optical rotation: +50°) does not contain chondroitin sulfate or dermatan sulfate contaminants as reported elsewhere [25].

Purified heparin was depolymerized by a controlled chemical process in the presence of free radicals. Fig. 1 shows the chromatographic profiles of some heparins with different M_r obtained by HPSEC as reported elsewhere [30]. Fig. 2 shows the electrophoretic profiles of the heparins with different M_r prepared by the chemical process. The figure shows the electrophoretic

TABLE I

RELATIVE MOLECULAR MASSES, SULFATE-TO-CARBOXYL RATIOS AND PERCENTAGES OF SLOW MOVING AND FAST MOVING COMPONENTS OF NATIVE AND DIFFERENT MOLECULAR MASS HEPARINS

Peak M_r (× 1000)	SO_3^-/COO^-	Fast moving percentage	Slow moving percentage
11.60[a]	2.37	65	35
7.56	2.29	70	30
6.30	2.22	81	19
5.43	2.18	91	9
4.56	2.11	100	0
3.80	N.D.[b]	95	5
3.64	N.D.	100	0
3.36	N.D.	97	3
3.03	N.D.	100	0
2.82	N.D.	100	0
1.62	N.D.	N.D.	N.D.

[a] Native.
[b] N.D. = Not detectable.

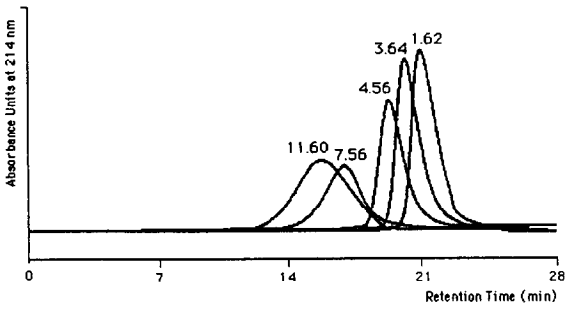


Fig. 1. High-performance size-exclusion chromatography (HPSEC) profiles of different relative-molecular-mass heparin fractions. The relative molecular masses are reported in kDalton.

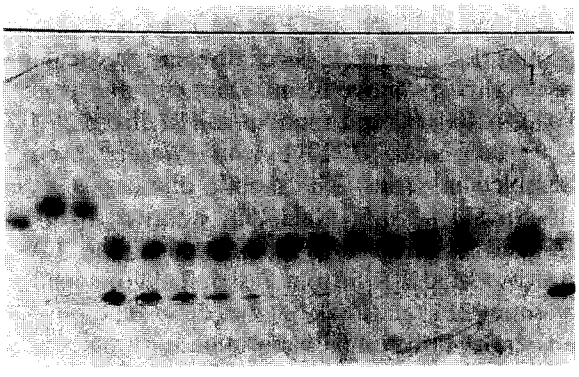


Fig. 2. Discontinuous agarose-gel electrophoresis performed with the Pharmacia Multiphor II electrophoretic cell. From left to right: beef mucosa dermatan sulfate, bovine trachea and shark cartilage chondroitin sulfates, commercial bovine heparin with 155 U.I., native heparin with $M_r = 11\,600$, heparin with $M_r = 7560$, heparin with $M_r = 6300$, heparin with $M_r = 5430$, heparin with $M_r = 4560$, heparin with $M_r = 3800$, heparin with $M_r = 3640$, heparin with $M_r = 3360$, heparin with $M_r = 3030$, heparin with $M_r = 2820$, heparin with $M_r = 1620$ (not detectable due to the very low molecular mass), fast moving species, and slow moving species prepared as reported elsewhere [25].

separation of fast moving and slow moving components of different heparins together with the separation of dermatan sulfate (from beef mucosa), chondroitin sulfates (from bovine trachea and shark cartilage) and the fast moving and slow moving heparin components purified as reported elsewhere [25]. The percentage of the two heparin components of heparins with different M_r (Table I) was evaluated by densitometric analysis and specific calibration curves obtained with purified fast moving and slow moving heparin components [25]. The densitometric profiles of several heparins with different M_r are illustrated in Fig. 3. Under the experimental conditions used, the lowest M_r heparin fraction detectable is that with $M_r = 2820$. In fact, no heparin fraction with $M_r = 1620$ is detectable (Fig. 2) after staining with toluidine blue.

