ELSEVIER

Journal of Chromatography A, 873 (2000) 269–282

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Analysis of glycosaminoglycan monosaccharides by capillary electrophoresis using indirect laser-induced fluorescence detection

V. Ruiz-Calero, L. Puignou*, M.T. Galceran

Departament de Química Analítica, Universitat de Barcelona, Avda. Diagonal 647, 08028 Barcelona, Spain

Received 26 August 1999; received in revised form 19 November 1999; accepted 3 December 1999

Abstract

Two methods for monosaccharide analysis by capillary electrophoresis (CE) using counterelectroosmotic and coelectroosmotic modes with indirect laser-induced fluorescence detection were optimised and compared. A mixture of seven glycosaminoglycan-derived hexoses was separated in alkaline fluorescein-based electrolytes and detected in both counterelectroosmotic and coelectroosmotic conditions. The fluorescein concentration and pH of the background electrolyte, and the influence of the reversal of electroosmotic flow by addition of hexadimethrine bromide on the separation were studied. Coelectroosmotic CE conditions provided better resolution and limits of detection. A 10^{-6} *M* fluorescein solution at pH 12.25 containing 0.0005% (w/v) hexadimethrine bromide was used as background electrolyte. Quality parameters such as run-to-run, day-to-day precision and limits of detection were calculated, and better figures of merit were obtained for the coelectrooosmotic conditions than for the counterelectroosmotic mode. The coelectroosmotic method was applied to the quantitation of the hexosamine contents in glycosaminoglycans after acid hydrolysis. The method proved to be suitable for the determination of dermatan sulfate in heparin down to 2% (w/w).

Keywords: Detection, electrophoresis; Indirect detection; Monosaccharides; Glycosaminoglycans; Heparin; Dermatan sulfate

1. Introduction

Glycosaminoglycans (GAGs) constitute a group of complex polydisperse carbohydrates that play an important role in many biological processes [1,2]. They are all linear polysaccharide chains composed of repeating disaccharide units, each disaccharide consisting of one hexosamine, D-galactosamine or D-glucosamine, and one uronic acid, β-D-glucuronic or α-L-iduronic, or a neutral hexose [3]. Therefore, monosaccharide analysis can be an effective method of identifying GAG types and also to determine the cross-contamination between GAGs in raw materials, e.g., dermatan sulfate can be found as contaminant in raw heparin materials [4–7]. The determination of the monosaccharide composition of complex carbohydrates is one of the main areas of interest among the wide variety of analytical problems related to carbohydrate characterisation. This determination is difficult due to the presence of isomeric forms and the possible presence of non-glycosyl substituents in their structures, such as acetyl, sulfate ester, or amine groups, thus requiring high separation efficiencies. Monosaccharide analysis has been conventionally performed by gas chromatography (GC) of volatile derivatives [8], high-performance anion-exchange

*Corresponding author.

0021-9673/00/$ – see front matter
PII: S0021-9673(99)01283-2

liquid chromatography with pulsed amperometric detection (PAD) [9–11], and polyacrylamide gel electrophoresis (PAGE) [12,13]. These methods usually require large amount of samples and, in the case of GC, time-consuming derivatization procedures.

In the last few years, capillary electrophoresis (CE) methods have been developed for the analysis of monosaccharides, providing high separation efficiencies and requiring only small amounts of samples [14–20]. However, CE separation and detection of carbohydrates is difficult because of their high pK_a values, which makes them difficult to ionize, and the lack of chromophoric or fluorophoric groups. Moreover, their hydrophilic properties prevent the use of micellar electrophoresis systems. To overcome these problems, different approaches have been used. Recently, extensive reviews focused on either the electrophoretic separation or the detection of different kinds of carbohydrates analysed by capillary electrophoresis have been published [21–24]. The most frequent strategy has been the use of counterelectroosmotic conditions, which implies the use of basic electrolytes for changing the sign of zeta potential on the capillary inner wall in order to force the migration by the high electroosmotic flow (EOF). On-column complexation with borate allowed direct absorbance at 195 nm of the polyols–borate complex of monosaccharides, which acquired a negative charge [25]. Electrochemical detection in highly alkaline electrolytes (pH 13) using copper or gold electrodes has also been reported [26–29]. However the lack of commercially available instrumentation prevents its use as a routine method. Another strategy for monosaccharide detection is labelling with UV or fluorescence tags [17–20,30–39]. Several procedures for labelling with chromophoric or fluorophoric reagents have been described and excellent detection limits down to attomole levels have been obtained [21–24]. However, due to the unavoidable microscale of working in the derivatization reaction, which involves small volumes as low as 4 μl, precision could be compromised, in addition, the variable yield of the reaction makes difficult to determine the derivatized products. In spite of the lack of selectivity of indirect detection methods, indirect UV and laser-induced fluorescence (ILIF) detection have also been tested for underivatized monosaccharides, avoiding the labelling procedure. Sorbate [14,40–42], *p*-nitrophenol [16], tryptophan [15] and 1-naphthylacetic acid [43] at high pH have been used as carrier electrolytes for the indirect UV detection, and the detection limits are in the pmol range. ILIF detection has been less applied, and few papers on this kind of detection have been published. The possibilities for indirect fluorescence detection in CE were studied by Yeung and Kuhr [44]. In the field of monosaccharide determination, Garner and Yeung used a He–Cd laser to analyse sucrose, glucose and fructose at fmol levels with coumarin 343 as the background electrolyte [45]. Fluorescein-based electrolytes have also been used to separate inorganic cations [46], organic anions [47,48] and some high-molecular-mass polysaccharides, such as dextrans and amyloses with an argon laser-induced fluorescence detector [49]. Up to now, this indirect detection method has not been applied to the analysis of the monosaccharide constituents of GAGs.

Monosaccharides are usually separated in counterelectrosmotic migration mode. Nevertheless, modification of EOF by the addition of a polycationic surfactant to the electrolyte, such as cetyltrimethylamonium bromide (CTAB) [50] and hexadimethrine bromide (HDMB) [14], and reversing the polarity of the power supply (reversed polarity) have also been used for the separation of oligo- and monosaccharides, with electrochemical and indirect UV detection, respectively. In these conditions, the dynamically coated surface of the capillary acquires a positive charge and the EOF is directed towards the cathode as well as the negatively charged analytes.

This paper reports the study of the parameters that influence the ILIF detection in the electrophoretic separation of seven substituted hexoses which are constituents of GAGs and other glycoforms. The constituent monosaccharides (uronic acids and hexosamines) of these carbohydrates can have sulfate substituents in various positions, and the hexosamine unit can be *N*-acetylated or sulfated. Depending on the hexosamine, the GAGs can be classified as glucosaminoglycans (heparin and heparan sulfate) and galactosaminoglycans (chondroitin sulfate and dermatan sulfate). Conditions for the acid hydrolysis of glycosaminoglycans have to be carefully controlled. Acid hydrolysis under mild conditions could neither cleave all glycosidic linkages nor release all

sulfate and acetyl groups, whereas acid hydrolysis under drastic conditions could cause degradation of uronic acids residues. The influence of several parameters, such as pH, fluorescein concentration, inner capillary diameter, and injection time, on the separation and detection in counterelectroosmotic and coelectroosmotic modes was studied. Further, quality parameters for both methods were established and compared. Finally, the developed coelectroosmotic method was applied to the analysis of monosaccharides in glycosaminoglycans after high-temperature acid hydrolysis [13], and to the determination of low contents of dermatan sulfate in heparin.

