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

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Abstract

A newly developed capillary electrophoretic method using laser-induced fluorescence detection (CE–LIF) for the analysis of monosaccharides released from acid hydrolysis of glycosaminoglycans was studied. The method was compared with a previously published method using indirect LIF detection (CE–ILIF). For the CE–LIF method, electrophoretic conditions for the separation of the monosaccharides derivatised with 8-aminopyrene-1,3,6-trisulfonate (APTS) were optimised. The best separations were obtained using 100 m*M* acetate at pH 4.5 as running buffer. The influence of the injection vial volume on the precision and stability of the sample in different conditions was studied. The detection limits of the CE–LIF method were found to be 0.4–0.6 n*M*, while those obtained by CE–ILIF ranged from 11.4 to 14.3 μ*M*. Other quality parameters of the method, such as run-to-run precision, day-to-day precision, and linearity were also determined. Finally, the new method was applied to the analysis of the acid hydrolysis products from a glucosaminoglycan (heparin) and a galactosaminoglycan (dermatan sulfate) and cross-contamination between the two solutions was determined. The high sensitivity of the new method allows the determination of dermatan sulfate contaminations in a heparin raw sample down to 0.04% (w/w) and broadens the practical applicability of CE–LIF for the quantitation of the endogenous levels of glycosaminoglycans in animal samples and for pharmacokinetic control after therapeutical heparin administration.
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1. Introduction

One of the main areas of interest in carbohydrate analysis is related to the determination of the monosaccharide composition of glycoconjugates and free carbohydrates. This determination requires high separation efficiencies due to the presence of isomeric forms and the presence of non-glycosidic substituents, such as acetyl, sulfate ester, or amine groups. Various electrophoretic methods based on different separation and detection strategies have been applied for this purpose, resulting in high efficiency separations with lower detection limits and requiring lower sample volumes than chromatographic methods [1–8].

Derivatisation of carbohydrates is a preferred approach for the electrophoretic determination of mono- and oligosaccharides at low levels, especially using sensitive laser-induced fluorescence (LIF) detection [1–3,6,9,10]. A large variety of charged and uncharged reagents have been used for UV or fluorescing labelling commonly by means of reductive amination. Derivatisation with 2-aminoacridone

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(2-AMAC) and 8-aminopyrene-1,3,6-trisulfonate (APTS) yields fluorescing derivatives that can be excited at the excitation line wavelength of Ar lasers (ex. 488 nm) [1,6,9,11–15]. Since these are the most available lasers in commercial CE instrumentation, the use of these derivatives is becoming increasingly popular for analysis of simple carbohydrates and the glycosidic moieties of glycoconjugates in biological samples [1,6,11–15].

Glycosaminoglycans (GAGs) are linear polydisperse and heterogeneous carbohydrates that play important roles in many biological processes [16,17]. The GAG chains are composed of repeating disaccharide units consisting of one hexuronic acid (β-D-glucuronic or α-L-iduronic) and one hexosamine (D-galactosamine or D-glucosamine). Both monosaccharide units can be variously sulfated and the hexosamine units can be either *N*-acetylated or sulfated [16–19]. GAGs are classified according to their hexosamine units as glucosaminoglycans (heparin and heparan sulfate) and galactosaminoglycans (chondrotin sulfate and dermatan sulfate). Thus, monosaccharide analysis after acid hydrolysis can be a convenient strategy for the identification of GAG types and for the determination of cross-contamination between GAGs in raw materials.

Recently, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) has been applied for the estimation of GAG contents in plasma and serum [20] and for the quantitation of heparin in intraocular lenses [21]. Both methods are based on the determination of hexosamine and hexose contents after acidic hydrolysis with hydrochloric acid and further extrapolation to GAG contents. The authors reported detection limits of the order of 1–5 pmol. Using similar hydrolysis conditions, Starr et al. [22] observed hexosamine and hexuronic acids released from GAGs using fluorophore-assisted carbohydrate electrophoresis (FACE) of the monosaccharides derived with AMAC. However, no quantification was performed [22]. Whitfield et al. [23] reported that partial degradation of uronic acids occurred during acidic hydrolysis, but the HPAEC–PAD method enabled their detection for qualitative analysis. Reduction of the hexuronic acids to alditols with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) and borohydride before acidic hydrolysis of the GAGs could prevent their possible loss due to instability in acidic conditions. However, this requires considerably laborious procedures. Karamanos et al. combined this strategy with reversed-phase liquid chromatography with UV detection of per-*O*-benzoyl derivatives [24]. The method has recently been applied to the characterisation of dermatan sulfate in the presence of chondroitin sulfate in animal tissues [25].