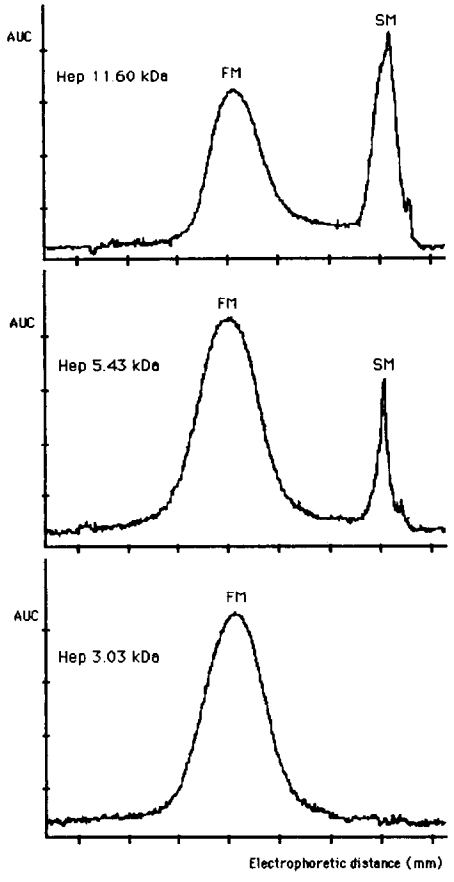


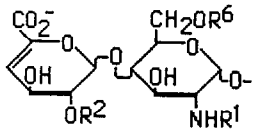
Fig. 3. Densitometric profiles of different relative-molecular-mass heparins. The area under the curve (AUC) of the two heparin components is illustrated. FM: fast moving; SM: slow moving.

Agarose-gel electrophoresis of glycosaminoglycans is generally performed using electrophoretic chambers not commercially available; two chambers are necessary: one for the run with barium acetate buffer, and one for the run with 1,2-diaminopropane buffer. However, under such conditions, it is not possible to analyze more than four to five samples per electrophoretic run. Under our experimental conditions, utilizing the Pharmacia Multiphor II electrophoretic cell, it is possible to analyze as many as twenty samples in a single run.

The disaccharide patterns of heparins with different M_r were evaluated by cleavage with heparinase I, II and III as previously reported [6]. Separation of the enzymatic products obtained on SAX-HPLC with a binary gradient [5,6] gives, under our experimental conditions, six heparin disaccharides with a different number and position of sulfate groups. The structures of the identified disaccharides and their relative amounts in heparins with different M_r are reported in Table II. The amount of trisulfated disaccharide decreases with the M_r of the heparin fractions, whilst there is an increase in mono- and disulfated disaccharides. For heparin fractions with an M_r smaller than *ca.* 4000–5000, it is not possible to assess precisely the percentages of the constituent disaccharides, owing to the incomplete cleavage of the LMW heparin fragments, as previously demonstrated [6]. In fact, the percentage of the various disaccharides obtained by cleavage with heparinase I, II and III is lower than 60% for heparin with an M_r of *ca.* 3500 and this percentage increases for very low-molecular-mass heparins. The sulfate-to-carboxyl ratio for heparins with different M_r was calculated considering the relative percentages of the disaccharides with a different number of sulfate groups. The evaluation of the charge density by enzymatic cleavage is more accurate than that obtained by potentiometric analysis due to the difficulty to evaluate small differences between the sulfate-to-carboxyl ratios of heparins with only small differences in their physico-chemical properties. The sulfate-to-carboxyl ratio decreases with the M_r of the heparin

TABLE II

STRUCTURAL FORMULAS AND PERCENTAGES OF DIFFERENT HEPARIN CONSTITUENT DISACCHARIDES FOR NATIVE HEPARIN AND SEVERAL DIFFERENT RELATIVE MOLECULAR MASS HEPARIN FRACTIONS. THE RELATIVE MOLECULAR MASSES ARE REPORTED IN kDALTON



	R^1	R^2	R^6	11.60	7.56	6.30	5.43	4.56
ΔDiH-OS	$COCH_3$	H	H	6.3	7.0	7.2	5.1	8.6
ΔDiH-NS	SO_3^-	H	H	3.8	4.1	6.8	7.1	5.7
ΔDiH-6S	$COCH_3$	H	SO_3^-	2.4	4.1	4.5	4.5	3.1
ΔDiH-2,NdiS	SO_3^-	SO_3^-	H	19.2	18.4	17.7	20.4	23.2
ΔDiH-N,6diS	SO_3^-	H	SO_3^-	12.5	14.7	16.5	22.6	22.2
ΔDiH,N,6TriS	SO_3^-	SO_3^-	SO_3^-	55.8	51.7	47.3	40.3	37.2