2. Experimental

2.1. Chemicals

Monosaccharides, D-glucosamine (GlcN), D-galactosamine (GalN), *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-galactosamine (GalNAc) were obtained from Sigma (St. Louis, MO, USA). D-(+)-Glucose (Glc) was purchased from Merck (Darmstadt, Germany), D-glucuronic acid (GlcA) as monohydrate sodium salt was from Janssen (Beerse, Belgium), and D-(+)-galactose (Gal) from Carlo Erba (Milan, Italy). Stock solutions of each monosaccharide were prepared at concentrations ranging from 1000 to 500 mg l^{-1}. Unfractionated heparin (sodium salt) from intestinal porcine mucosa, chondroitin 4-sulfate and dermatan sulfate were kindly provided by Bioibérica (Palafolls, Barcelona, Spain).

Reagents used for the preparation of buffer solutions were of analytical grade. Fluorescein (acid), sodium hydroxide, methanol and acetone were from Merck. HDMB was from Fluka (Ronkonkoma, NY, USA). Water purified using a Milli-Q water-purification system (Millipore, Bedford, MA, USA) was used for all solutions.

2.2. Capillary electrophoresis conditions

The experiments were performed on a P/ACE system 5500 (Beckman Instruments, Fullerton, CA, USA) equipped with a laser-induced fluorescence (LIF) detection system using a 4 mW argon-ion laser with an excitation wavelength of 488 nm and a 520 nm emission wavelength notch filter. The indirect fluorescence detection mode was selected to acquire reversed signals. The fluorescence intensity was calibrated following the instructions of the supplier [51]. Data were collected using the Beckman P/ACE Station software system version 1.0.

The electrophoretic separations were carried out using uncoated fused-silica capillaries of either 50 μm or 25 μm I.D., with total length (L) ranging from 57 to 107 cm (length to detector, l, of 50–100 cm) 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 at 3447.5 Pa (0.5 p.s.i.) for 2–10 s. Running electrolytes were filtered through a 0.45-μm nylon membrane, and degassed before use. The pH of electrolyte solutions was adjusted to alkaline values with 1 *M* sodium hydroxide. The temperature was held at 25°C.

2.3. Hydrolysis procedure

Solutions of GAG samples were prepared at 1000 μg ml^{-1} in ultrapure water. A 50-μl volume of 8 *M* hydrochloric acid was added to aliquots of 50 μl containing 10 μg heparin, chondroitin sulfate or dermatan sulfate in a 1-ml conical glass vial. The solution was homogenised in a vortex mixer before the hydrolysis step. The vials were then placed in a sand heat block, where the solutions were incubated at 100°C for 30 min. After hydrolysis, the mixtures were dried in a centrifugal vacuum evaporator. Finally, the dried pellet was resuspended in 250 μl of water before injection in the CE system. Hydrolysis products were stored in the reaction vials at −18°C.

3. Results and discussion

3.1. Counterelectroosmotic separation

Counterelectroosmotic methods can be used to analyse negatively charged species providing that the

EOF allows an apparent mobility towards the cathodic end of the silica capillary. In this work, suitable counterelectroosmotic conditions were achieved using alkaline electrolyte solutions, which ensured high enough EOF to detect the anionic monosaccharides at the cathode. For the optimisation, a standard solution of ca. 100 μg ml^{-1} of each monosaccharide was hydrodynamically injected for 2 s and the separations were carried out at +12 kV. This voltage was chosen after a previous test using 10^{-6} *M* fluorescein electrolyte in a *L*: 107 cm fused-silica capillary of 50 μm I.D.

3.1.1. Influence of pH

The separation of the seven monosaccharides was studied under the general conditions mentioned above at pH higher than 11. Due to the high pK_a values of these carbohydrates (e.g., pK_a of the glucose is 12.35) the pH of the running electrolyte was adjusted at highly alkaline values to achieve their selective ionisation. The pH was varied between 11.2 to 12.6, using sodium hydroxide solutions. It was observed that at pH values lower than 11.9, only GlcA, which carries a carboxylate group (pK_a: 3.20), was detected due to its negative charge. In contrast, the other monosaccharides remained electrically neutral in these conditions and only were detected at higher pH. Migration times (t_m) and resolutions (R_s) increased with the alkalinity of the running electrolyte. However, in some cases the improvement of resolution of one pair led to a reduction on the resolution of the other pair, for example GalN–Glc and GlcN–GalNAc at pH 12.40 (Fig. 1). Two system peaks were detected in all the electropherograms, the first, corresponding to the EOF appeared as a decrease in fluorescence intensity and the second, which migrated after the monosaccharides corresponding to the fluoresceinate anion, was detected as an increase in fluorescence intensity. The appearance of this system peak was due to the charge balance which produced the displacement of background electrolyte by the analytes [15]. This phenomenon has been also described by other authors [52] using indirect UV detection. A pH of 12.25 was selected because resolution values were not significantly improved using more alkaline electrolytes while migration times increased and produced an important decrease in peak areas.

3.1.2. Influence of capillary

The separation of the monosaccharides was studied in 50 μm I.D. capillaries with total length (*L*) ranging from 67 to 107 cm. Shortening the capillary reduced the resolution between analytes, as well as run time (Table 1). Migration times and ''separation window'', i.e., the time interval limited by the migration of both system peaks where all the analytes migrate, decreased when short capillaries were used, which resulted in slightly worse resolutions for the monosaccharides. A capillary length of *L*: 77 (*l*: 70) cm was chosen as total run time was less than 20 min and the resolution values were satisfactory (higher than 1.0 with the exception of GalN–Glc which was not significantly improved in longer capillaries).

The effect of the capillary inner diameter was also studied. There is a relationship between low-frequency noise and baseline disturbance in indirect detection and a deficient Joule heat dissipation [42]. As Joule heat is proportional to the electrophoretic current an improvement of baseline disturbance was expected from the use of 25 μm I.D. capillaries. Nevertheless, in our case using 2 s hydrodynamic injection the decrease of the inner diameter produced lower current but the baseline noise and signal-to-noise ratios were similar to those obtained in 50 μm I.D. capillaries, which could be related to the high efficiency of the liquid cooling system. To increase the signal higher injection times up to 10 s were used. A decrease in resolution at high injection times occurred and similar values were obtained for both capillaries for the resolution between Glc–GlcN and GalNAc–GlcNAc at injection times from 2 to 10 s (Fig. 2). As the use of 25 μm I.D. capillaries did not produce a higher signal-to-noise ratio, and required longer flushing and conditioning times, the standard 50 μm I.D. capillaries using 2 s hydrodynamic injection was chosen.