In a previous work, we developed a coelectroosmotic electrophoretic method using indirect LIF (ILIF) detection with an Ar laser for the determination of seven monosaccharides (galactose, glucose, galactosamine, glucosamine, *N*-acetylglucosamine, *N*-acetylgalactosamine and glucuronic acid), which was applied for the determination of hexosamines released from GAGs by acid hydrolysis [8]. The aim of the present work was to develop a more sensitive CE–LIF method involving reductive amination with APTS for the determination of neutral and aminated sugars related to GAGs using the same CE instrumentation. The experimental conditions were studied and quality parameters were established. The performance of both CE–ILIF and CE–LIF methods was compared, in terms of sensitivity, linear range, and precision. Finally, the CE–LIF method was applied to the quantitation of dermatan sulfate contamination in heparin.

2. Experimental

2.1. Chemicals

Monosaccharides standards, D-glucosamine (GlcN), D-galactosamine (GalN), *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-galactosamine (GalNAc) were purchased from Sigma–Aldrich (Steinheim, Germany). D-(+)-Glucose (Glc) was obtained from Merck (Darmstadt, Germany), D-(+)-galactose (Gal) was from Carlo Erba (Milan, Italy) and D-glucuronic acid (GlcA), as monohydrate sodium salt, was from Janssen (Beerse, Belgium). Stock solutions of each monosaccharide were prepared at concentrations ranging from 1000 to 400 mg l^{-1}.

APTS was purchased from Fluka (Buchs, Switzerland), sodium cyanoborohydride (solid and 1.0 *M*

solution in tetrahydrofuran) was from Sigma–Aldrich. Tetrahydrofuran Chromosolv was obtained from Riedel-de Haën (Seelze, Germany). Citric acid was from Carlo Erba and sodium hydrogencarbonate and acetic anhydride were from Merck. Fluorescein (acid) was obtained from Merck.

Unfractionated heparin (sodium salt) obtained from intestinal porcine mucosa was kindly provided by Bioibérica (Palafolls, Barcelona, Spain). Dermatan sulfate (Chondroitin sulfate B) was obtained from Sigma–Aldrich.

Analytical-grade reagents were used for the preparation of CE buffer solutions. Acetic acid, formic acid and sodium hydroxide were obtained from Merck. Hexadimethrine bromide (HDMB) was from Fluka. Water purified using a Milli-Q water-purification system (Millipore, Bedford, MA, USA) was used for all solutions.

2.2. Instrumentation

The experiments were carried out on a P/ACE System 5500 (Beckman Coulter, Fullerton, CA, USA) equipped with an 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 fluorescence intensity was calibrated following the instructions of the supplier using a fluorescein solution (10^{-9} *M*) as fluorescence calibrator solution [26]. Data were collected using the Beckman P/ACE Station software system version 1.0.

2.3. Electrophoretic procedures

Uncoated fused-silica capillaries of 47 cm (length to the detector 40 cm)×50 μm I.D., from Beckman Coulter were used. 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 3.45 kPa (0.5 p.s.i.) for 5 s. Running electrolytes were filtered through a 0.45 μm nylon membrane, and degassed before use. The pH of electrolyte solutions was adjusted with 1 *M* sodium hydroxide. The temperature was held at 25 °C. Polypropylene minivial (20–200 μl) and polyolefin microvial (5–30 μl) inserts were used.

2.4. Derivatisation and acetylation procedures

The labelling procedure is based on the derivatisation method developed by Chen and Evangelista [12], Evangelista et al. [13] and Guttman and co-workers [6,14]. Aliquots (2 μl) of 10 m*M* individual monosaccharide standards solutions, corresponding to 20 nmol, were labelled by addition of 2 μl 0.2 *M* APTS in 25% citric acid and 4 μl of 1 *M* sodium cyanoborohydride solution in tetrahydrofuran (THF). The reaction mixtures were incubated at 37 °C overnight. The labelling reactions were stopped by addition of 42 μl of water. Ten- and 500-fold dilutions were prepared and injected for identification in the CE system. Standard mixtures of approx. 1.44 μ*M* of each monosaccharide derivative were prepared from the individual diluted solutions. The rest of the standards (down to $2.27 \cdot 10^{-9}$ *M*) were prepared by further dilution of these mixtures. The derivatives were stored at −18 °C and no degradation was observed during the 3-month period of use.