ΔDiH-OS: 2-acetamido-2-deoxy-4-O-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose. ΔDiH-NS: 2-deoxy-2-sulfamino-4-O-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose. ΔDiH-6S: 2-acetamido-2-deoxy-4-O-(4-deoxy-α-*threo*-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose. ΔDiH-2,NdiS: 2-deoxy-2-sulfamino-4-O-(4-deoxy-2-O-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose. ΔDiH-N,6diS: 2-deoxy-2-sulfamino-4-O-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose. ΔDiH-TriS: 2-deoxy-2-sulfamino-4-O-(4-deoxy-2-O-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucosc.

fractions (Table I) and eventually it reaches the value calculated for the fast moving component purified from native heparin (2.12 for the fast moving fraction *versus* 2.11 for the heparin fraction of M_r 4560 composed of 100% of the fast moving species) [25].

DISCUSSION

The heterogeneity of the primary structure and the physico-chemical properties of heparin depends on the source from which this heteropolysaccharide is purified. Heparins from various species and tissues have different percentages of oligosaccharides, as analyzed by cleavage with heparinase I [31]. Heparin purified from human tissue is structurally similar to that purified from porcine intestinal mucosa and different from that extracted and purified from bovine lung [32]. Heparin extracted and purified from bovine intestinal mucosa gives under the experimental conditions used an $M_r = 11\,600$, about the same order of magnitude as for commercial heparin ($M_r = 10\,000$–$14\,000$) [33], and it gives a higher percentage of fast moving component than commercial bovine heparin. The different ratio between the two components of heparin, i.e. the highly sulfated high-molecular-mass species and the less sulfated low-molecular-mass species, is another important characteristic for the heterogeneity of commercially available heparin purified from different sources [34].

LMW heparins are heparin derivatives that typically consist of polysaccharide chains having molecular masses ranging from 2000 to 8000 with an average of 5000 [35]. LMW heparins can be produced by different processes which on the one hand provide derivatives with low M_r and on the other hand do not cause desulfation and deacetylation. LMW heparins can be produced by enrichment of LMW fractions of commercial heparin, by controlled chemical depolymerization with nitrous acid, chemical β-elimination and utilization of peroxides and redox systems [36]. Chemical synthesis and enzymatic depolymerization can also be used for preparation of

LMW heparins. Moreover, acid hydrolysis in the presence of sulfuric acid and 5% chlorosulfonic acid produces a supersulfated LMW heparin with pharmacological activity [37]. The different preparation processes produce heparin derivatives with different structures and with different molecular-mass distributions, different anticoagulant activities and pharmacological properties [20,38].

The depolymerization process used in this study produces LMW heparin fractions that have structures and physico-chemical properties similar to those of the fractions obtained by gel-permeation enrichment of fractions of native heparin [6]. No marked desulfatation effect is observed. Moreover, it is possible to obtain heparin fractions with different M_r by stopping the chemical depolymerization process at different times. In fact, the chemical depolymerization reaction is apparently a first order reaction under the selected conditions, as has also been reported for the degradation of chondroitin sulfate and dermatan sulfate [28,30].

The molecular masses and the dispersion of the different M_r heparin fractions produced by chemical degradation were analyzed by HPSEC. The fractions with small M_r were further fractionated with preparative size-exclusion chromatography to obtain very low M_r heparins with a HPSEC profile with low dispersion (Fig. 1). These fractions were analyzed for their composition in slow and fast moving components by agarose-gel electrophoresis. The percentage of slow moving component decreases with the molecular mass and the fractions with M_r smaller than 3640 are composed of 100% of the fast moving component. This is related to degradation of the high-molecular-mass chains of the slow moving component by the chemical process and production of polysaccharide chains with low molecular mass.

