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Journal of Chromatography A, 828 (1998) 497–508

JOURNAL OF CHROMATOGRAPHY A

Use of reversed polarity and a pressure gradient in the analysis of disaccharide composition of heparin by capillary electrophoresis

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Abstract

A capillary electrophoresis method with reversed polarity, combining both the application of a voltage and a pressure gradient between the buffer vials, was developed for the analysis of eight heparin-derived Δ-disaccharides obtained by enzymatic depolymerization. A 60 m*M* formic acid buffer at pH 3.40 was selected as running electrolyte, with an applied voltage of −15 kV and an overimposed pressure gradient ($3.45 \cdot 10^{-3}$ MPa) for 6 min from inlet to outlet starting at 20 min. Figures of merit such as run-to-run and day-to-day precision, and limits of detection were established. The electrophoretic method was applied to the analysis of depolymerization products of different kinds of heparins. The composition of the depolymerization buffer was selected in order to reduce baseline distortions in the electrophoretic separation, thus a buffer solution containing 20 m*M* Tris, 50 m*M* sodium chloride, and 3 m*M* calcium chloride at pH 7.10 was used. Percentages of molar disaccharide compositions for unfractionated heparins from porcine, bovine and ovine intestinal mucosa, and bovine lung were determined. In addition, low-molecular-mass heparins from bovine and porcine intestinal mucosa were analysed as well.

Keywords: Disaccharides; Heparin

1. Introduction

Heparin, a widely used anticoagulant agent, is a polydisperse highly sulphated glycosaminoglycan (molecular mass range: 3000–40 000), which is biosynthesised as a proteoglycan consisting of a small core protein to which polysaccharide side-chains are attached [1]. It is composed of repeating disaccharide units of uronic acid; α-L-iduronic (IdoA) or β-D-glucuronic acid (GlcA), and β-D-glucosamine (GlcN) residues linked by α(1→4) bonds [2]. The glucosamine unit can be either *N*-acetylated or *N*-sulphated. The disaccharide units can be *O*-sulphated at the C-6 and/or C-3 of glucosamine and also at C-2 of the acid residues [3,4]. It is known that the molecular mass distribution and degree of sulphation are closely related to the biological properties of heparin [5]. Since the 1980s low-molecular-mass heparins (molecular mass range: 2000–8000) have been extensively studied because they show better antithrombic activity and bioavailability [6–8].

Heparin is depolymerized through an eliminative mechanism by heparin lyases, isolated from *Flavobacterium heparinium*, giving compositional Δ-disaccharides and oligosaccharides with unsaturated uronic acid residues (ΔUA) at their nonreducing termini [9,10]. Heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (heparitinase II, no assigned EC number) and heparin lyase III (heparitinase I, EC 4.2.2.8) act on specific linkages, and thus suitable mixtures of these three heparin lyases are needed to

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00662-1

produce characteristic Δ-disaccharides from different kinds of heparin [9–11]. Heparin lyase I cleaves heparin sequences at the glycosidic linkage: GlcNS(±6S)α(1→4)IdoA(±2S), while heparin lyase III cleaves heparin and heparin sulphate sequences containing GlcNS or GlcNAc which are not sulphated at C-6. Heparin lyase II activity is less specific, as it can act on disaccharide sequences containing two or three sulphates per disaccharide unit [9–11]. The analysis of the products obtained by enzymatic degradation provide a compositional pattern to characterize the origin of heparin samples.

High-performance liquid chromatography (HPLC) [9,12–17] and capillary electrophoresis (CE) methods have been applied to the separation and identification of heparin-derived Δ-disaccharides. In particular, CE is an effective technique for the analysis of these disaccharides at normal and reversed polarity with UV detection [11,18–26]. Borate buffers are usually used at normal polarity [11,19–22]. Reversed polarity methods are based on the use of phosphate electrolyte systems at a pH close to 3.50 [22–26]. To date, most of these CE electrolyte systems provide long run times for the migration of all the Δ-disaccharides, moreover these electrolytes are not suitable for coupling to a mass spectrometer for the selective detection of heparin digestion products. In order to obtain reliable comparison of heparin compositional patterns, reproducibility of Δ-disaccharides determination should be improved. In this work, the influence of the electrolyte composition and other electrophoretic conditions in the precision of Δ-disaccharides determination was studied.

With the aim of improving separation efficiency, running buffers with better buffering characteristics at acidic pH values were studied. Solutions of formic acid buffers at different pH values were used in order to optimize the electrophoretic separation of heparin-derived Δ-disaccharides with photodiode array detection. The volatility of these buffers could enable the coupling of this CE separation to a mass spectrometer, thus enhancing the reliability of the heparin characterization. Electrophoretic separation combining both electrical and pressure gradients to reduce analysis time was also evaluated. The separation method was applied to the depolymerization product mixture of heparin samples of different origins. The composition of the depolymerization buffer was also selected to reduce matrix effects in the analytical separation. The disaccharide molar composition of porcine, bovine and ovine heparins and low-molecular-mass heparins (LMMHs) from different tissues were determined following the optimum experimental conditions of the method.

2. Experimental

2.1. Chemicals

Eight heparin standard Δ-disaccharides (sodium salts) were obtained from Sigma (St. Louis, MO, USA): ΔUA2S-(1→4)-D-GlcNS6S (IS), ΔUA-(1→4)-D-GlcNS6S (IIS), ΔUA2S-(1→4)-D-GlcNS (IIIS), ΔUA-(1→4)-D-GlcNS (IVS), ΔUA2S-(1→4)-D-GlcNAc6S (IA), ΔUA-(1→4)-D-GlcNAc6S (IIA), ΔUA2S-(1→4)-D-GlcNAc (IIIA), ΔUA-(1→4)-D-GlcNAc (IVA). Their structures are shown in Fig. 1.