Assessment of the *N*-acetylation procedure was performed as follows. An 18-μl volume of acetic acid anhydride–0.2 *M* sodium hydrogencarbonate, pH 9.5 (adjusted with 1 *M* NaOH) (1:8, v/v) was added to 2-μl aliquots of 10 m*M* individual GalN and GlcN solutions. The solutions were incubated at 0 °C for 20 min. Finally, the acetylated solutions were dried and derivatised following the above described procedure.

2.5. GAG hydrolysis and derivatisation procedures

Solutions of GAG samples were prepared at 400 μg ml^{-1} in Milli-Q water. A 50-μl volume of 8 *M* hydrochloric acid was added to aliquots of 50 μl containing 20 μg of GAGs in a 1-ml conical glass vial. The solutions were 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 vacuum evaporator. The pellet was re-suspended in 2 μl of water and further *N*-acetylated and derivatised following the proce-

dures previously described. The derivatisations were stopped by addition of 242 μl of water. Twenty-five- and 250-fold dilutions were prepared and injected for quantitation in the CE system. Derivatised products were stored in the reaction vials at −18 °C.

3. Results and discussion

3.1. Electrophoretic separation of APTS-monosaccharides

In a preliminary study, different electrophoretic systems were tested for the separation of the derivatised monosaccharides. The optimisation was performed using freshly prepared $7.3 \cdot 10^{-8}$ *M* solutions of each monosaccharide and by the injection of individual solutions of $7.3 \cdot 10^{-7}$ *M*. Counterelectroosmotic systems using a borate buffer (100 m*M* at pH 8.35), coelectrosmotic systems with a borate buffer (100 m*M*) or potassium bromide (10–20 m*M*) and hexadimethrine bromide (HDMB, 0.0005%), and reverse polarity systems using formate or acetate buffers at pH 3.0–5.0 as running electrolytes were used. A significant number of intense peaks due to the derivatising agent or possible contaminants of reagents were observed in addition to those of the analytes. Cleaner blank electropherograms were obtained using the commercial 1.0 *M* solution of sodium cyanoborohydride in THF than preparing it from the individual reagents. In reverse polarity systems using acidic electrolytes such as formate or acetate buffers, these peaks were detected at the beginning of the electropherogram, while the analytes were clearly detected at longer migration times. On the other hand, when basic electrolytes were used, these peaks appeared distributed in different zones of the electropherogram. Narrower peaks and better resolutions for the analytes (Glc, Gal, GlcNAc and GalNAc) were obtained in acidic conditions. Studies performed by other authors [6] indicate that the yield of the derivatisation procedure of hexosamines (<50%) is lower than that of the acetylated hexosamines and, as a consequence, instead of the direct determination of the derivatised hexosamines, the introduction of an acetylation step was recommended to obtain higher sensitivities. Thus, assignation of APTS derived hexosamine peaks in the electropherograms of the standards was not reported in these works. In our case, concentrated standard solutions of derivatised hexosamine and glucuronic acid (up to 10^{-5} *M*) were injected but, as in the previous work [6], unequivocal assignation of the signals was not accomplished, as they did not present peaks that could be distinguished from the blank electropherogram.

The optimisation of the separation was performed using acidic electrolytes at different concentrations with different concentration and pH values at various voltages. Running buffers based on 50–100 m*M* formic acid or acetic acid solutions adjusted with 1 *M* NaOH to a pH ranging from 3.0 to 4.8, and from 4.0 to 5.0, respectively, were tested. The use of 75 and 50 m*M* formic acid and acetic acid electrolytes and pH buffers lower than 4.0 did not enable good resolution. Migration times and resolutions between the hexoses and the *N*-acetylhexosamines using 100 m*M* running buffers at −25 kV are given in Fig. 1a and b. Higher resolutions with lower intensity current values were obtained using acetate buffers. These values ranged from 100.0 to 128.5 μA in formate buffers and from 20.4 and 70.0 μA in acetate buffers. Lower applied voltage considerably increased analysis time but there was no significant improvement in resolution. On the basis of higher resolution and shorter analysis time, a 100 m*M* acetate buffer at pH 4.50 was selected for the electrophoretic separation of the APTS-monosaccharides at −25 kV. Fig. 2 shows the electropherograms obtained under the selected conditions.

