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Chromatographic and electrophoretic applications for the analysis of heparin and dermatan sulfate[1]

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Abstract

Heparin and dermatan sulfate are highly sulfated polydisperse glycosaminoglycans. The methods to determine such compounds include chromatographic and electrophoretic techniques. Here we report on the performances of various analytical methods for the characterization and the determination of GAGs. Heparin, low-molecular-mass heparins, dermatan sulfate and low-molecular-mass dermatan sulfate were analyzed. High-performance size exclusion chromatography was used to determine the molecular mass, polydispersity, absorbance and the area under the absorbance–time curve. Polyacrylamide gel electrophoresis was used to determine the average molecular mass and the polydispersity. Heparin and dermatan sulfate preparations were analyzed by capillary electrophoresis using reversed polarity. The results obtained reflect different performances of various analytical methods used to characterize GAGs.

Keywords: Heparin; Dermatan sulfate

1. Introduction

Sulfated polysaccharides such as heparin and dermatan sulfate are commonly known glycosaminoglycans (GAGs) [1]. The biosynthesis of heparin and dermatan sulfate takes place in several mammalian and non-mammalian tissues [2]. The analysis of these heterogeneous compounds requires the use of different spectroscopic methods such as NMR spectroscopy or chromatography and electrophoresis [3]. The measurement of the molecular mass and the size distribution of heparins and dermatan sulfate is usually performed using size-exclusion high-performance chromatography (HPSEC) with different columns and eluents [4]. Polyacrylamide gel electrophoresis resolves complex mixtures of heparin-fragments or dermatan sulfate-fragments [5]. This method is useful to determine the molecular mass and polydispersity of the compound.

Enzymatic elimination of heparin leads to unsaturated disaccharides. The resulting disaccharides with the general structure 4-deoxy-α-L-threo-hex-4-enoxy-pyranose (Δ UA) 2X (1→4)-D-GlcNY6X (X=H, S and Y=H, S) for heparin and heparan sulfate [6] and Δ UA 2X (1→3)-D-GalNY6X (X=H, S and Y=H, S) for chondroitin sulfate [7] absorb specifically at 232 nm. Strong anion-exchange chromatographic methods were described for their characterization. In

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the last years high-performance capillary electrophoresis (HPCE) methods were developed for the composition analysis of chondroitin sulfates, heparin and heparan sulfate. A 20 m*M* phosphate buffer (pH 3.5) heparin and dermatan sulfate can also be analyzed by capillary electrophoresis [8]. They show a migration profile that is different from the elution in HPSEC because the compounds are separated according to their charge density.

Here we describe the performances of various analytical methods for the characterization and the determination of GAGs.

2. Experimental section

The following unfractionated and low-molecular-mass GAGs were used: dermatan sulfate and low molecular mass dermatan sulfate (Derven) were obtained from Alfa Wassermann (Bologna, Italy). Unfractionated- (UFH) and low-molecular-mass sodium–heparin (LMMH) was obtained from Braun Melsungen (Melsungen, Germany). Clexane (LMMH) was from Rhone Poulenc Rorer (Köln, Germany). Fragmin (LMMH) was obtained from Kabi Pfrimmer (Erlangen, Germany). Fraxiparin was provided from Sanofi-Wintrop (Munich, Germany). LMMH-Merckle was from Merckle (Ulm, Germany). Mono-Embolex (LMMH) was generously provided by Sandoz AG (Nürnberg, Germany). Reviparin (LMMH) was provided from Nordmark (Uetersen, Germany).

2.1. Standards for mass calibration

Heparin–oligosaccharides prepared by synthesis ranging from di- to decasaccharide were generously provided by Dr. M. Petitou and Dr. L. Lormeau from the Institute Choay (Paris, France). Dermatan sulfate (batch OP 435, $M=25.3\cdot10^3$), low-molecular-mass dermatan sulfate (batch OP LL 105/2, $M=5.5\cdot10^3$), fast moving heparin (batch OP 1117, $M=8.4\cdot10^3$), slow moving heparin (batch OP 045-25, $M=14\cdot10^3$) and heparin–oligosaccharides (batch OP-684-6, $M=2.1\cdot10^3$), which were characterized by NMR spectroscopy were generously provided by Dr. Mascellani from Opocrin (Bologna, Italy).