Stock solutions of each Δ-disaccharide were prepared at concentrations ranging from 400 to 1000 mg l^{-1} on the basis of their absorbance at 232 nm (ϵ_{232}=5500 M cm^{-1}) [20,21].

Unfractionated and LMMHs (sodium salts) from different sources were used. Heparin and LMMH (peroxide depolymerized) from intestinal porcine mucosa were kindly provided by Bioibérica (Palafolls, Barcelona, Spain). Bovine lung, bovine intestinal and ovine intestinal unfractionated heparins, and a bovine intestinal LMMH (peroxide depolymerized) were purchased from Sigma.

Heparin lyase I (50 Sigma units), heparin lyase II (10 Sigma units) and heparin lyase III (5 Sigma units) were obtained from Sigma. One unit produces 0.1 μmol of unsaturated uronic acid per hour at pH 7.5 at 25°C. One international unit (I.U.) is equivalent to approx. 600 Sigma units.

Sodium hydroxide, tri(hydroxymethyl)-aminomethane, sodium chloride, formic acid 98%, and ammonia solution 25% were from Merck (Darmstadt, Germany). Potassium dihydrogenphosphate was from Fluka (Ronkonkoma, NY, USA).

Water purified using a Culligan water-purification system (Barcelona, Spain) was used for all solutions.

2.2. Capillary electrophoresis conditions

The experiments were performed on a P/ACE

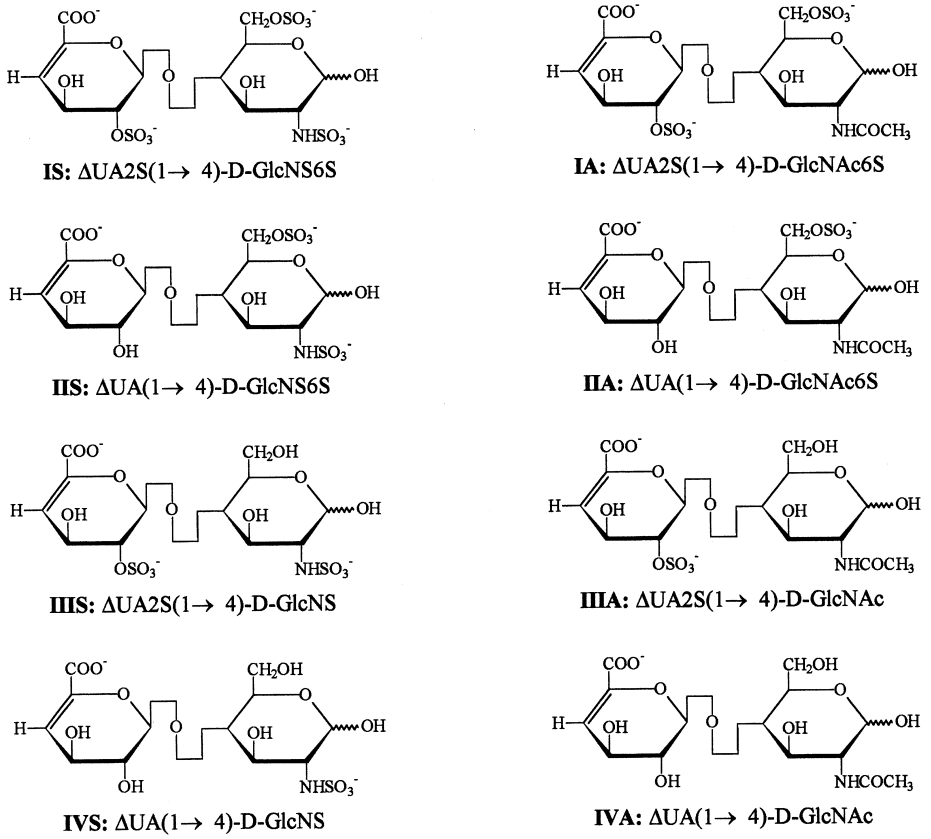

Fig. 1. Chemical structure of the eight heparin Δ-disaccharides obtained by enzymatic depolymerization.

System 5500 (Beckman Instruments, Fullerton, CA, USA) equipped with a photodiode array detector.

The temperature was held at 25°C. Running electrolytes were filtered through a 0.45-μm membrane filter, and degassed before use.

The electrophoretic separations were carried out using uncoated fused-silica capillaries, with a total length of 57 cm (separation length 50 cm)×75 μm I.D. from Supelco (Bellefonte, PA, USA). New capillaries were pre-treated with 1 *M* sodium hydroxide for 30 min, then rinsed with ultrapure water for 20 min. The capillary was conditioned with the running electrolytes for 60 min before the first run and for 5 min in-between runs. Samples were loaded by pressure injection for 3 s at $3.45 \cdot 10^{-3}$ MPa (0.5 p.s.i.). The separations were performed at a voltage in the range of −25 kV and −12 kV.

An overimposed pressure gradient, in addition to the electrophoresis voltage, was applied in some electrophoretic separations. It consisted in the application of a pressure value of 0.5 p.s.i. between the inlet and the outlet buffer vials. The conditions applied for the pressure gradient separations were: 0–20 min (0 p.s.i.), 20–26 min (0.5 p.s.i.).