The stability of the sample solutions was also studied. Due to the small volume of standards and samples, the use of vial inserts of 30 μl (microvial inserts) and 300 μl (minivial inserts) was mandatory. Additional peaks, which might be due to the degradation of the labelling reagent, appeared in both the control standard mixture and the blank when these were kept for more than 36 h in the polypropylene and polyolefin injection inserts, even when stored at −18 °C. This phenomenon can be seen in Fig. 2, where the signals of the initial peaks are shown beside the new electrophoretic profile. This effect was not observed when solutions were kept in the glass vials used for preparation instead in the polypropylene inserts. On the other hand, the reduced volume of the inserts promoted solvent evaporation

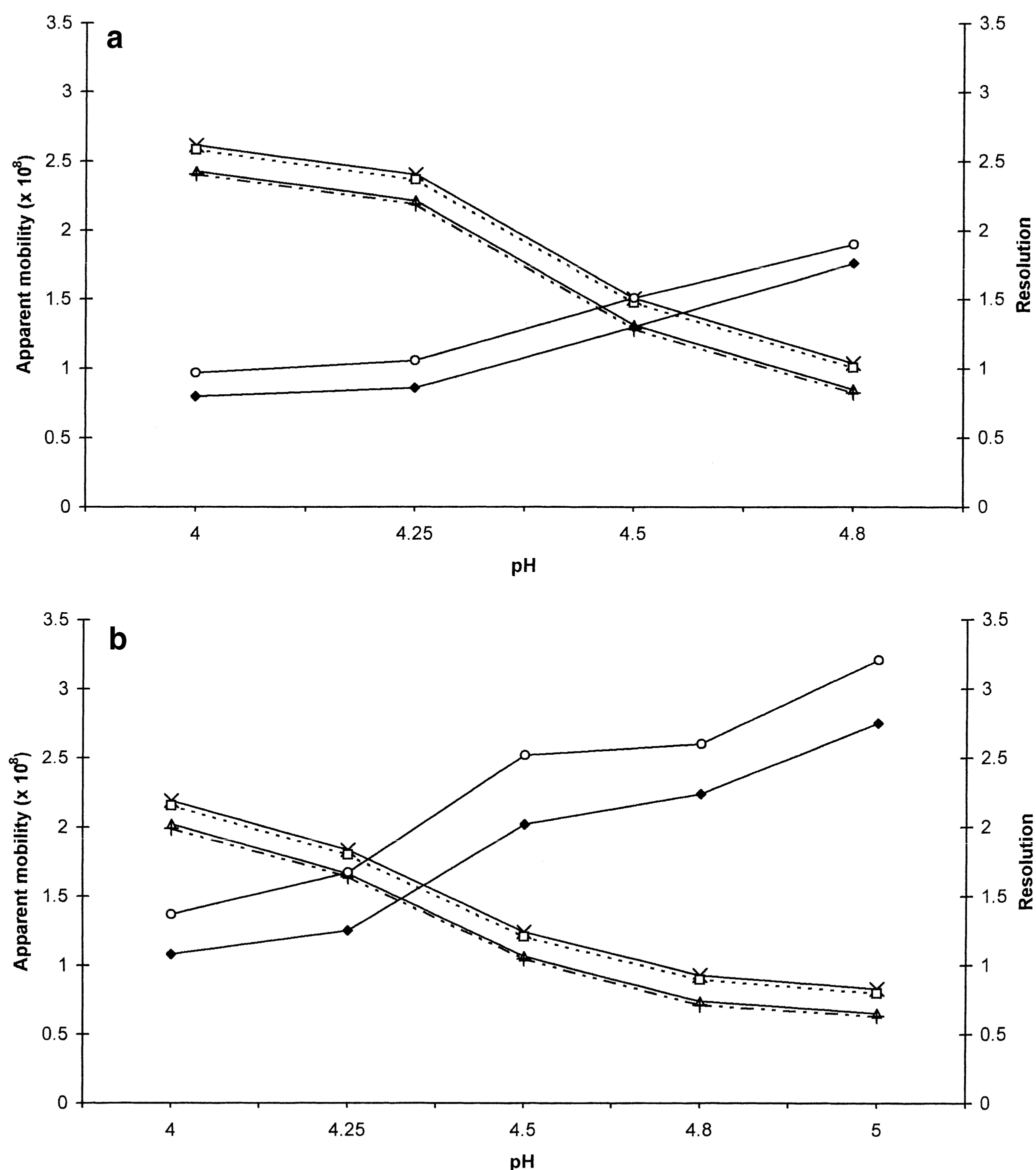

Fig. 1. Apparent mobility ($m^2\ V^{-1}\ s^{-1}$) and resolution of the hexoses and hexosamines vs. pH in (a) formic buffers and (b) acetic buffers. Voltage applied: −25 kV, injection time (0.5 p.s.i.): 5 s. Peak assignments as in Table 1. Apparent mobility of peaks (×) 1, (□) 2, (△) 3 and (+) 4. Resolution of (○) 1–2 and (♦) 3–4.