2.2. Chemicals and enzymes

Boric acid and Tris-HCl were of analytical grade and obtained from Sigma (Deisenhofen, Germany). Glycerol was from Merck (Darmstadt, Germany) and 3-dimethylamino-propionitrile (DNPN) from Fluka (Neu Ulm, Germany). Acrylamide, N,N″-methylene-bisacrylamide, ethylene diamine tetraacetic acid and glycine of research grade were obtained from Serva (Heidelberg, Germany).

2.3. High-performance size exclusion chromatography

A system consisting of a multisolvent delivery system (Model 600 from Millipore-Waters, Eschborn, Germany) with a Waters 600 E system controller, an injector (Model U6K from Millipore-Waters), 20-μl sample loop, a photodiode array detector (Model Waters 991) with a computer (NEC Power Mate SX Plus) and the 990/991 foreground/background software (Millipore-Waters) were used.

An Ultropac TSK G 2000 SW column (600×7.5 mm I.D., particle size 10 μm) and an Ultrapac TSK SWP precolumn (75×7.5 mm I.D.) from LKB (Broma, Sweden) were connected between the injector and the pump. Sodium chloride (0.1 *M*) (filtered and de-gased before use) at a rate of 1.0 ml/min was used as eluent and the detector was set at a wavelength range from 190 to 300 nm. The sample concentration was 10 mg/ml and the injection volume was 20 μl.

For calibration five glycosaminoglycan preparations from 2.1 to $26.3\cdot10^3$ and six synthesized heparin–oligosaccharides ranging from disaccharide to heparin–decasaccharide were used. The log molecular mass of each calibrant was plotted against the retention time. A third order regression equation ($y=-0.0058x^3+0.2923x^2-4.9517x+32.206$) was fitted to the data points. The correlation coefficient was 0.98.

The average molecular mass of the GAGs was calculated as $M_m=(\Sigma n_i M_i^2)/\Sigma n_i M_i$ where n_i and M_i

are the quantity of material and the molecular mass of the material in slice ($i = 1$ mm) respectively. The term M_w with average molecular mass is equivalent to M_m.

3. Polyacrylamide gel electrophoresis

A linear gradient polyacrylamide resolving gel (8×7 cm, 1.5 mm thick consisting of 20% T and 30% T and 10% C) was prepared. Samples (10–20 μg) containing 10% glycerol (v/v) were loaded into the wells in a volume of 20 to 30 μl. Electrophoresis was performed at 140 V while the samples concentrated and migrated through the stacking gel; the voltage was decreased to 70 V after the samples had entered the resolving gel. The gels were cooled at 10°C by a circulating tap water. Electrophoresis was terminated after about 3–4 h. The gels were stained by a 1% aqueous solution of acridine orange. The gels were scanned by an Elscript densitometer from Hirshmann (Taufkirchen, Germany) at 548 nm. The average molecular mass was calculated as $M_m = (\Sigma n_i M_i^2)/\Sigma n_i M_i$ where n_i and M_i are the quantity of material and the molecular mass of the material in slice ($i = 1$ mm), respectively.

3.1. Separation of glycosaminoglycan-fragments by HPCE

The experiments were performed on a PACE 2050 from Beckmann Instruments (Fullerton, CA, USA) equipped with a variable-wavelength absorbance detector. System operation and data management were controlled using Gold-software from Beckmann Instruments running on a IBM personal computer. The samples were injected using a 50 cm×75 μm I.D. capillary cartridge from Beckmann Instruments. The concentrations of the stock solutions were 1 mg/ml for heparin–disaccharides and 10 mg/ml for heparin–oligosaccharides and heparin preparations, respectively. The samples were injected by high-pressure injection for 15 s.