2.3. Enzymatic depolymerization conditions

Heparin lyase I, heparin lyase II and heparin lyase III were dissolved in 100 μl 40 m*M* Tris, 100 m*M* sodium chloride solution adjusted to pH 7.02 with 1 *M* hydrochloric acid. Ten aliquots of 30 μl containing 7.8 units of heparin lyase I, 1.0 unit of heparin lyase II, and 0.9 units of heparin lyase III were stored in single Eppendorf vials at −18°C until use.

Solutions of heparin samples were prepared at a concentration of 2000 μg ml^{-1} in 8 m*M* Tris, 20 m*M* sodium chloride, 4.8 m*M* calcium chloride solution buffered to pH 7.10 with 1 *M* hydrochloric acid. Fifty μl of this solution was added to a lyase

aliquot and homogenized. The final concentration of the depolymerization buffer was 20 m*M* Tris, 50 m*M* sodium chloride and 3 m*M* calcium chloride.

Digests were performed in a water bath at 37°C with agitation for 24 h. The enzymatic reactions were stopped by boiling for 1 min. Depolymerized samples were stored frozen at −18°C.

3. Results and discussion

3.1. CE optimization

Reversed polarity methods in which the sample is applied at the cathode and detected at the anodic end of the capillary, are usually used to analyse negatively charged species. In reversed polarity a low pH electrolyte is used, which ensures that the silanol residues on the capillary are uncharged. At a low pH, electroosmotic flow (EOF) towards the cathode is reduced, and thus the anions migrate to the detector quickly. Phosphate (pK_{a1} 2.1), acetate (pK_a 4.8) and formate (pK_a 3.8) buffers are frequently used as running buffers at low pH values. CE separation of heparin derived Δ-disaccharides in reversed polarity has mostly been carried out using running buffers consisting of phosphate solutions with concentrations ranging from 15 to 200 m*M* adjusted to pH values close to 3.50 [22–26]. At this pH, sulphated Δ-disaccharides are negatively charged due to the ionisation of their *O*- and *N*-sulphate groups. However, the carboxylic group of the uronic acid is only partially dissociated. Thus, Δ-disaccharide IVA, which is nonsulphated, has low anionic characteristics in these conditions (pK_a values=3.1–3.2 [26]). Formic acid buffers cover a range of pH between 2.8 and 4.8 with better buffering properties than other buffers that are frequently used in these separations such as phosphate. In this study, in order to compare the suitability of the buffer, 20 m*M* potassium dihydrogenphosphate (KH_2PO_4) and formic acid solutions with concentrations ranging from 50 to 130 m*M* at pH values close to 3.50 were used as running buffers.

The pH of formic buffers were adjusted either with 1 *M* NaOH or with ammonia (12%). Separations carried out with both kinds of buffers were similar. Ammonia-adjusted buffers could be used for coupling to a mass spectrometer due to their volatility. However, buffers adjusted with ammonia produced higher CE currents for the same voltage as those adjusted with NaOH, due to the different counterion. As an example, 80 m*M* formic acid buffer (pH 3.40) adjusted with 1 *M* NaOH gave a current value of 63 μA, while the same buffer adjusted with ammonia (12%) gave a value of 87 μA. In order to minimize current values, buffers adjusted with sodium hydroxide were used for this study.

3.2. pH

The effect of the pH value of a formic buffer on the electrophoretic migration and resolution of disaccharides was studied. The pH was varied between 3.0 and 4.0, using a 60 m*M* formic buffer. Fig. 2 shows the influence of pH on migration times and resolution of disaccharides IIS–IA and IIIA–IIA, applying −20 kV. The elution order was consistent with the anionic character of the Δ-disaccharides, and it did not depend on the composition of these running electrolyte solutions. Δ-Disaccharide IS, which has three sulphate substituents, was the first peak detected. Disulphated Δ-disaccharides (IIS, IIIS and IA) migrate before monosulphated Δ-disaccharides (IVS, IIA and IIIA). Sulphated Δ-disaccharides migrated in less than 18.5 min, while Δ-disaccharide IVA, the anionic characteristics of which are provided by the uronic acid group, was detected at migration times higher than 43–50 min with low reproducibility. The effect of pH on monosulphated Δ-disaccharides (IVS, IIA and IIIA) and non-sulphated Δ-disaccharide was greater than the effect on di- and trisulphated Δ-disaccharides. At high pH values monosulphated Δ-disaccharides migrate at lower migration times because of their higher dissociation, which is responsible for their anionic character. As their migration times decreased, a loss of resolution was observed between monosulphated Δ-disaccharides IIIA and IIA (1.33 at pH 3.00, 0.63 at pH 4.00), whereas it increased for disulphated IIS and IA (1.10 at pH 3.00, 2.86 at pH 4.00). Migration of tri- and disulphated Δ-disaccharides was not affected by pH changes, probably due to their pK_a values. A pH value of 3.40 was chosen because it provided the best buffering features, allowing res-