and consequent concentration when the samples were left in the autosampler tray. In particular, using microvial inserts (maximum volume 30 μl) and injecting from partially filled vials (less than 50% maximum volume) after sequences longer than 12 h produced erratic injection, which was reflected in peak distortion and loss of resolution. When minivial inserts were used, although solvent evaporation was not visually observed the increase in areas after 8, 16 and 24 h was estimated as 16.6±1.8, 29.2±2.3 and 47.0±1.3%. In addition, the appearance of degradation peaks was occasionally observed after 24 h. For these reasons, samples were prepared and stored at −18 °C in glass vials and it was necessary to

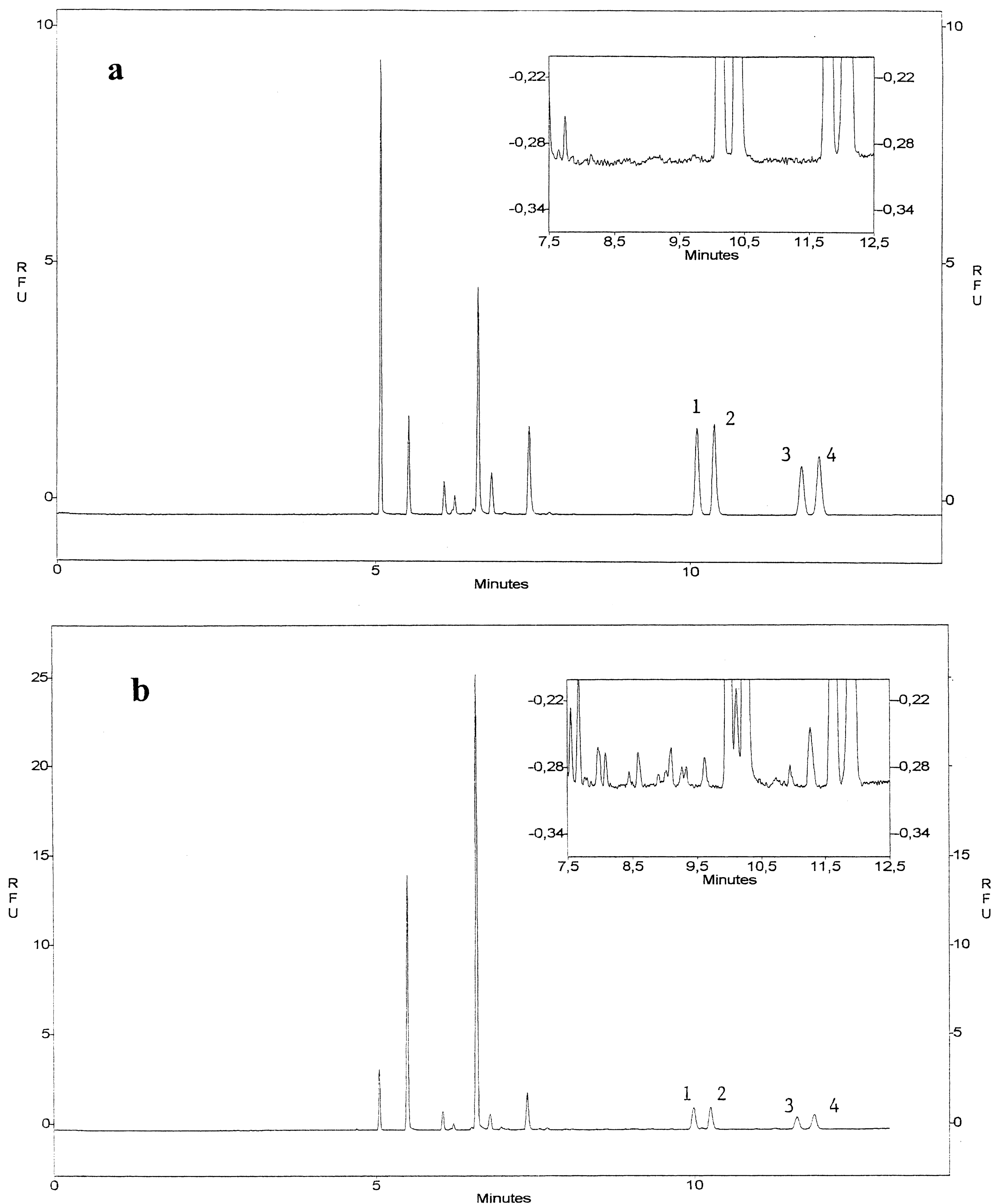

Fig. 2. CE–LIF electropherograms of APTS-derivatised monosaccharides (approx. 13–16 μg l^{-1}). (a) Freshly prepared dilutions and (b) solutions kept 48 h at −18 °C in polypropylene injection inserts. Experimental conditions: 100 m*M* CH_3COOH, pH 4.50 (NaOH adjusted), voltage applied: −25 kV, injection time (0.5 p.s.i.): 5 s. Peak assignments as in Table 1.

replace the injection vials and discard the solutions after run sequences longer than 8 h.

3.2. Quality parameters. Comparison of CE–ILIF and CE–LIF methods

The limits of detection (LODs) based on a 3:1 signal-to-noise ratio are given in Table 1. Concentration LODs are on the order of 0.6 n*M*, which corresponds to 0.1 $\mu g\ l^{-1}$ for GalNAc and GlcNAc, and 0.4 n*M* for Gal and Glc. These values are $2\cdot10^{4}$ times lower than those obtained using ILIF detection (approx. $13\cdot10^{-6} M$). Approximately 6 nl is injected (5 s, 0.05 p.s.i.), 4 amol for GalNAc and GlcNAc, and it was possible to detect 3 amol for Gal and Glc, respectively. Good linearity ($r^2>0.9996$) was observed in the range from approx. $1.5\cdot10^{-6}$ to $1.8\cdot10^{-9}$ *M* for the monosaccharides, which corresponds to 270–0.32 and 322–0.40 $\mu g\ l^{-1}$ for the hexoses and *N*-acetylhexosamines, respectively. The linearity range of this CE–LIF method encompasses three orders of magnitude while in indirect mode linearity was observed in two orders of magnitude (approx. 10^{-4}–10^{-3} *M*).