Electrophoresis was run using a 20 m*M* sodium phosphate buffer adjusted with hydrochloric acid to pH 3.5. The other conditions were: data rate, 5 Hz; rise time, 1 s; range (adsorbtion unit, AU), 0.2; polarity, indirect; wavelength, 230 nm; time, 60 min; voltage, 12 kV; temperature, 25°C.

4. Results

Heparin and dermatan sulfates and low-molecular-mass derivatives respectively were analyzed using HPSEC, PAGE and HPCE.

4.1. High-performance size exclusion chromatography

Heparin and dermatan sulfate preparations were analyzed by high-performance size-exclusion chromatography (HPSEC) in 5 repetitive runs according to Ref. [4]. Unfractionated heparins and dermatan sulfate and their low-molecular-mass derivatives show different distribution of their molecular masses resulting in different elution profiles (Fig. 1) and retention times. A calibration curve is displayed in Fig. 2. The intra-day coefficients of variation of the absorbance were between 0.0009 and 0.097% at 203 nm. One of the LMMH absorbed also at 230 nm (Clexane), which is due to its terminal unsaturated uronic residue. Therefore some of the LMM–GAGs can be detected by their specific UV spectra. The intra-day coefficients of variation for the retention time ranged from 0.016 to 3.90%, whereas the area under the curve showed higher coefficients of variation from 4.17 to 21.15% (Table 1).

The intra-day coefficients of variation of the average molecular mass M_m are different for unfractionated and low-molecular-mass compounds. They ranged for unfractionated compounds from 2.37 to 7.43% and for the low-molecular-mass compounds from 0.95 to 20.28%. The polydispersity (P) of unfractionated preparations varied from 2.91 to 3.02%, whereas low-molecular-mass preparations were between 0.92 and 6.96% (Table 2).

4.2. Polyacrylamide gel electrophoresis

The molecular mass of the GAG preparations was assayed by polyacrylamide gel electrophoresis (Fig. 3) according to Ref. [8]. The bands of the com-