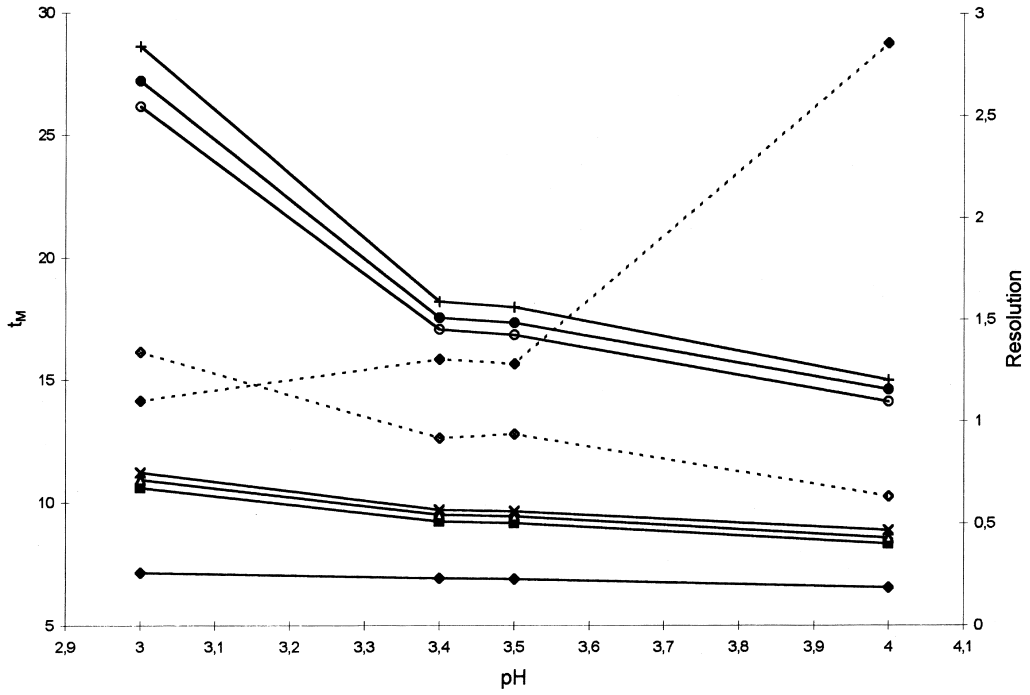

Fig. 2. Influence of pH on Δ-disaccharide migration times and resolution between Δ-disaccharides IIS–IA and Δ-disaccharides IIIA–IIA ($t_{m\ IVA}$ is higher than 50 min). Straight lines (migration times): ♦ dis IS, ■ dis IIIS, △ dis IIS, × dis IA, ○ dis IVS, ● dis IIIA, + dis IIA. Dashed lines (resolution): ♦ resolution IIS–IA, ◇ resolution IIIA–IIA. Experimental conditions: 60 m*M* formic acid buffer, V: −20 kV, injection time (0.5 p.s.i.): 3 s. Δ-Disaccharide (sodium salt) concentrations: IS: 74 μg ml^{-1}; IIS: 71 μg ml^{-1}; IIIS: 68.2 μg ml^{-1}; IVS: 66.5 μg ml^{-1}; IA: 80.2 μg ml^{-1}; IIA: 68.6 μg ml^{-1}; IIIA: 66 μg ml^{-1}; IVA: 70.7 μg ml^{-1}.

olutions higher than 1.0 for monosulphated Δ-disaccharides and a reasonable analysis time.

3.3. Concentration

The effect of formic buffer concentration on electrophoretic separation was studied at pH 3.40, −20 kV, using formic buffers at 130, 100, 80, 60 and 50 m*M*. Migration times were similar for all buffers, and the resolution was sufficient in all cases. An increase in concentration produced higher electrophoresis current values. Moreover, peaks of *N*-acetylated disaccharides presented distortions as shoulders at high electrolyte concentrations as can be seen in Fig. 3. This may be due to the occurrence of two anomeric forms (α and β) for these disaccharides, as found by other authors [13,27] using HPLC. However, this phenomenon was not observed for *N*-sulphated disaccharides. It seems that the presence of the *N*-sulphate group allowed the occurrence of only one anomeric form. The use of high concentration buffers seems to enhance resolution of these anomeric forms, which migrate as a slightly broader but single peak with low concentration buffers, being more suitable for quantification purposes. A concentration of 60 m*M* was selected, because migration times using 50 m*M* formic buffer were less reproducible.

3.4. Voltage

The linear velocity of the flow of the analyte through the capillary is determined by the electric field strength. Therefore, an increase in the voltage enhances the velocity of electrophoretic migration and decreases migration time. Separations were carried out at a voltage in the range of −25 and −12 kV. As expected, an increase in peak areas was observed at low voltages. However, peaks corresponding to N-acetylated disaccharides were dis-

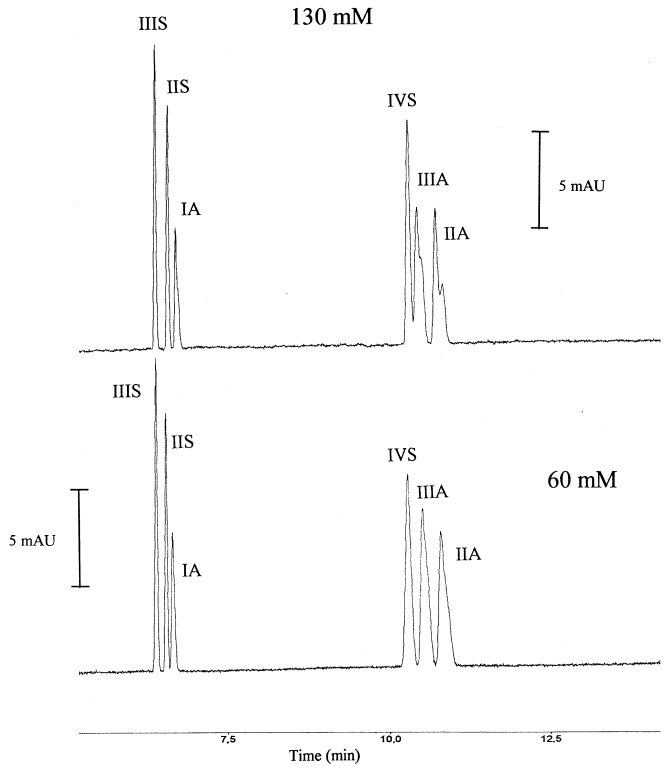

Fig. 3. Sections of the electropherograms of Δ-disaccharides in 130 and 60 m*M* formic acid buffer, pH 3.40. Experimental conditions as in Fig. 2.