The influence of the volume of the injection vial on precision was studied. The data for this are also given in Table 1. Run-to-run precision was determined using the microvial and minivial inserts for the standards. Relative standard deviations (RSDs) were calculated on both migration time ($n=6$) and areas ($n=6$) using a standard test sample at the $7.3\cdot10^{-8}$ *M* (13–16 $\mu g\ l^{-1}$) level. Migration times and area values were satisfactorily reproducible (RSDs lower than 0.8 and 5.62%, respectively) using both inserts. However, better precision was observed using microvial inserts of 300 μl, the RSDs of area values being lower than 1.21%. Day-to-day precision was calculated using minivial inserts and data corresponding to 3 days. The accuracy of the method was estimated by comparison of the actual and calculated concentration values of the standard test sample using minivials. In all cases, standard deviations were lower than 5%.

3.3. Application to the analysis of hydrolysed GAG samples

The CE–LIF conditions were applied to the analysis of the APTS derivatised products from the acid hydrolysis of heparin and dermatan sulfate, and the results were compared to those obtained using the CE–ILIF method. The hydrolysis, acetylation and derivatisation procedures are described in the Experimental section. While in CE–ILIF the determination of hexosamines was used as an analytical tool for the characterisation of glucosaminoglycans and galactosaminoglycans, the direct CE–LIF method required the acetylation and derivatisation with APTS of the samples for the determination of APTS-derived *N*-acetylhexosamines. Quantitation of the GlcNAc and GalNAc contents in heparin and dermatan sulfate by CE–LIF was compared to that corresponding to the hexosamine contents determined by CE–ILIF, using the same acid hydrolysis conditions. For the comparison, the results of *N*-acetylhexosamine quantitation were expressed as hexosamine percentages.

Table 1
CE–LIF quality parameters

Compound		Detection limits			Run-to-run precision				Day-to-day precision minivial. RSD area (%)	Accuracy[b] minivial. (% Bias)
		$\mu g\ l^{-1}$	n*M*	Amount injected[a] (mol)	μvial		Minivial			
					RSD t_m (%)	RSD area (%)	RSD t_m (%)	RSD area (%)		
1	Glc	0.072	0.40	$2.52\cdot10^{-18}$	0.67	5.58	0.17	1.07	5.53	2.39
2	Gal	0.080	0.44	$2.80\cdot10^{-18}$	0.70	5.16	0.11	1.08	3.59	5.06
3	GlcNAc	0.140	0.63	$3.99\cdot10^{-18}$	0.77	5.62	0.23	1.21	3.08	5.83
4	GalNAc	0.134	0.61	$3.82\cdot10^{-18}$	0.79	4.80	0.20	0.96	3.40	4.88

[a] Pressure injection (0.5 p.s.i.) 5 s.
[b] Concentration of monosaccharide standard solution: 13–16 $\mu g\ l^{-1}$.