The different M_r heparin fractions were also analyzed for their disaccharide pattern by specific enzymatic cleavage with heparinases. The fraction were either treated with heparinase I alone or simultaneously with heparinase I, heparinase II and heparinase III. The degradation of heparin fractions with heparinase I produces *ca.* 50% of two unsaturated disaccharides [2-deoxy-2-sulfamino-4-O-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose and 2-deoxy-2-sulfamino-4-O-(4-deoxy-2-O-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose] and *ca.* 50% of oligosaccharides, as reported by Linhardt *et al.* [5]. The simultaneous cleavage of fractions with heparinase I, II and III produces *ca.* 75% of unsaturated disaccharides (six identified disaccharides with a different number and position of sulfate groups, as illustrated in Table II) and 25% of heparin oligosaccharides still resistant to treatment with heparinases under the conditions used. The pattern of the identified disaccharides is different for the various M_r heparin fractions. The amount of trisulfated disaccharide decreases with the M_r of the fractions, and the percentage of mono- and disulfated disaccharides generally increases. This difference in the constituent disaccharides is shown by the decrease of the sulfate-to-carboxyl ratio with the M_r of the heparin fractions. Thus, the different M_r heparin fractions produced by the radical depolymerization process show a decrease in the slow moving component, a decrease in the percentage of trisulfated disaccharide (and an increase of less-sulfated disaccharides) and a decrease in the sulfate-to-carboxyl ratio. This is related to an increase in heteropolysaccharide chains with low M_r and low charge density. On the other hand, the biological activities, such as anti-factor Xa activity and activated partial thromboplastin time, also decrease with the M_r of the heparin fractions [6]. The fat-clearing activity is higher for the slow moving component [24] and proportional to the charge density of the heparins [18].

LMW heparin preparations used for pharmaceutic purposes are heterogeneous in chemical composition, M_r and particle size range [38] due to the different sources of the native heparin and to the various degradation processes used. Moreover, other factors for the heterogeneity are the various percentages of slow and fast moving components, the different disaccharide patterns and charge densities that could be related to the different biological and pharmacological activities of LMW heparins.

ACKNOWLEDGEMENTS

This research was supported by a 60% grant from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (M.U.R.S.T.).