torted. At high voltages, resolution between disulphated Δ-disaccharides IIS–IA and monosulphated Δ-disaccharides IIIA–IIA decreased from 1.10 (−15 kV) to 0.9 (−25 kV), and 0.97 (−15 kV) to 0.80 (−25 kV), respectively. Resolution between monosulphated Δ-disaccharides IVS–IIIA was constant and close to 0.80. A voltage of −15 kV was selected as optimum because it provided resolutions close to 1.0 for most of the separations, while analysis run time remained similar, as migration of Δ-disaccharide IVA occurred at 44.41 min at −18 kV, and at 51.45 min at −15 kV, with low reproducibility (relative standard deviation, R.S.D. 10–15%).

3.5. Pressure gradient

The high migration time of Δ-disaccharide IVA and its low reproducibility was due to its reduced anionic character, caused by the partial ionization of this Δ-disaccharide which leads to a low effective charge. This was also observed in formic buffers, and has been reported to occur in phosphate buffers [23,25]. While an increase in pH could enhance its anionic character, the consequent increase in silanol ionisation would increase EOF towards the injection inlet, preventing its detection at the anodic outlet.

In order to reduce run time, a 6-min pressure gradient (0.5 p.s.i.) from inlet to outlet was applied after the detection of the sulphated Δ-disaccharides (20 min). Electropherograms obtained with and without a pressure gradient are shown in Fig. 4. A significant reduction in run time was observed for Δ-disaccharide IVA. Run times and peak areas for the eight Δ-disaccharides were reproducible, even without replenishing buffer vials for 10 runs. Table 1 shows the migration times of the eight Δ-disaccharides, using two running electrolytes (pH 3.40), a 20 m*M* KH_2PO_4 solution and a 60 m*M* formic acid solution, applying the pressure gradient at a constant voltage (−15 kV). Electrophoresis current values