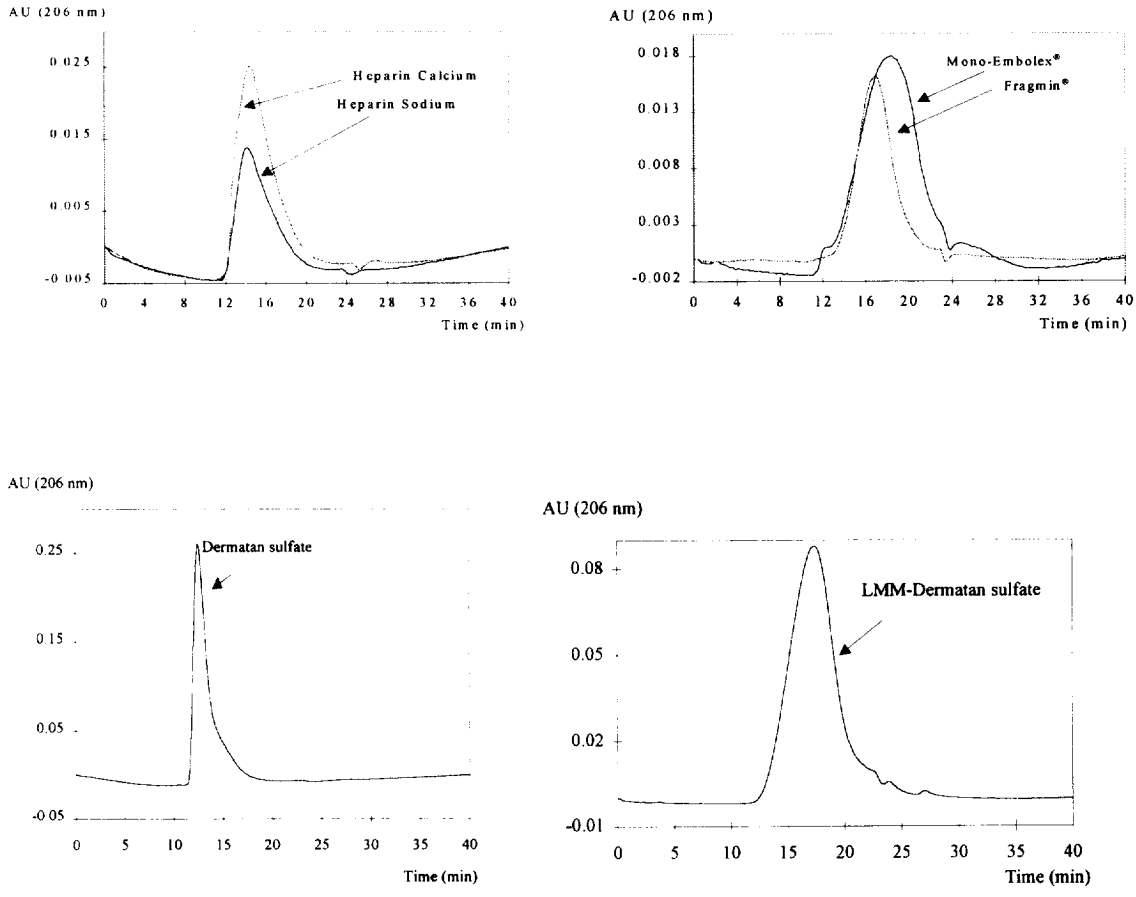

Fig. 1. HPSEC of heparin (calcium and sodium salt), low-molecular-mass heparins (Mono-Embolex and Fragmin) dermatan sulfate and low-molecular-mass dermatan sulfate.

pounds were visualized using acridine orange. The coefficients of variation of the average molecular mass M_m ranged from 3.36 to 19.78% and that of the polydispersity (P) from 6.67 to 14.49 (Table 2).

4.3. Comparison of HPSEC and PAGE

The mean values of the molecular mass obtained by PAGE and HPSEC are somewhat different and show a correlation coefficient of the average molecular mass M_m of $r^2 = 0.73$. Oligosaccharides were resolved better by PAGE than by HPSEC. The polydispersity P of the GAGs is lower using PAGE compared with HPSEC.

4.4. HPCE of heparin and dermatan sulfate

Negatively charged glycosaminoglycans like heparin and chondroitin sulfates show similar electropherograms using high-performance capillary electrophoresis (Fig. 4). The resolution of these compounds is increased when the reversed polarity mode is used. The higher the charge density of the molecule the faster the compounds migrate. Therefore highly sulfated low-molecular-mass heparins with a range from 9.69 min to 11.82 min show shorter migration times than heparin (12.54 min, see Table 3). The differences between heparin and low-molecular-mass heparin and dermatan sulfate and low-molecular-mass dermatan sulfate are not pro-

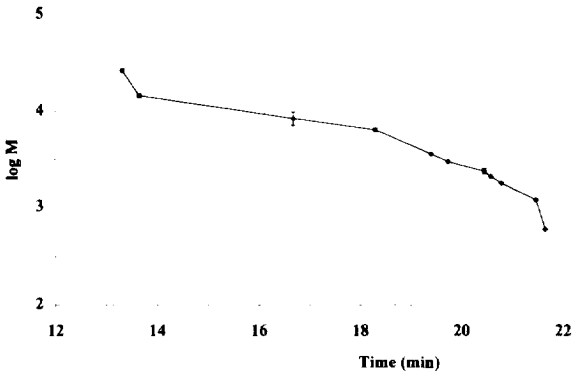

Fig. 2. The calibration curve of the Ultropac TSK G 2000 SW column is displayed. Heparin–oligosaccharides (di-, tetra, penta, hexa-, octa-, decasacharide) and heparin and dermatan sulfates of known molecular mass were analyzed in 3 runs within one day. The y-axis denotes the logarithm of the molecular mass (M_m) and the x-axis, the retention time. An equation of third order was fitted to the data points. The correlation coefficient was 0.98.