3.1.3. Influence of fluorescein concentration

In general, a low fluorophore concentration is required for the application of ILIF detection as the sensitivity can be improved by a decrease in the background signal [46,53]. Desbène and co-workers used 10^{-5} *M* fluorescein electrolyte to separate cations [46] and organic anions [47], however, in our case when this concentration was tested, neither

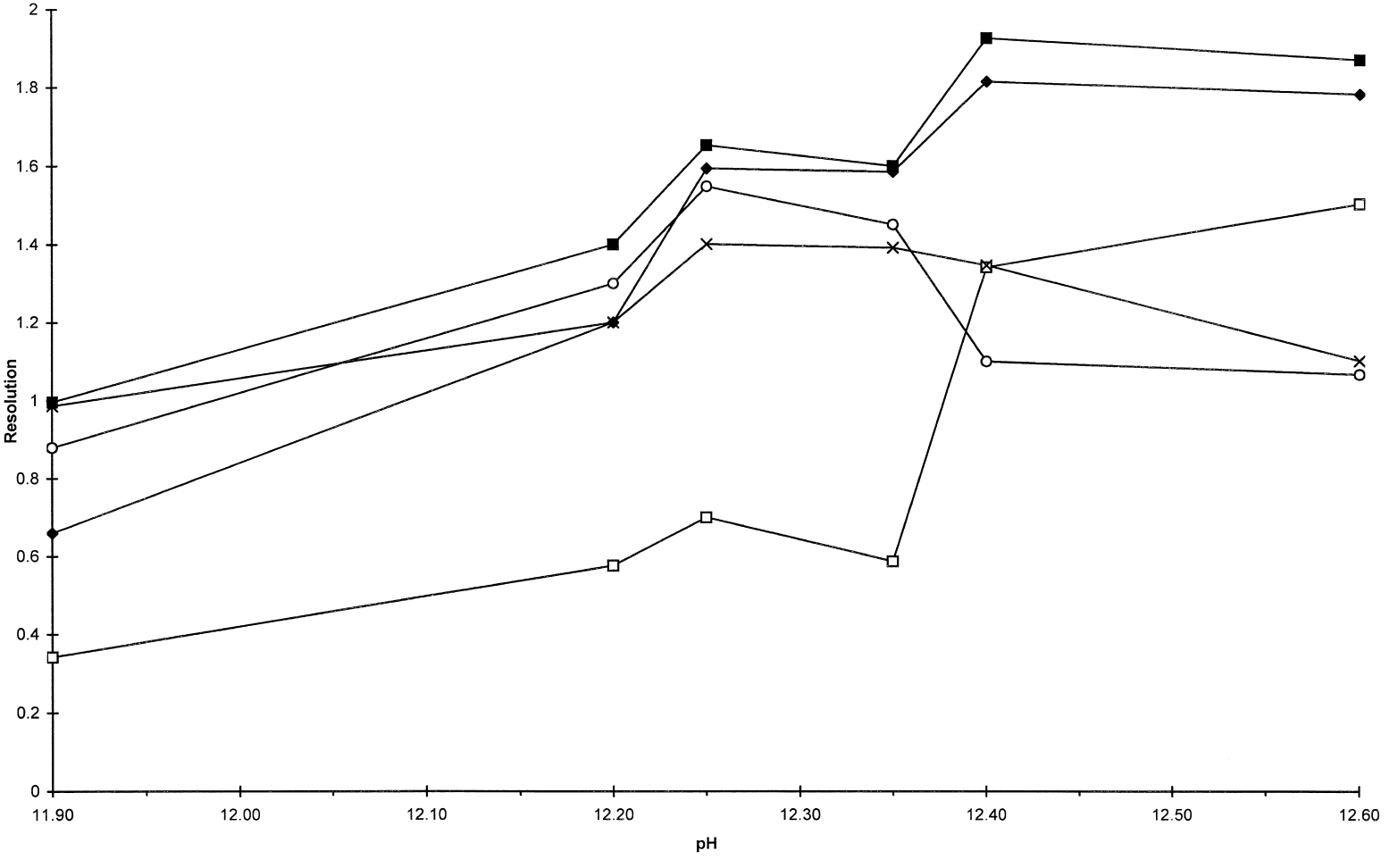

Fig. 1. Influence of pH on the resolution of monosaccharides. Carrier electrolyte: 10^{-6} *M* fluorescein. Injection time: 2 s, 100 μg ml^{-1} monosaccharide. Capillary: 107 cm (effective length 100 cm)×50 μm I.D. Voltage applied: +12 kV. ♦ Gal–GalN, □ GalN–Glc, ■ Glc–GlcN, ○ GlcN–GalNAc, × GalNAc–GlcNAc.

Table 1
Influence of capillary length in monosaccharide separation in counterelectroosmotic conditions[a]

	Capillary total length (and length to detector)							
	107 (100) cm		97 (90) cm		77 (70) cm		67 (60) cm	
	t_m	R_s	t_m	R_s	t_m	R_s	t_m	R_s
Monosaccharides								
Gal	22.87		20.79		13.11		10.20	
		1.59		1.61		1.55		1.20
GalN	23.15		21.06		13.29		10.34	
		0.63		0.88		0.85		0.89
Glc	23.29		21.23		13.40		10.44	
		1.65		1.74		1.45		1.29
GlcN	23.65		21.58		13.63		10.60	
		1.55		1.36		1.16		0.90
GalNAc	23.94		21.83		13.80		10.73	
		1.40		1.46		1.17		0.87
GlcNAc	23.18		22.07		13.94		10.84	
GlcA	33.28		31.16		19.93		15.76	
System peaks								
1	20.89		18.53		11.32		8.91	
2	37.16		33.63		20.50		16.50	

[a] Injection time: 2 s (0.5 p.s.i.); concentration of monosaccharide standard solution: 100 μg ml^{-1}. Other conditions are given in the text.