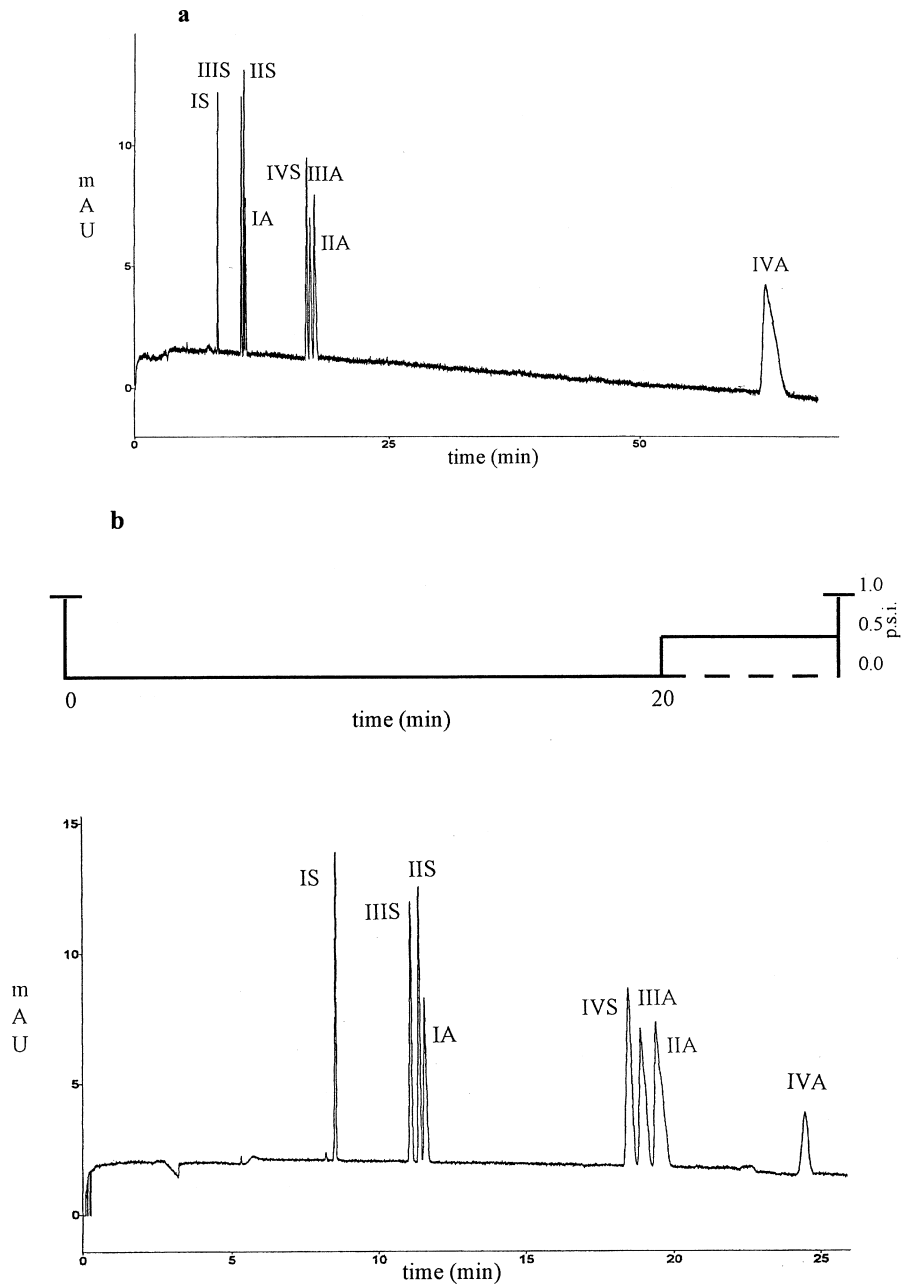

Fig. 4. (a) Electropherogram of Δ-disaccharide standard without pressure gradient. Experimental conditions: 60 m*M* formic buffer, pH 3.40, V applied: −15 kV, injection time (0.5 p.s.i.): 3 s. Δ-Disaccharide (sodium salt) concentrations: IS: 74 μg ml^{-1}; IIS: 70.3 μg ml^{-1}; IIIS: 70.7 μg ml^{-1}; IVS: 83.3 μg ml^{-1}; IA: 64.3 μg ml^{-1}; IIA: 70 μg ml^{-1}; IIIA: 68 μg ml^{-1}; IVA: 67.4 μg ml^{-1}. (b) Electropherogram of Δ-disaccharide standard with pressure gradient (0.5 p.s.i.) from inlet to outlet applied at 20 min. Rest of experimental conditions as in (a).

Table 1
Influence of buffer and pressure gradient on run-to-run precision

Disaccharide	With pressure gradient						Without pressure gradient		
	20 m*M* KH_2PO_4 (pH 3.40)			60 m*M* HCOOH buffer (pH 3.40)			60 m*M* HCOOH buffer (pH 3.40)		
	t_m (min)	R.S.D. (t_M) (%)	R.S.D. (area) (%)	t_m (min)	R.S.D. (t_M) (%)	R.S.D. (area) (%)	t_m (min)	R.S.D. (t_M) (%)	R.S.D. (area) (%)
IS	7.70	0.58	8.17	8.56	0.32	1.54	8.16	1.38	10.38
IIIS	9.59	0.73	5.60	11.11	0.17	1.46	10.86	1.81	19.97
IIS	9.83	0.72	6.58	11.38	0.14	1.09	10.70	1.74	20.63
IA	9.97	0.75	7.73	11.58	0.18	1.47	10.86	2.84	20.50
IVS	14.32	1.23	5.83	18.54	0.32	1.54	16.79	2.75	20.64
IIIA	14.55	1.24	6.58	18.96	0.32	1.80	17.12	2.84	20.50
IIA	14.85	1.26	6.48	19.48	0.32	2.46	17.54	2.94	19.63
IVA	22.68	0.22	8.49	24.52	0.15	2.55	60.63	8.78	28.24

were similar in both running electrolytes (≈32 μA). The R.S.D. of migration times and peak areas was obtained from six replicates of a standard mixture of the Δ-disaccharides with concentrations ranging from 64 to 83 mg l^{-1} (as sodium salts) carried out the same day. Data obtained using the formic buffer without the application of a pressure gradient are also included. A significant reduction in analysis time (60 min to 25 min) was obtained by the application of a pressure gradient. In addition, run-to-run precision of both migration times and peak areas significantly improved for all the compounds, because the shorter analysis time improved the conditioning and equilibrating properties of the capillary. Furthermore, the use of formic acid buffer improved the precision of both migration times and areas, which could be related to its better buffering capacity at this pH in comparison to phosphate buffer.

As a result of these studies, a 60 m*M* formic acid–NaOH buffer at pH 3.40 was selected as the most adequate running buffer for the separation of the eight Δ-disaccharides, with an applied voltage of −15 kV and an overimposed pressure gradient (0.5 p.s.i.) for 6 min from inlet to outlet starting at 20 min.

3.6. Day-to-day precision and limits of detection

The day-to-day precision of migration times and peak areas expressed as the R.S.D. of four replicates of the standard Δ-disaccharide solution on four different days is given in Table 2. While migration times were satisfactorily reproducible (R.S.D. lower

Table 2
Day-to-day precision of migration times, areas and concentration (R.S.D.), and limits of detection

Disaccharide (sodium salt)	Day-to-day precision		Detection limits	
	R.S.D. (t_m) (%)	R.S.D. (conc) (%)	μg ml^{-1}	μ*M*
IS	1.91	18.20	1.58	2.38
IIIS	2.24	16.72	1.62	2.88
IIS	2.38	15.25	1.41	2.51
IA	2.29	18.18	2.77	4.58
IVS	3.24	16.47	1.33	2.88
IIIA	3.30	15.51	2.21	4.39
IIA	3.38	15.68	2.04	4.05
IVA	1.03	12.12	5.05	12.5

Pressure injection (0.5 p.s.i.): 3 s.
Concentration of disaccharide standard with 40 μg ml^{-1}

than 3.40%), peak areas obtained from injections without an internal standard varied considerably. A contributing factor to this variation could be the use of low-volume (30 μl) inserts for the standard vials. For this reason, a daily calibration is essential for quantitative purposes, as indicated by the difference between the deviations of areas and concentrations calculated for a standard with 40 μg ml^{-1} for each anion.

The limits of detection (LODs) based on a 3:1 signal-to-background noise ratio ranging from 1.33 to 5.05 μg ml^{-1} for each Δ-disaccharide at $\lambda=232$ nm are given in Table 2. These values are consistent with published data [23,28]. At 200 nm, the signals of N-acetylated Δ-disaccharides (IA, IIA, IIIA and IVA) increased more than those of N-sulphated disaccharides (IS, IIS, IIIS and IVS). However, the LODs did not improve at this wavelength because baseline noise considerably increased.