Table 2
Determination of the molecular mass and the polydispersity of GAGS using PAGE

Compound	M_m PAGE	Polydispersity PAGE
Dermatansulfate	n.d.	n.d
Heparin	12 760±757	1.23±0.16
LMMH-Merckle	8458±566	1.46±0.11
Fragmin	5948±476	1.23±0.10
Mono-Embolex	5675±692	1.38±0.20
LMMH-Braun	5550±1098	1.35±0.10
Fraxiparin	4807±841	1.20±0.09
LMM-Dermatan sulfate	4675±308	1.35±0.09
Clexane	4370±369	1.35±0.14
Reviparin	4311±145	1.30±0.07

The componds are displayed in descending order of the average molecular mass. Heparin and dermatan sulfate were unfractionated compounds, the other GAGs were low-molecular-mass compounds. The polydispersity is calculated according to Refs. [4,5] (mean±S.D., $n=5$).

nounced using a 75 μm capillary. Dermatan sulfate (12.57 min), however, has a shorter migration time than low-molecular-mass dermatan sulfate (14.45 min). The peak height ranged from 0.36 to 2.45 AU·cm^{-2}. The peak area was measured between 2.75 and 37.2 AU·min·cm^{-2} with high values for dermatan sulfate, low-molecular-mass dermatan sulfate and Clexane.

5. Discussion

Heparin belongs to the glycosaminoglycans and is a polysaccharide which consists of derivatives of the disaccharide unit 2-amino-2-deoxy-D-glucopyranose-(1→4). The most frequent glucosamine unit is *N*-sulfated (Glc*N*SO$_3$) although occasionally it is *N*-acetylated (Glc*N*Ac). Heparin is widely used as an antithrombotic agent and is an anionic polydisperse

Table 1
Determination of the molecular mass, polydispersity, area under the curve (AUC) and spectral absorbance of heparins and dermatan sulfates performed by HPSEC

Compound	M_m HPSEC	Polydispersity (P) HPSEC	AUC (AU min)	Spectral absorbance (nm)
Dermatan sulfate	22 145±372	1.16±0.035	0.486±0.041	203.2±0.053
Heparin	11 231±837	1.48±0.043	0.052±0.011	203.1±0.197
LMMH-Merckle	9084±186	1.37±0.018	0.072±0.003	202.9±0.065
LMM-Dermatan sulfate	6607±63	1.41±0.013	0.414±0.039	203.0±0.002
Fragmin	6441±403	1.35±0.094	0.047±0.007	202.7±0.069
Mono-Embolex	5558±186	1.57±0.091	0.091±0.019	202.8±0.107
Fraxiparin	5453±1106	1.23±0.019	0.108±0.012	202.9±0.137
Reviparin	4903±111	1.30±0.033	0.062±0.004	202.7±0.131
LMMH-Braun	4772±447	1.40±0.027	0.046±0.004	203.1±0.034
Clexane	4424±201	1.27±0.034	0.387±0.049	203.2±0.026 232.0±0.002

The compounds are displayed in descending order of the average molecular mass. Heparin and dermatan sulfate were unfractionated compounds, the other GAGs were low-molecular-mass compounds. The polydispersity is calculated according to Refs. [4,5]. The mean±S.D. of five runs is displayed.