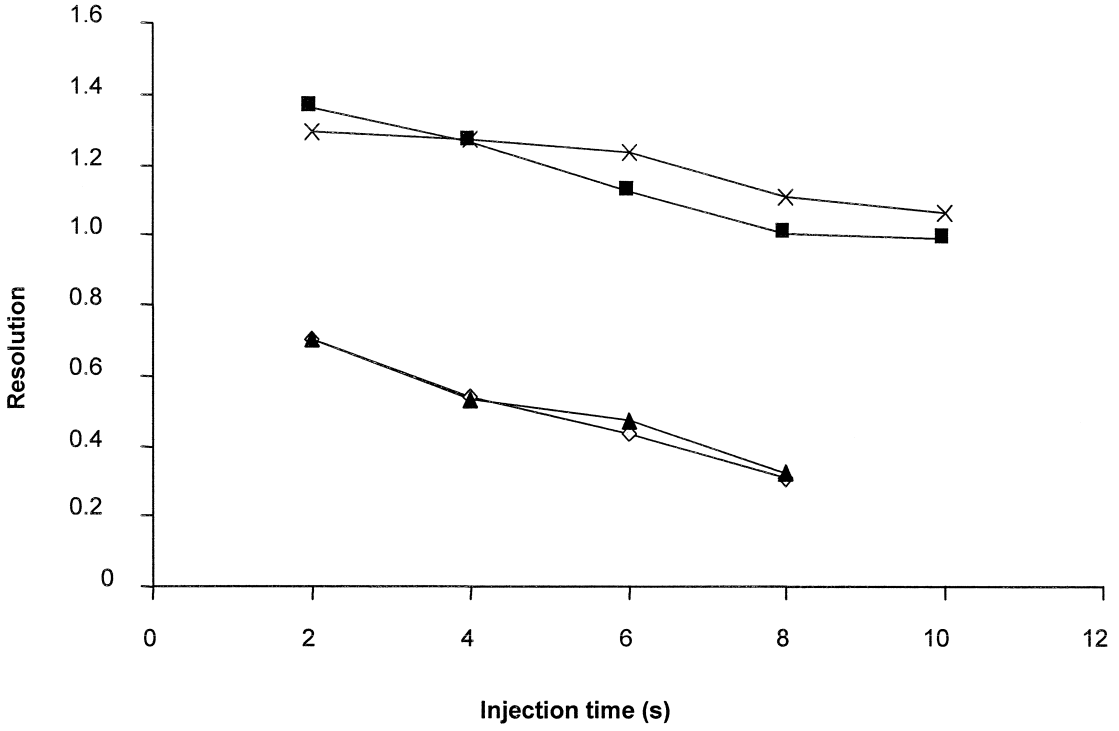

Fig. 2. Influence of capillary inner diameter and injection time on the resolution of Glc–GlcN and GalNAc–GlcNAc. ◇ Glc–GlcN (50 μm), ▲ Glc–GlcN (25 μm), ■ GalNAc–GlcNAc (50 μm), × GalNAc–GlcNAc (25 μm).

monosaccharides nor baseline noise were observed, which indicated that the detector was saturated by the high fluorescence intensity of the electrolyte. Modification of the photomultiplier value did not show any improvement. This limited the fluorescein concentration to values lower than 10^{-5} *M*, in this study the concentration was varied from 10^{-5} to 10^{-8} *M*. Fig. 3 shows the electropherograms acquired at 10^{-6}, 10^{-7} and 10^{-8} *M*. As can be seen peak intensities decreased with the concentration of the fluorophore. Although peak intensity was lower at 10^{-7} *M*, the baseline noise improved significantly with respect to 10^{-6} *M*. At 10^{-8} *M* fluorescein, the signal decreased and background increased (Fig. 3), which could be due to capillary wall interactions [53]. As the best signal-to-noise ratio was obtained with 10^{-7} *M* fluorescein, this concentration was used for the remainder of this study.

3.1.4. Addition of organic solvents

The influence of organic solvents as modifiers of the carrier electrolyte was studied. Electrolytes with 5% (v/v) methanol and acetone were used, but in both cases, a reduction of peak intensity was observed, while resolution was worsened. Addition of methanol increased migration times and decreased the resolution between Gal–GalN and GalNAc–GlcNAc peaks, whereas the critical separation between GalN–Glc was not improved. Similar behaviour was observed when acetone was used as organic modifier.

As a result of these studies, a running electrolyte consisting of 10^{-7} *M* fluorescein at pH 12.25 was used for the separation of the monosaccharides in 50 μm I.D. capillaries of *L*: 77 (*l*: 70) cm length at a separation voltage of +12 kV.

3.2. Coelectroosmotic separation

The addition of a polycationic surfactant such as HDMB, which reversed the zeta potential sign on the silica capillary wall, and the use of reversed polarity were tested. Among the different substances used to reverse the EOF direction, HDMB was selected because both the low concentration needed to coat the capillary surface and the low baseline noise produced.

The running electrolyte was the same used for the counterelectroosmotic mode but adding to the electrolyte HDMB at a concentration of 0.0005% (w/v). In previous studies concentrations in the range of 0.0001–0.001% (w/v) were successfully used to reverse EOF direction [14,54]. The other electrophoretic conditions were also the same as those optimised for counterelectroosmotic mode with the exception of the polarity of the power supply which was reversed. In this conditions, migration order of monosaccharides and system peaks was reversed and run time was decreased to less than 17 min. To study the pH influence on the separation it was changed from 11.80 to 12.40. In this mode, better resolution between all pair of compounds was observed for the range tested, which was especially important for GalN and Glc, that increased to 1.0 at pH 12.25. At higher pH a decrease in the resolution occurred which might be due to the defficient coating of the capillary surface at these highly alkaline conditions. For these reasons the pH selected was 12.25 as for the counterelectroosmotic mode.

The significant reduction of baseline instability in the coelectroosmotic conditions contrasted to what was observed in counterelectroosmotic mode, and a concentration of 10^{-6} *M* fluorescein gave the best signal-to-noise ratio. In Fig. 4 the electropherogram acquired in the best conditions, 10^{-6} *M* fluorescein, pH 12.2, 0.0005% (w/v) HDMB, is given.

3.3. Quality parameters

The limits of detection (LODs) based on a 3:1 signal-to-noise ratio obtained using the counter- and coelectroosmotic conditions with injection times of 2 s are given in Table 2. Lower concentrations, in the order of 2.5 μg ml^{-1} corresponding to 11.40 μ*M* (20 fmol) to 14.56 μ*M* (25 fmol), were obtained using the coelectroosmotic conditions for hexoses, hexosamines and *N*-acetylhexosamines, whereas GlcA could be detected at lower concentrations up to 0.54 μg ml^{-1} (2.76 μ*M*, i.e., 5 fmol). The higher response of the hexuronic acid in both counter- and coelectroosmotic modes can be explained by the migration near the fluoresceinate system peak. The influence of the relative mobilities of the solute to the marker ion when they are close together in the peak areas has also been reported by other authors using UV detection [15,52]. Lower detection limits