3.7. Application

Heparin depolymerization by heparin lyases, such as heparin lyase I, heparin lyase II and heparin lyase III, can be performed in buffers of different compositions at a pH ranging from 7.00 to 7.50, and with a great variety of temperatures, times, and substrate/enzyme ratios [10,11]. Experimental conditions for heparin depolymerization with the three heparin lyases are selected in order to allow optimum activity for the three enzymes. Phosphate [9–11,19,20,24,25], acetate [10,13,17,26,29] and Tris·HCl [10,30] buffers at pH values 7.0–7.5 have been used to perform heparin depolymerization. Calcium is added to acetate and Tris·HCl buffers at a low concentration, usually less than 10 m*M*.

In this study, the composition of the depolymerization buffer was selected in order to reduce baseline distortions in the electrophoretic separation. Four depolymerization buffers selected from these studies, as well as buffered solutions of both heparin and mixtures of the disaccharides, were injected into the electrophoretic system and analysed. The buffers tested were sodium hydrogenphosphate (5 m*M* and 50 m*M*) with 200 m*M* sodium chloride at pH 7.10; 80 m*M* sodium acetate with 2 m*M* calcium acetate at pH 7.10; and 20 m*M* Tris with 50 m*M* sodium chloride and 3 m*M* calcium chloride at pH 7.10. The pH values were adjusted with 1 *M* hydrochloric acid in all cases. Buffers containing phosphate and chloride showed positive and negative baseline distortions, while acetate buffer increased baseline absorbance and background noise. The Tris·HCl buffer was selected because it did not affect baseline absorbance, and background noise was not increased. In addition, the composition of this buffer is similar to the buffer used in Sigma activity assays for heparin lyase I and heparin lyase III. Depolymerization of heparin samples was carried out following the procedures described in Section 2.3.

The optimized electrophoretic conditions for the analysis of the disaccharides were used to analyse the depolymerization products of different kinds of heparins. Electropherograms showed different profiles for heparins from different sources, as shown in Fig. 5 where the electropherograms for porcine intestinal mucosa and bovine lung heparins are given. Quantification of the molar concentration of each disaccharide was carried out by external calibration using Δ-disaccharide standards at five or six concentrations. Owing to the different concentration of each disaccharide in the depolymerized mixture, the use of appropriate concentration ranges for each disaccharide was necessary. Working linear ranges ($r^2=0.998$) for disaccharides IIS, IA, IVS, IIIA, IIA and IVA, were from 83 mg l^{-1} to the corresponding limits of detection whereas for the major disaccharide IS the higher limit was 883 mg l^{-1}, and 310 mg l^{-1} for disaccharide IIIS. However, as the response factors of each disaccharide were constant in these concentration ranges, one-level calibration could be use for routine work. The compositional disaccharide patterns were calculated as the molar percentage of each disaccharide in the sample. The results are given in Table 3. Percentages of disaccharide for porcine intestinal mucosal, bovine lung and LMMHs from porcine and bovine intestinal mucosa are consistent with those published by other authors [13,17,19,20,26,31]. For instance, the molar percentage of the major disaccharides IS and IIS typically range from 50 to 68% and from 10 to 14%, respectively, for porcine mucosal heparins [13,19,26,31]. In addition, in this study, the disaccharide composition of ovine intestinal mucosa was determined.