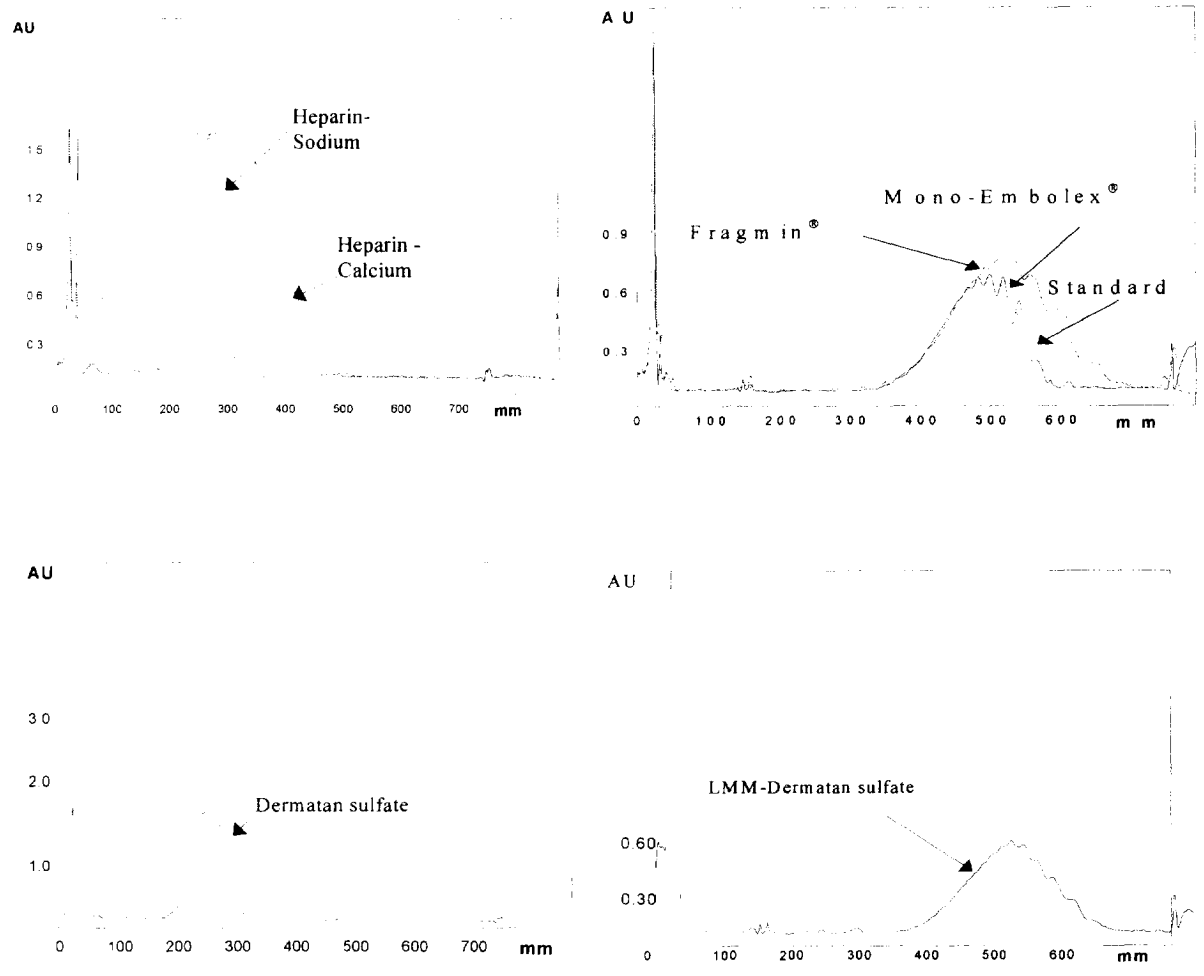

Fig. 3. Electropherograms of heparin (calcium and sodium salt), low-molecular-mass heparins (Mono-Embolex and Fragmin) dermatan sulfate and low-molecular-mass dermatan sulfate are displayed. Reviparin was used as standard compound for the calculation of the molecular mass, see Ref. [8]. The absorbance wavelength was 548 nm.

macromolecule with a wide range of average molecular masses from 3000 to 30 000. Heparin and low-molecular-mass heparin act as well as an anticoagulant drug by binding antithrombin III and accelerating the rate at which the protein complex inactivates the serine proteases of the haemostatic system. Dermatan sulfate belongs to the family of galactosaminoglycans [1]. It is a co-polymer of following disaccharide repeats: D-glucuronate-*N*-acetylgalactosamine which can be *O*-sulfated at C-4 or C-6 and L-iduronate-*N*-acetylgalactosamine, which is almost exclusively 4-*O*-sulfated. The antithrombotic action of dermatan sulfate is related to a catalysis of inhibition of thrombin by heparin cofactor II.