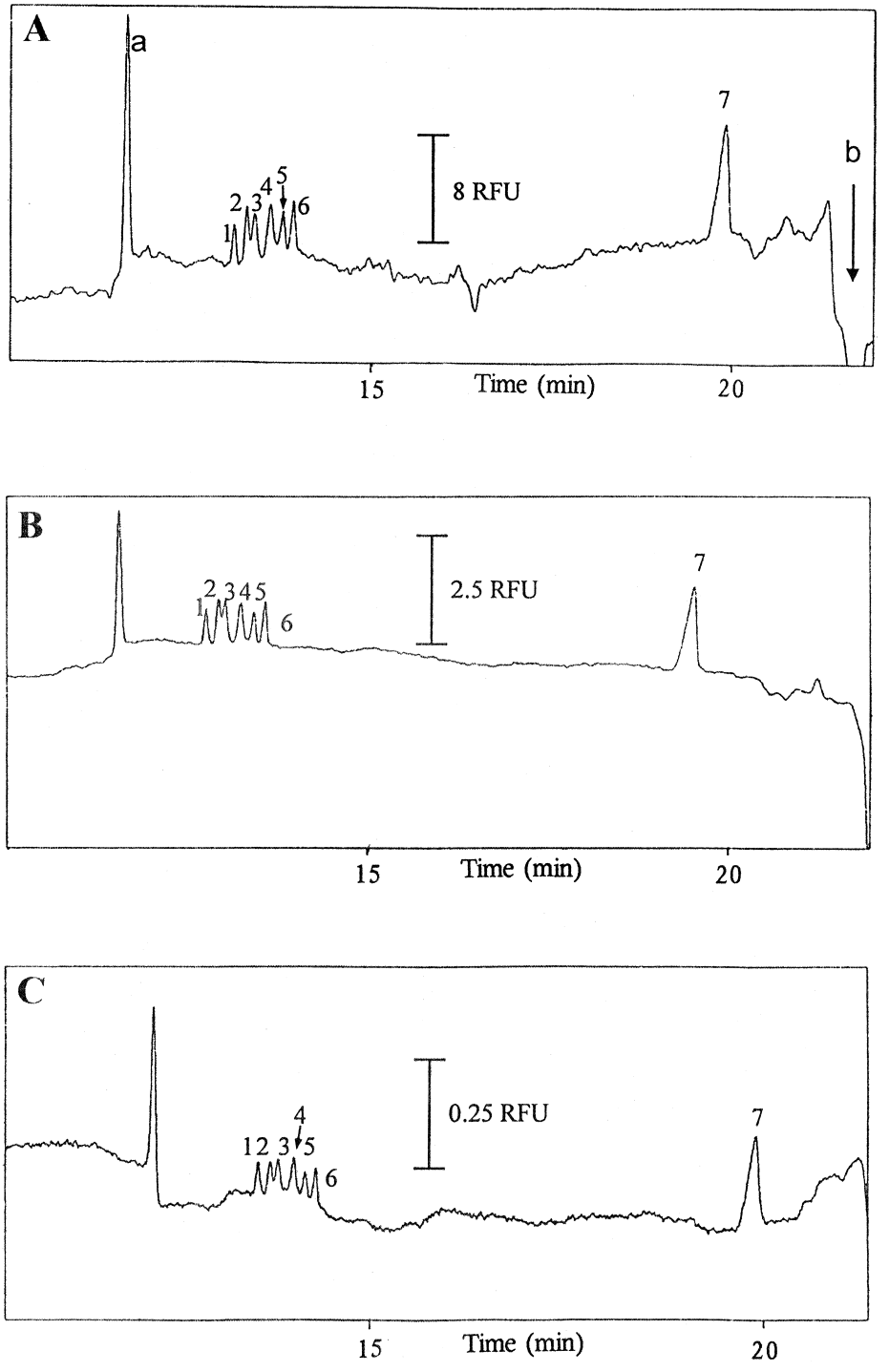

Fig. 3. Electropherograms with different fluorescein concentration at pH 12.2: (A) 10^{-6} *M*, (B) 10^{-7} *M* and (C) 10^{-8} *M*. Other electrophoretic conditions as in Fig. 1. Peaks: 1=Gal, 2=GalN, 3=Glc, 4=GlcN, 5=GalNAc, 6=GlcNAc, 7=GlcA, system peaks a and b.

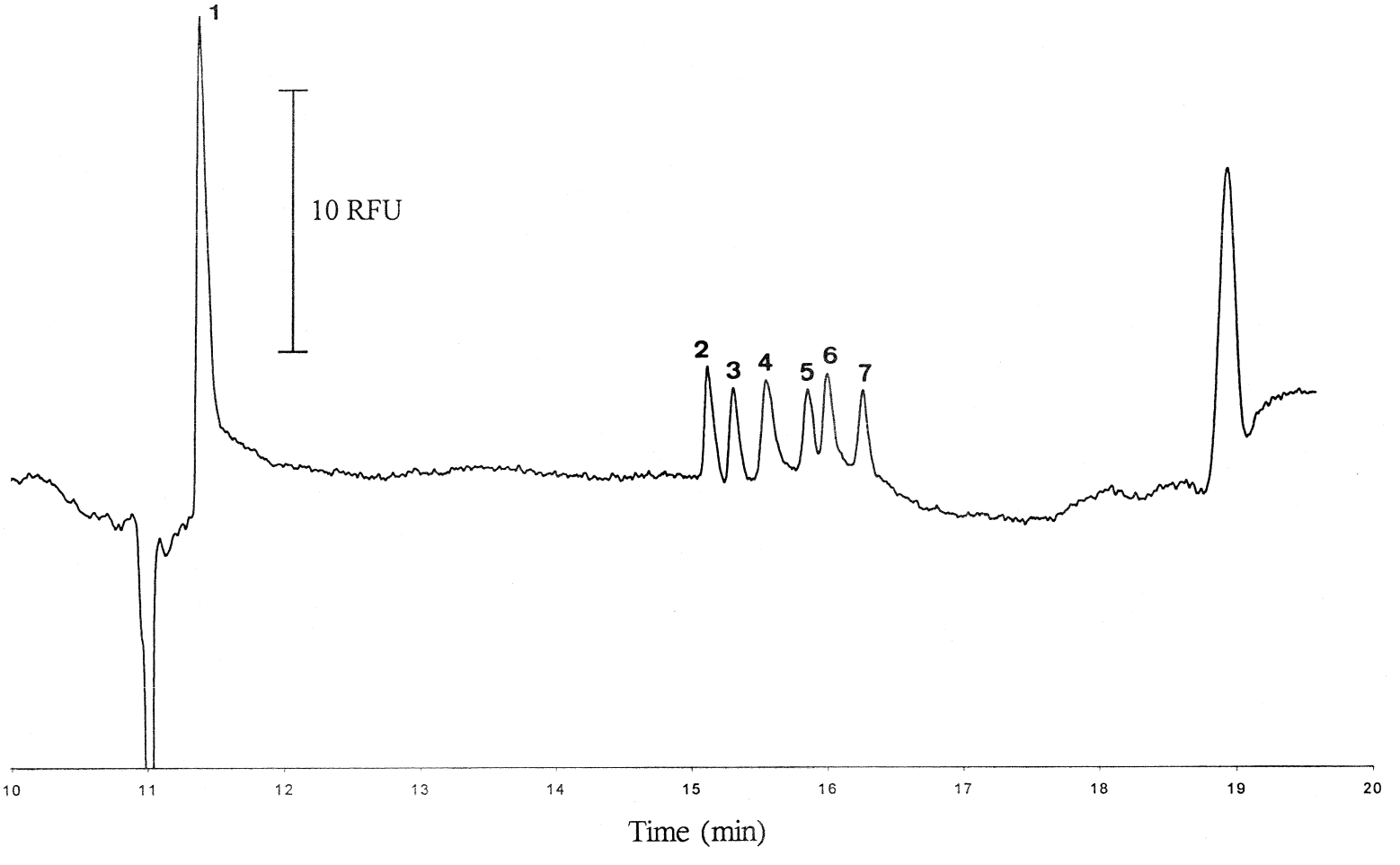

Fig. 4. Electropherogram of a 80 μg ml^{-1} monosaccharide standard obtained at coelectroosmotic mode. Conditions: 10^{-6} *M* fluorescein, pH 12.25, 0.0005% (w/v) HDMB, injection time: 2 s, −12 kV (reversed polarity), capillary: 67 cm (effective length 60 cm)×50 μm I.D. Peaks: 1=GlcA, 2=GlcNAc, 3=GalNAc, 4=GlcN, 5=Glc, 6=GalN, 7=Gal.