In a preliminary study, heparin and dermatan samples were quantitated using the calibration functions obtained with the GalNAc and GlcNAc standards. The hexosamine contents in the hydrolysed, *N*-acetylated and derivatised samples thus calculated were lower than expected (compared to those of the CE–ILIF method), this effect could be caused by the influence of the experimental conditions of both the acid hydrolysis and the acetylation step on the yield of the formation of the APTS-derived *N*-acetylated hexosamines. In fact, the acetylation yield, estimated from the quantitation of the APTS derivatives of the *N*-acetylated hexosamine standards using the calibration built with the derivatives of *N*-acetylhexosamines in the aforementioned linearity range, was 85.5% (±3.6). Therefore, the GAG samples were quantified using GalN and GlcN standards that were manipulated as the samples during the acid hydrolysis, acetylation, and derivatisation processes. The linearity was established in the range from approx. $1.5 \cdot 10^{-6}$ to $1.8 \cdot 10^{-9}$ *M*, which corresponds to 323–0.34 $\mu g\ l^{-1}$ for the hexosamines ($r^2 > 0.998$). Run-to-run precision of area values (RSD) was lower than 2%. The results obtained in this way are similar to those of the CE–ILIF method. The GlcN and GalN contents thus calculated were 17.39±0.97 and 24.28±1.3% (w/w) in heparin and dermatan sulfate, while the values obtained using the indirect method were 16.8±1.2 and 25.1±1.9% (w/w), respectively. The confidence limits are expressed as the standard deviation corresponding to four determinations. In contrast to the ILIF, the CE–LIF electropherograms of the heparin and dermatan sulfate present detectable peaks of both *N*-acetylhexosamines. Percentages of GalN in heparin and GlcN in dermatan sulfate were 0.164±0.011 and 0.736±0.06% (w/w), respectively. Hence, in this case, cross-contamination can be evaluated. The previously established percentage of GalN in dermatan sulfate (DS) standard was used to calculate DS contents in heparin. Thus, residues of dermatan sulfate in the heparin sample were of 0.65% (w/w).

The feasibility of the CE–LIF method to determine contamination of heparins with galactosaminoglycans was established using spiked heparin samples. Four heparin standard solutions were spiked with dermatan sulfate at levels corresponding to 6.0, 10.0, 19.9, and 29.8% (w/w) of DS in heparin. The DS percentages calculated in the samples (*n*: 4) were, respectively, 6.84±0.803, 10.0±0.252, 19.3±0.576 and 29.1±1.19%. Fig. 3 shows the electropherograms corresponding to the hydrolysed, acetylated and derivatised heparin, dermatan sulfate, and spiked samples of 10 and 20% (w/w). Using the LOD and limit of quantitation (LOQ) values of GalNAc, a DS contamination of 0.13% could be detected and 0.40% quantified. However, if the injection of a 10-fold concentrated solution of heparin (3.2 $\mu g\ ml^{-1}$) was performed, the method could be useful to quantify levels down to 0.040% (w/w) with electrophoretic resolution good enough for analytical purposes. This is a considerable improvement in relation to the ILIF method applicability, which enabled quantitation of levels of 2% (w/w).

4. Conclusions

A CE–LIF method for the determination of APTS derived hexoses and *N*-acetylhexosamines has been described. The influence of injection vial volumes and the stability of the derivatives were evaluated and, in comparison to the CE–ILIF method previously described, the new procedure provides better figures of merit such as superior sensitivity and reproducibility and longer linearity range. In contrast, the CE–LIF method requires additional acetylation and derivatisation steps. Finally, the CE–LIF method was applied to the determination of hexosamine contents in hydrolysed sulfated glycosaminoglycans samples and mixtures. Minor contamination of dermatan sulfate (0.016%, w/w) in heparin samples could be detected. The sensitivity of this method (0.4–0.6 n*M*) is considerably lower than that of the HPLC–PAD methods commonly applied for glycosaminoglycan determination as hexosamines in biological samples such as plasma and serum. Therefore, this CE–LIF method seems to be particularly convenient for future applications in this field, such as the determination of the endogenous levels of GAGs and pharmacokinetic studies, where submicroscale analysis is mandatory because of both the sample restrictions regarding amounts and because of the low content of GAGs.