For the analysis of the molecular mass and polydispersity of glycosaminoglycans different column materials are available [9,10]. For the determination of the molecular mass a small standard deviation of the retention time from run to run and a broad sieving range of the column material are necessary.

An international study for the determination of the molecular mass of LMMHs was described by van Dedem and Nielsen [9]. The method used a polycomponent mixture of heparin oligosaccharides

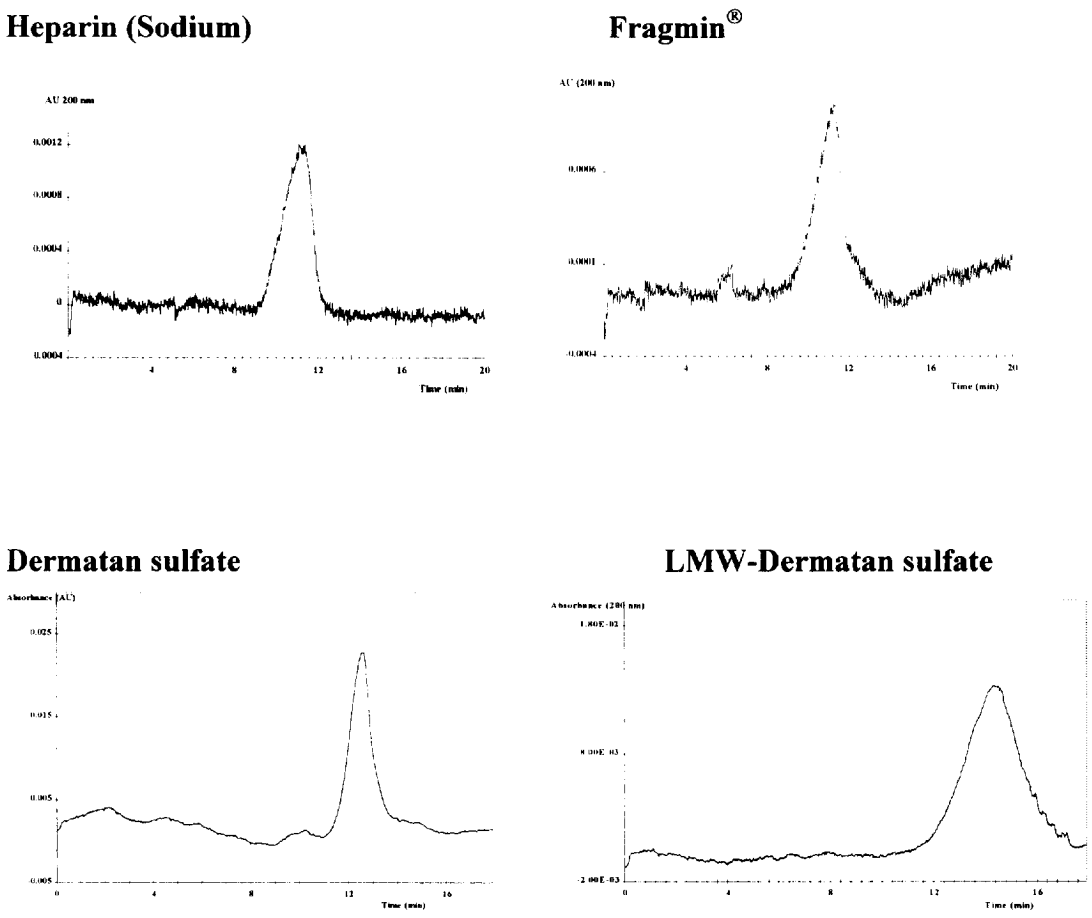

Fig. 4. High-performance capillary electrophoresis of heparin (sodium salt), low-molecular-mass heparin (Fragmin) dermatan sulfate and low-molecular-mass dermatan sulfate. The conditions are summarized in Section 2.