Table 2
Quality parameters obtained in counter- and coelectroosmotic modes[a]

	Limits of detection				Precision[b]							
	Counterelectroosmotic mode		Coelectroosmotic mode		Run-to-run						Day-to-day	
					Counterelectroosmotic mode			Coelectroosmotic mode			Coelectroosmotic mode	
	μg ml^{-1}	μ*M*	μg ml^{-1}	μ*M*	t_m (min)	RSD t_m (%)	RSD area (%)	t_m (min)	RSD t_m (%)	RSD area (%)	RSD t_m (%)	RSD area (%)
Gal	8.73	48.5	2.50	2.76	13.2	0.23	3.76	16.2	0.60	2.46	1.13	9.8
GalN	8.94	49.9	2.55	11.40	13.4	0.20	5.96	15.9	0.60	5.26	1.04	14.7
Glc	8.78	48.8	2.51	11.48	13.5	0.36	2.87	15.8	0.58	4.29	1.05	14.6
GlcN	9.13	50.9	2.61	14.56	13.7	0.22	5.50	15.4	0.56	4.92	1.15	13.6
GalNAc	8.89	40.2	2.54	13.93	13.9	0.27	12.40	15.2	0.58	4.50	1.16	12.9
GlcNAc	8.83	39.9	2.52	14.26	14.0	0.26	6.42	15.0	0.60	1.98	1.13	12.8
GlcA	2.30	11.8	0.54	13.85	20.0	0.54	6.60	11.2	0.64	6.41	1.22	12.2

[a] Injection time: 2 s (0.5 p.s.i.).
[b] Concentration of monosaccharide standard solution: 80 μg ml^{-1}.

in the range of 2–10 fmol can be achieved increasing the injection time to 8 s.

Run-to-run precision of migration time and area expressed as the relative standard deviation (RSD) of six replicates of approx. 80 μg ml^{-1} monosaccharide standard for both methods are included in Table 2. Migration times showed a high reproducibility in both modes with values ranging from 0.20 to 0.64% for the run-to-run precision, but slightly better area precisions were obtained for the coelectroosmotic method, ranging from 2.0 to 6.4%. The day-to-day precision of the migration time and the area of the coelectroosmotic method are also given in Table 2. It could be observed that even though migration times were satisfactorily reproducible (RSD lower than 1.2%), peak areas obtained from injections in three days without an internal standard varied giving RSD values of 9.8–14.7%. The counterelectroosmotic method provided less reproducibility for the migration times and the concentrations.

3.4. Application

The coelectroosmotic conditions optimised were applied to the analysis of three different GAGs; heparin (Hep), dermatan sulfate (DS) and chondroitin 4-sulfate (C4S) after acid hydrolysis. Hexosamine determination was used as an analytical tool for the characterization of glucosaminoglycans and galactosaminoglycans.

GAGs were hydrolysed following the procedure described in the Experimental section. After acid hydrolysis, the samples were injected in the CE system operating in the coelectroosmotic conditions. Hexosamine concentration was calculated by external calibration using GalN, GlcN, GalNAc and GlcNAc, as standards at six concentration levels ranging from 20 to 200 μg ml^{-1}. In Fig. 5 the electropherograms corresponding to the injection of the Hep and C4S hydrolysis solutions and the 80 μg ml^{-1} standard are shown. Only the hexosamine peaks were detected in the electropherograms of the hydrolysis solutions, which indicated that the substituted hexosamines were converted to nonsubstituted hexosamines after the acid hydrolysis of these sulfated GAGs in this conditions. A high distortion of the baseline observed at approx. 11 min near the migration time of glucuronic acid. This important distortion could be due to the presence of the high amounts of chloride and sulfate in the hydrolysed sample, which, in addition, can produce a higher system peak of fluoresceinate. Apparently, hexuronic acids were not observed in the hydrolysates due to the instability of hexuronic acids after the acid hydrolysis for 0.5 h, which is in contrast to the results observed by Starr et al. for the fluorophore-assisted carbohydrate electrophoresis analysis of monosaccharides released from these compounds [13]. A previous reduction of hexuronic acids before the hydrolysis procedure could prevent their possible loss [54].

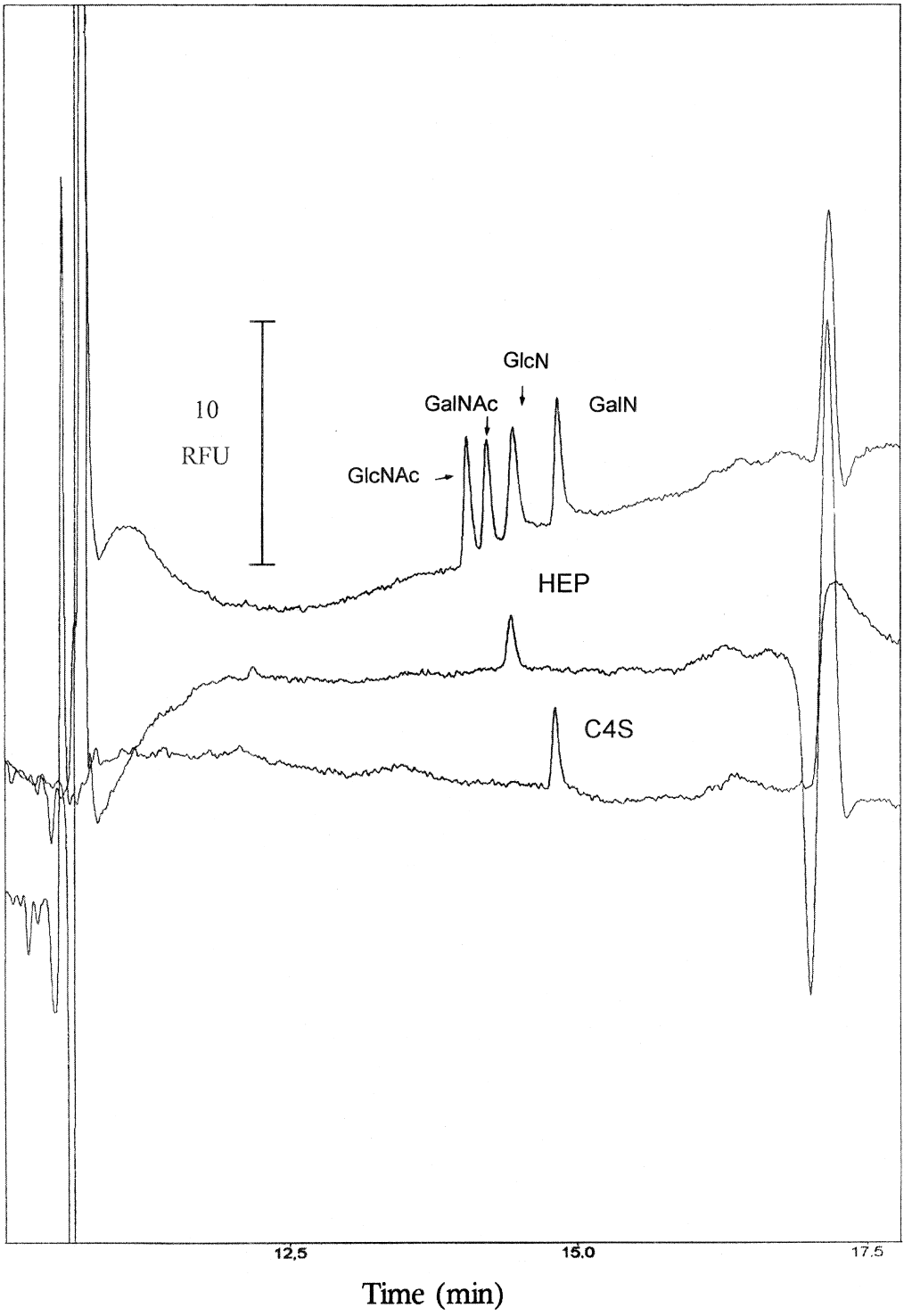

Fig. 5. Electropherogram of a 80 μg ml^{-1} monosaccharide standard solution and the hydrolysed products of Hep and C4S. Electrophoretic conditions as in Fig. 4.