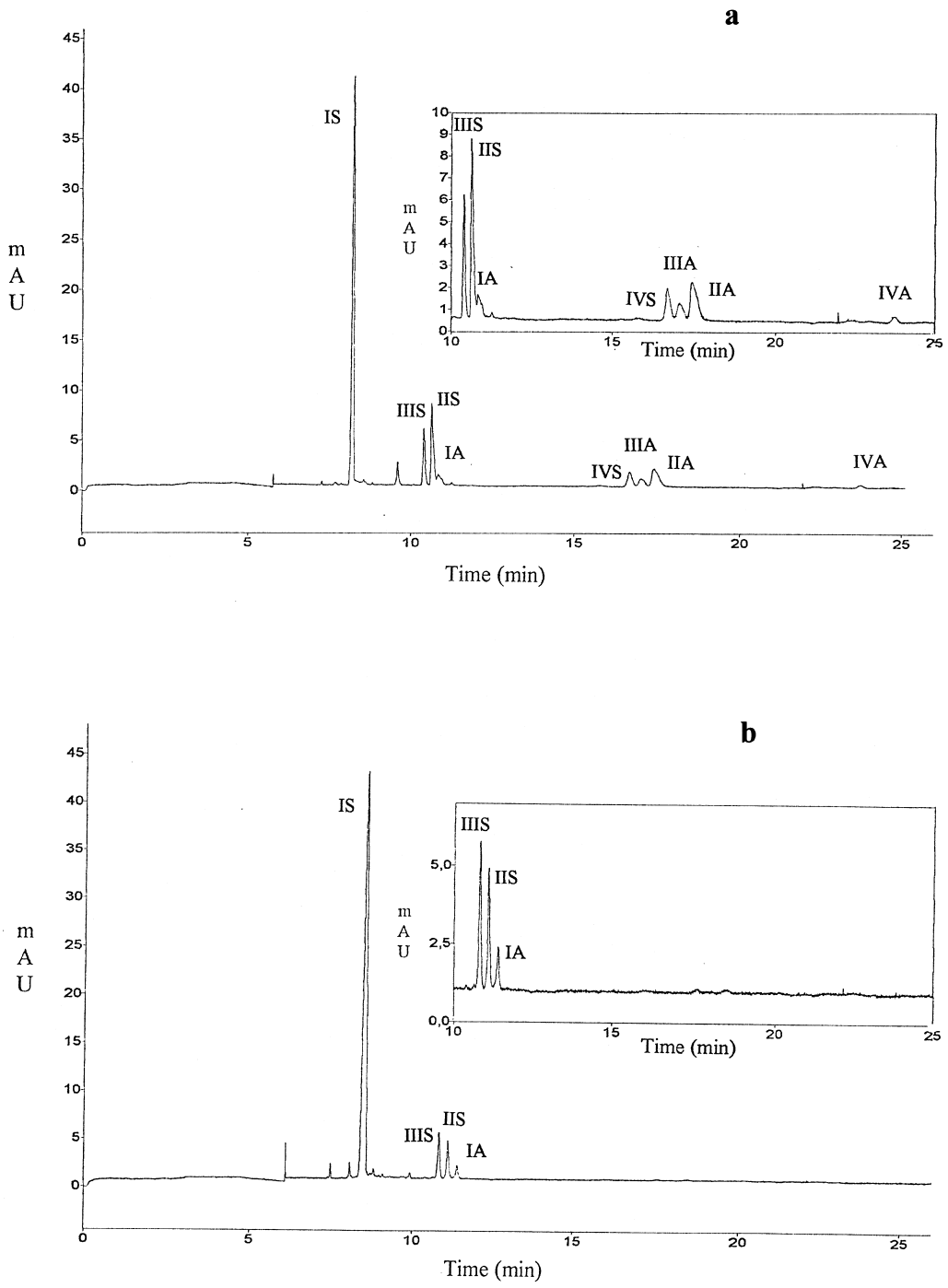

Fig. 5. Electropherograms of the depolymerized samples of (a) a porcine mucosal heparin and (b) a bovine lung heparin. Electrophoretic conditions as in Fig. 4b. Insets show peaks of minor disaccharides.

Table 3
Δ-Disaccharide molar composition (in %) of the depolymerized heparin samples

Disaccharide	Molar percentage (mol%)					
	Porcine mucosal	LMMH porcine mucosa	Bovine mucosal	Bovine lung	LMMH bovine mucosa	Ovine mucosal
IS	65.8	80.4	55.4	88.5	68.7	76.0
IIS	13.5	7.3	8.9	4.26	9.9	12.0
IIIS	7.5	4.55	30.5	5.08	16.5	7.4
IVS	2.63	0.60	2.74	n.d.	1.56	1.44
IA	3.51	5.86	0.51	2.19	2.64	1.22
IIA	4.14	1.03	0.24	n.d.	0.48	0.82
IIIA	1.18	0.28	1.53	n.d.	0.14	0.30
IVA	1.68	n.d.	0.23	n.d.	n.d.	0.89

n.d.=Not detected.

Heparin from porcine, bovine and ovine intestinal mucosa mainly differed in the presence of the trisulphated disaccharide IS, and the disulphated IIIS. Among these mucosal heparin samples, ovine heparin contained the highest percentage of trisulphated disaccharide IS (76.0%), while bovine heparin contained the lowest molar percentage for this disaccharide (55.4%) and the highest amount of disulphated disaccharide IIIS (30.5%). A high percentage of disaccharide IIIS (16.3%) was found in bovine LMMHs produced by peroxide depolymerization, and also in the corresponding unfractionated heparin. In contrast, for porcine LMMH low percentages (4.5%) were obtained, indicating that the origin of the initial native heparin might be distinguished in spite of the fractionation process. Bovine heparins from lung and intestinal mucosa showed different disaccharide profiles, which could be used for their identification. Lung bovine heparin consisted mainly of disaccharide IS (88.5%) with a low percentage of IIIS (5.1%), while mucosal bovine heparin had lower percentages of the trisulphated disaccharide IS and more disaccharide IIIS. It must be pointed out that in order to compare percentages of molar disaccharide compositions of different heparin samples, enzymatic depolymerizations and electrophoretic separation must be performed in the same experimental conditions because disaccharide abundance, especially that of minor disaccharides, can be significantly affected by experimental conditions, which can thus alter the disaccharide profile.

4. Conclusions

A CE method combining both electrical and pressure gradients was optimised for heparin disaccharide determination. The composition of the running buffer was optimised in order to improve buffering capacity, which proved to be related to migration time and area reproducibility. A 60 m*M* formic acid–NaOH buffer at pH 3.40 with an applied voltage of −15 kV was selected for the separation of the eight disaccharides studied. Good run-to-run precision (R.S.D. better than 2.55%) and day-to-day precision (R.S.D. ranging from 12.11 to 18.18%) were obtained. Limits of detection between 1.33 and 5.05 μg ml^{-1}, corresponding to 2.38–12.5 μ*M*, were obtained. This method proved to be effective for the separation and determination of the disaccharides in the buffered depolymerization mixtures. To show its applicability, depolymerized mixtures of porcine, bovine and ovine heparin from different tissues were analysed and their disaccharide compositions were determined, showing that the major disaccharides profile can be used to distinguish between samples of different origins. Unlike other disaccharide profile comparisons described in the literature, in this study disaccharide compositions of different heparins were determined using the same depolymerization conditions and a single analytical technique, removing variation other than that due to the origin of the heparin. Thus, reliable comparisons were possible. In addition, adjustment of the pH value of the formic

buffer with ammonia could enable coupling to a mass spectrometer, which could be used to identify possible interferences and oligosaccharides that are resistant to heparin lyase degradation. Research on the implementation of this coupling is currently in progress.

Acknowledgements

The authors wish to thank Bioibérica S.A. for kindly providing the heparin samples, disaccharide standards and enzymes used in this work. The authors also thank Dr. M. Mora (University of Barcelona) for her helpful assistance.This work has been partially supported by the Comissionat per Universitats i Recerca de la Generalitat de Catalunya (CIRIT SGR97-394).

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