REFERENCES

1 B. Casu, in F.A. Ofosu, I. Danishefsky and J. Hirsh (Editors), *Heparin and Related Polysaccharides. Structure and Activities, Ann. New York Acad. Sci.*, 556 (1989) 2.
c 2 B. Casu, *Adv. Carb. Chem. Biochem.*, 43 (1985) 51.
3 H.E. Conrad, in F.A. Ofosu, I. Danishefsky and J. Hirsh (Editors), *Heparin and Related Polysaccharides. Structure and Activities, Ann. New York Acad. Sci.*, 556 (1989) 18.
4 K. Murata and Y. Yokoyama, *Anal. Biochem.*, 149 (1985) 261.
5 R.J. Linhardt, K.G. Rice, Y.S. Kim, D.L. Lohse, H.M. Wang and D. Loganathan, *Biochem. J.*, 254 (1988) 781.
6 N. Volpi, G. Mascellani and P. Bianchini, *Anal. Biochem.*, 200 (1992) 100.
7 C.F. Moffat, W. McLean, W.F. Long and F.B. Williamson, *Eur. J. Biochem.*, 202 (1991) 531.
8 Y. Guo and H.E. Conrad, *Anal. Biochem.*, 176 (1989) 96.
9 M.J. Bienkowski and H.E. Conrad, *J. Biol. Chem.*, 260 (1985) 356.
10 F.A. Ofosu, I. Danishefsky and J. Hirsh (Editors), *Ann. New York Acad. Sci.*, 556 (1989).
11 G.L. Bergonzini, P. Bianchini, G. Mascellani, B. Osima, B. Parma and N. Volpi, *Arzneim.-Forsch./Drug Res.*, 42 (1992) 1322.
12 R.D. Hull and G.F. Pineo, *Coagulation Disorders*, 6 (1992) 1095.
13 M.N. Levine, J. Hirsh and J.G. Kelton, in D.A. Lane and U. Lindhal (Editors), *Heparin. Chemical and Biological properties. Clinical Applications*, Edward Arnold, London, 1989, pp. 517–531.
14 N. Volpi, G.L. Bergonzini, B. Parma, P. Bianchini and L. Bolognani, *Thromb. Haemorrh. Dis.*, 4 (1991) 43.
15 H.C. Godal, in D.A. Lane and U. Lindahl (Editors), *Heparin. Chemical and Biological properties. Clinical Applications*, Edward Arnold, London, 1989, pp. 533–548.
16 J. Porter and J. Hershel, *J. Am. Med. Assoc.*, 237 (1977) 879.
17 H. Engelberg, in E.F. Mammen, J.M. Walenga and J. Fareed (Editors), *Seminars in Thrombosis and Hemostasis*, Theme Medical Publishers, New York, vol. 17 (Suppl. 1, 2), 1991, p. 5.
18 B. Casu, E.A. Johnson, M. Mantovani, B. Mulloy, P. Oreste, R. Pescador, G. Prino, G. Torri and G. Zoppetti, *Arzneim.-Forsch./Drug Res.*, 33 (1983) 135.
19 B. Ljungberg, M. Blomback, H. Johnsson and L.-E. Lins, *Clin. Nephrol.*, 27 (1987) 31.
20 E. Holmer, in D.A. Lane and U. Lindahl (Editors), *Heparin. Chemical and Biological properties. Clinical Applications*, Edward Arnold, London, 1989, pp. 575–595.
21 J. Albada, H.K. Nieuwenhuis and J.J. Sixma, in D.A. Lane and U. Lindahl (Editors), *Heparin. Chemical and Biological properties. Clinical Applications*, Edward Arnold, London, 1989, pp. 417–431.
22 D.A. Lane, in D.A. Lane and U. Lindahl (Editors), *Heparin. Chemical and Biological properties. Clinical Applications*, Edward Arnold, London, 1989, pp. 363–393.
23 P. Prandoni, A.W.A. Lensing, H.R. Büller, M. Carta, A. Cogo, M. Vigo, D. Casara, A. Ruol and J.W. Ten Cate, *The Lancet*, 9 (1992) 401.
24 P. Bianchini, B. Osima, B. Parma, H.B. Nader, C.P. Dietrich, B. Casu and G. Torri, *Arzneim.-Forsch./Drug Res.*, 35 (1985) 1215.
25 N. Volpi, *Carb. Res.*, 247 (1993) 263.
26 H.K. Takahashi, H.B. Nader and C.P. Dietrich, *Arzneim.-Forsch./Drug Res.*, 35 (1985) 1620.
27 P. Bianchini, H.B. Nader, H.K. Takahashi, B. Osima, A.H. Straus and C.P. Dietrich, *J. Chromatogr.*, 196 (1980) 455.
28 N. Volpi, G. Mascellani, P. Bianchini and L. Liverani, *Il Farmaco*, 47 (suppl. 5) (1992) 841.
29 R. Cappelletti, M. Del Rosso and V.P. Chiarugi, *Anal. Biochem.*, 99 (1979) 311.
30 N. Volpi and L. Bolognani, *J. Chromatogr.*, 630 (1993) 390.
31 D. Loganathan, H.M. Wang, L.M. Mallis and R.J. Linhardt, *Biochemistry*, 29 (1990) 4362.
32 R.J. Linhardt, S.A. Ampofo, J. Fareed, D. Hoppensteadt, J.B. Mulliken and J. Folkman, *Biochemistry*, 31 (1992) 12441.
33 K.G. Jacobsson, U. Lindahl and A.A. Horner, *Biochem. J.*, 240 (1986) 625.
34 H.B. Nader, H.K. Takahashi, J.A. Guimaraes, C.P. Dietrich, P. Bianchini and B. Osima, *Int. J. Biol. Macromol.*, 3 (1981) 356.
35 R.J. Linhardt, D. Loganathan, A. Al-Hakim, H.M. Wang, J.M. Walenga, D. Hoppensteadt and J. Fareed, *J. Med. Chem.*, 33 (1990) 1639.
36 R.J. Linhardt, H.M. Wang and S.A. Ampofo, in D.A. Lane, I. Björk and U. Lindahl (Editors), *Heparin and Related Polysaccharides. Advances in Exp. Med. and Biol.*, Plenum Press, New York, Vol. 313, 1992, p. 37.
37 A. Naggi, G. Torri, B. Casu, J. Pangrazzi, M. Abbadini, M. Zametta, M.B. Donati, J. Lansen and J.P. Maffrand, *Biochem. Pharm.*, 36 (1987) 1895.
38 G.A. Neville, F. Mori, T.J. Racey, P. Rochon, K.R. Holme and A.S. Perlin, *J. Pharm. Sci.*, 79 (1990) 339.