The results of the quantitation are given in Table 3. As can be seen, the percentage of GlcN in heparin is lower than the percentage of GalN obtained for DS and C4S. This could be explained by the higher sulfation of heparin which confers a highly anionic character to this GAG, thus, heparin preparations contain more sodium ions than the galac-

Table 3
Quantitation of hexosamine contents in GAGs (%, w/w)[a]

	Hep	DS	C4S
GlcN	16.8±1.2%	n.d.	n.d.
GalN	n.d.	25.1±1.9%	22.7±1.7%

[a] Confidence limits expressed as the standard deviation corresponding to four determinations.

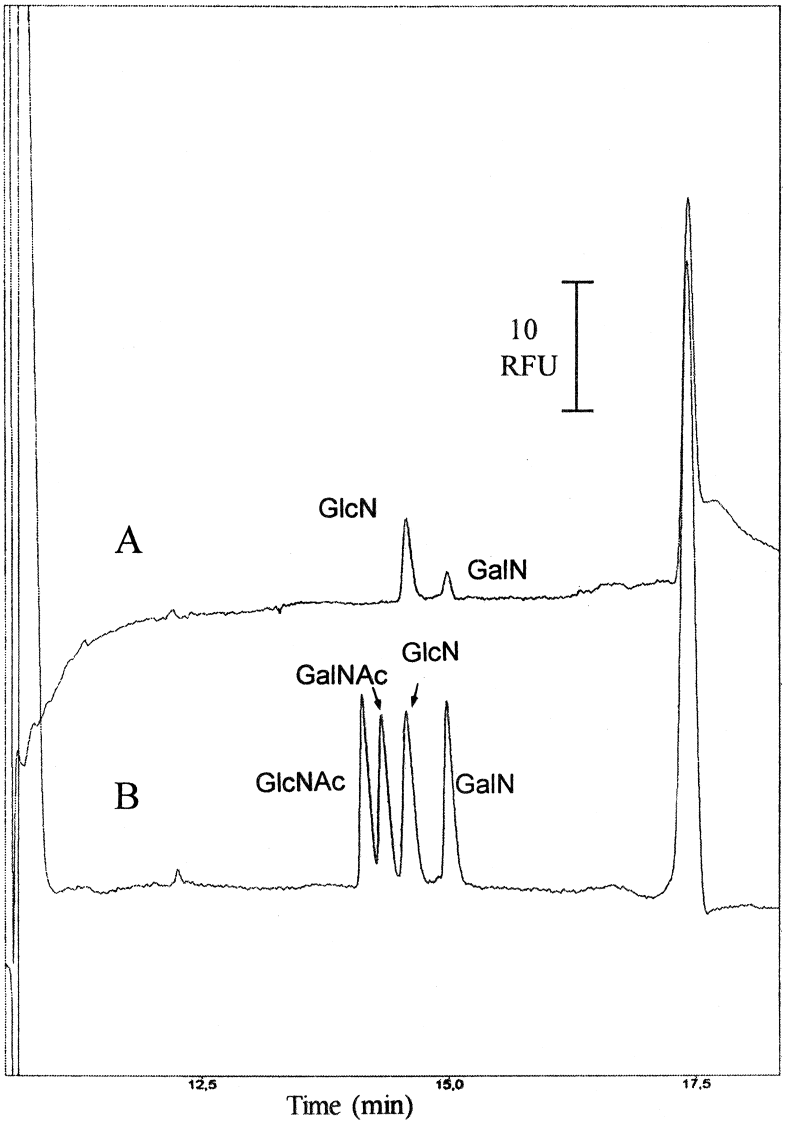

Fig. 6. Electropherogram of (A) an heparin sample spiked with dermatan sulfate at DS/total GAGs ratio of 21.5% (w/w) and (B) a 80 μg ml^{-1} monosaccharide standard solution. Injection time: 8 s. Other electrophoretic conditions as in Fig. 4.

Table 4
Evaluation of dermatan sulfate contamination in heparin (%, w/w)

	M1	M2	M3	M4
GalN found (μg)	<0.08[a]	1.07	2.51	4.03
%DS/total GAGs found[c]	<2.0[b]	8.4±2.0	19.6±1.3	31.2±1.0
%DS/total GAGs spiked	0	10.8	21.5	31.9

[a] Corresponding to LOD (8 s) in the hydrolysed samples.
[b] Corresponding to LOQ (8 s) in the hydrolysed samples.
[c] Confidence limits expressed as the standard deviation corresponding to four determinations.

tosaminoglycans, lowering the monosaccharide percentage. In order to test whether the hexosamine yield increased with hydrolysis time longer than 0.5 h, acid hydrolysis was carried out for 1 h and 3 h, and we did not observe an increase in the hexosamine peaks indicating that their recoveries did not improve with longer hydrolysis times. Thus, the shorter hydrolysis time (0.5 h) was selected. This fact confirmed that the hydrolysis conditions (4 *M* HCl, 100°C, 0.5 h) were effective for releasing hexosamine units from sulfated GAGs, such as heparin, dermatan sulfate and chondroitin sulfates, whereas they should be probably modified for the hydrolysis of hyaluronic acid, a non-sulfated GAG.

The extent of the contamination of heparin preparations by dermatan sulfate is an important parameter for the characterisation of heparin and levels ranging from 2 to 12% (w/w) have been reported. In order to evaluate this contaminations the coelectroosmotic method was applied to spiked heparin samples. Four heparin standard solutions were spiked with dermatan sulfate at levels corresponding to DS/total GAGs ratio of 0, 10, 21.5 and 32% (w/w), which were identified as M1, M2, M3 and M4, respectively. As only GalN and GlcN were detected in the hydrolysed mixtures the resolution between peaks was not critical, thus the injection time should be increased to 8 s in order to obtain higher GalN signals. Fig. 6 shows the electropherograms corresponding to the 8-s injection of the hydrolysed sample with DS/total GAGs ratio 21.5% and a standard of 80 μg ml^{-1} of the hexosamines and *N*-acetylhexosamines. Galactosamine was not detected in the non-spiked heparin sample (see Fig. 5), which indicated that the dermatan content in the heparin preparation analysed was lower than 2.0% (w/w). This value corresponds to the limit of quantitation (LOQ) of GalN based on 10 times signal-to-noise ratio for an injection time of 8 s, expressed as the equivalent dermatan content. The ratios of DS/total GAGs spiked and obtained by the CE determination of hexosamines using the compositional percentages of Table 3 are given in Table 4. It can be seen that the ratios obtained from the electrophoretic determination of hexosamines are in agreement with the GAG ratios of the spiked samples.

4. Conclusion

A coelectroosmotic CE method was developed for the separation of seven underivatized monosaccharides with ILIF detection. The addition of the HDMB to the carrier electrolyte and the polarity reversal provided better resolution and limits of detection than those obtained without the cationic modifier at normal polarity. Although the detection limits for hexosamines obtained using ILIF detection were only about 14 μ*M*, the method optimised provides satisfactory results for the applications studied. The method proved to be suitable for the determination of hexosamine contents in hydrolysed sulfated glycosaminoglycans samples and mixtures, thus enabling the evaluation of minor contamination of dermatan sulfate (2%, w/w) in heparin. Work is being carried out to improve the determination of hexuronic acids, and modifications of the hydrolysis procedure are being studied.