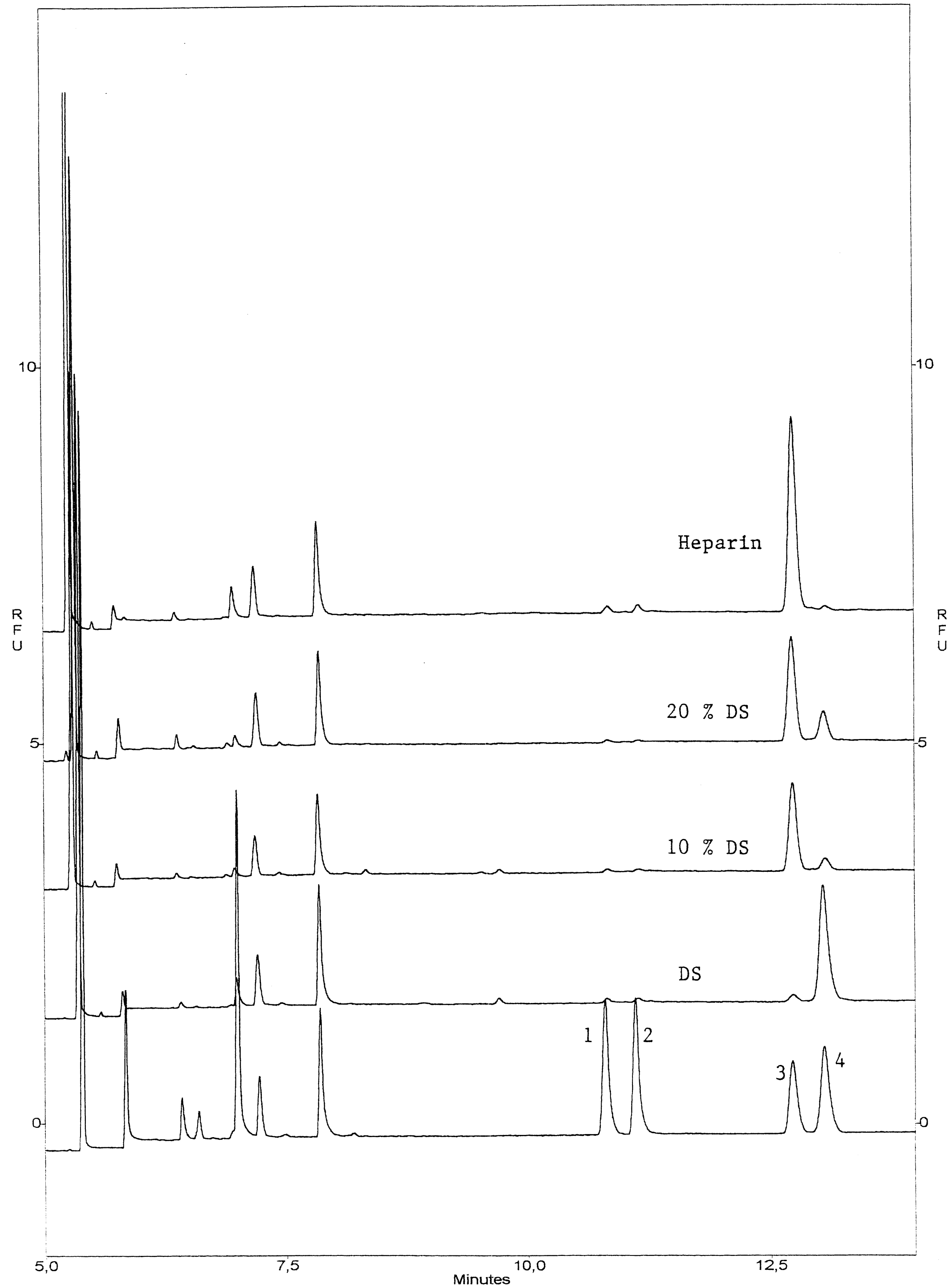

Fig. 3. CE–LIF electropherograms of APTS-monosaccharide standard solution and derivatised hydrolysates of heparin, dermatan sulfate and heparin spiked samples with dermatan sulfate at DS/total GAG ratios of 10.0 and 20.0% (w/w). Hydrolysed samples are acetylated before derivatisation. Electrophoretic conditions as in Fig. 2. Peak assignments as in Table 1.

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