Table 3
Determination of the migration time, area under the curve and spectral absorbance of heparins and dermatan using capillary electrophoresis

Compound	Migration time (min)	Peak height ($AU \times 10^{-2}$ cm)	Peak area ($AU\ min \times 10^{-2}$ cm)
Dermatan sulfate	12.57	n.d.	29.14
Heparin	12.54±0.45	2.45±0.876	5.42
Clexane	11.50±0.08	0.99±0.759	37.20
Fragmin	9.69±0.49	0.36±0.071	3.41
Fraxiparin	10.28±0.06	2.29±0.027	2.75
LMM-Dermatansulfate	14.45	n.d.	29.14
Mono-Embolex	11.82±0.74	0.36±0.071	3.12

Heparin and dermatan sulfate were unfractionated compounds, the other GAGs were low-molecular-mass compounds. Capillary electrophoresis was run 3 times using reversed polarity. (The mean±S.D. of three runs is displayed).

chains produced by heparinase which catalyze partial degradation of UFH. The calibrator contained a series of peaks which differ by disaccharide units which have an assumed molecular mass of 600. The range of the column was between 1.2 and $12 \cdot 10^3$ which is especially efficient for LMMH.

For our calibration, synthesized heparin oligosaccharides ranging from di- to decasaccharides and GAG-preparations with a molecular mass ranging from 2.1 to $26.3 \cdot 10^3$ were used. The x-axis denotes the elution time in min and the y-axis the logarithm of the molecular mass.

The limitations of the HPSEC method for the determination of the molecular mass are: the absence of high-molecular-mass oligosaccharide standards and the difficulty to have enough 'LMM' oligosaccharide standards available and the different structures and origins of calibrators which were used.

The differences in sulfation and mass of heparins and low-molecular-mass compounds influences also their pharmacological profiles [11]. Therefore the methods used for the measurement of activity and mass should be comparable to data obtained by other laboratories.

Electrophoresis of heparin and dermatan sulfate can be performed by agarose gel electrophoresis in barium trimethylenediamine and polyacrylamide gel electrophoresis [12]. For the resolution of low-molecular-mass heparins and dermatan sulfate PAGE was found to be better. The macromolecules are separated in polyacrylamide gels according to their molecular mass together with their shape and charge. UFH and unfractionated dermatan sulfates can not be resolved to defined bands whereas low-molecular-mass compounds show defined bands. The results of their molecular masses were similar to those obtained by HPSEC but showed a larger standard deviation which is mainly due to the hand made preparation of the gels from day to day.

Using capillary zone electrophoresis, the analysis of heparin and dermatan sulfate fragments have been performed. Capillary zone electrophoresis of heparin–disaccharides was first described by Ampofo et al. [6]. The composition of heparin and heparan sulfate was analyzed with heparin–disaccharides which were standardized by fast atom bombardment mass spectroscopy. A complete resolution of the highly charged glycosaminoglycans into chains of different size and charge is not yet possible. Reversing the polarity of the system gives a better resolution of longer oligosaccharides [8]. Differences in the resolution are also recorded using capillaries of different lengths and diameters. The heparins used were of research quality and contain a small proportion of ethanol (0.5 to 1%) and dermatan sulfate (<1%). The measurement of heparins and dermatan sulfates and their low-molecular-mass compounds may be also used to measure different concentrations of the drug in a formulation.

The chromatographic and electrophoretic methods discussed offer a variety of possibilities to analyze the heterogeneous matrix called heparin or dermatan sulfate. An international standard for the calibration of the molecular mass of heparins and dermatan sulfate and low-molecular-mass heparins and dermatan sulfate should be found as a calibrator in HPLC and HPCE methods.

6. List of abbreviations

GAGs	Glycosaminoglycans
HPCE	High-performance capillary electrophoresis
HPSEC	High-performance size exclusion chromatography
LMMH	Low-molecular-mass heparin
P	Polydispersity
M_m	Average molecular mass
M_n	Number average molecular mass
M_z	z-Average molecular mass
Δ UA	4-Deoxy-α-L-threo-hex-4-enoxypyranose
GlcNAc	2-Deoxy-2-acetamidoglycopyranose
AU	Absorption unit
Da	Dalton

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