Acknowledgements

Thanks are due to Bioibérica S.A. (Palafolls, Spain) for supplying us the monosaccharide standards and GAG samples and for the financial support

in the acquisition of the CE equipment. This work has been partially supported by the Comissionat per Universitats i Recerca de la Generalitat de Catalunya (CIRIT SGR99-49). V.R.-C. thanks the University of Barcelona for a grant.

References

[1] U. Lindahl, in: D.A. Lane, U. Lindahl (Eds.), Heparin, Chemical and Biological Properties, Clinical Applications, Edward Arnold, London, 1989, p. 159.
[2] L. Kjellen, U. Lindahl, Annu. Rev. Biochem. 60 (1991) 443.
[3] B. Casu, Adv. Carbohydr. Chem. Biochem. 43 (1985) 51.
[4] G.A. Neville, J. Pharm. Sci. 78 (1989) 101.
[5] K.R. Holme, A.S. Perlin, Carbohydr. Res. 186 (1989) 301.
[6] D.H. Atha, B. Coxon, V. Reipa, A.K. Gaigalas, J. Pharm. Sci. 84 (1995) 360.
[7] R. Malsch, J. Harenberg, Electrophoresis 17 (1996) 401.
[8] R.A. Laine, W.J. Esselman, C.C. Sweeley, Methods Enzymol. 28 (1972) 159.
[9] Y.C. Lee, Anal. Biochem. 189 (1990) 151.
[10] A. Klockow, A. Paulus, V. Figuereido, R. Amadò, H.M. Widmer, J. Chromatogr. A 680 (1994) 187.
[11] R.R. Townsend, in: Z. El Rassi (Ed.), Carbohydrate Analysis – High Performance Liquid Chromatography and Capillary Electrophoresis, Journal of Chromatography Library, Vol. 58, Elsevier, Amsterdam, 1995, p. 181.
[12] P. Jackson, Anal. Biochem. 216 (1994) 243.
[13] C.M. Starr, R.I. Masada, C. Hague, E. Skop, J.C. Klock, J. Chromatogr. A 720 (1996) 295.
[14] A. Zemann, D.T. Nguyen, G. Bonn, Electrophoresis 18 (1997) 1142.
[15] B. Lu, D. Westerlund, Electrophoresis 17 (1996) 325.
[16] J. Plocek, J. Chmelik, Electrophoresis 18 (1997) 1148.
[17] S. Honda, K. Togashi, K. Uegaki, A. Taga, J. Chromatogr. A 805 (1998) 277.
[18] S.A. Perez, L.A. Colón, Electrophoresis 17 (1996) 352.
[19] R.A. Evangelista, A. Guttman, F.A. Chen, Electrophoresis 17 (1996) 347.
[20] A. Guttman, J. Chromatogr. A 763 (1997) 271.
[21] S. Suzuki, S. Honda, Electrophoresis 19 (1998) 2539.
[22] Z. El Rassi, High-Performance Capillary Electrophoresis of Carbohydrates, Beckman Instruments, Fullerton, CA, 1996.
[23] N.K. Karamanos, A. Hjerpe, Electrophoresis 19 (1998) 2561.
[24] A. Paulus, A. Klockow, J. Chromatogr. A 720 (1996) 353.
[25] S. Hofstetter-Kuhn, A. Paulus, E. Gassmann, H.M. Widmer, Anal. Chem. 63 (1991) 1541.
[26] T.J. O'Shea, S.M. Lunte, W.R. LaCourse, Anal. Chem. 65 (1993) 948.
[27] W. Lu, R.M. Cassidy, Anal. Chem. 65 (1993) 2878.
[28] L.A. Colón, R. Dadoo, R.N. Zare, Anal. Chem. 65 (1993) 476.
[29] X. Hung, W.Th. Kok, J. Chromatogr. A 707 (1995) 335.
[30] P.J. Oefner, A.E. Vorndran, E. Grill, C. Huber, G.K. Bonn, Chromatographia 34 (1992) 308.
[31] S. Honda, S. Iwase, A. Makino, S. Fujiwara, Anal. Biochem. 176 (1989) 72.
[32] A. Rydlund, O. Dahlman, J. Chromatogr. A 738 (1996) 129.
[33] E. Grill, C. Huber, P. Oefner, A. Vorndran, G.K. Bonn, Electrophoresis 14 (1993) 1004.
[34] S. Honda, K. Yamamoto, S. Suzuki, M. Ueda, K. Kakehi, J. Chromatogr. 588 (1991) 327.
[35] C. Chiesa, P.J. Oefner, L.R. Zieske, R.A. O'Neill, J. Cap. Electrophoresis 2 (1995) 175.
[36] A. Klockow, H.M. Widmer, R. Amadò, H.M. Widmer, A. Paulus, Electrophoresis 17 (1996) 110.
[37] J.Y. Zhao, P. Diedrich, Y. Zhang, O. Hindsgaul, N.J. Dovichi, J. Chromatogr. B 657 (1996) 307.
[38] R.A. Evangelista, M. Liu, F.A. Chen, Anal. Chem. 67 (1995) 2239.
[39] F.A. Chen, R.A. Evangelista, Anal. Biochem. 230 (1995) 273.
[40] A. Bergholdt, J. Overgaard, A. Colding, R.B. Frederiksen, J. Chromatogr. 644 (1993) 412.
[41] A.E. Vorndran, P.J. Oefner, H. Scherz, G.K. Bonn, Chromatographia 33 (1992) 163.
[42] X. Xu, W.T. Kok, H. Poppe, J. Chromatogr. A 716 (1995) 231.
[43] Y.-H. Lee, T.-I. Lin, J. Chromatogr. B 681 (1996) 87.
[44] E.S. Yeung, W.G. Kuhr, Anal. Chem. 63 (1991) 275A.
[45] T.W. Garner, E.S. Yeung, J. Chromatogr. 515 (1990) 639.
[46] P.L. Desbène, C.J. Morin, A.M. Desbène Monvernay, R.S. Groult, J. Chromatogr. A 689 (1995) 135.
[47] A.M. Desbène, C.J. Morin, N.L. Mofaddel, R.S. Groult, J. Chromatogr. A 716 (1996) 279.
[48] Q. Xue, E.S. Yeung, J. Chromatogr. A 661 (1994) 287.
[49] M.D. Richmond, E.S. Yeung, Anal. Biochem. 210 (1993) 245.
[50] W. Zhou, R.P. Balwin, Electrophoresis 17 (1996) 319.
[51] Calibration Instructions for the P/ACE LIF Detector, Beckman Instruments, Fullerton, CA, 1992.
[52] J.L. Beckers, J. Chromatogr. A 679 (1994) 153.
[53] P.E. Andersson, W.D. Pfeffer, L.G. Blomberg, J. Chromatogr. A 699 (1995) 323.
[54] M.T. Galceran, L. Puignou, M. Diez, J. Chromatogr. A 732 (1996) 167.