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Separation of natural and synthetic heparin fragments by high-performance capillary electrophoresis

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ABSTRACT

The application of capillary electrophoresis (CE) for the analysis of natural and synthetic low-molecular-mass heparin fragments at low pH is described. It is demonstrated that under the applied conditions the separation is based on charge, charge distribution and molecular mass of the heparin molecules, yielding a high resolution. It is shown that the presence of sodium chloride in the sample solution has hardly any effect on the CE performance. However, the pH of the electrophoresis buffer is a critical parameter. The resolutions obtained with CE and high-performance anion-exchange chromatography (HPAEC) are compared for various heparin fragments and it is concluded that, at least for this type of molecule, CE forms an attractive alternative to HPAEC.

INTRODUCTION

Heparin occurs as a proteoglycan consisting of a small protein core to which multiple large glycosaminoglycan side-chains are attached. The glycosaminoglycan chains vary in length and consist of repeating uronic acid glucosamine disaccharide sequences in which the uronic acid may be either D-glucuronic acid or L-iduronic acid and the glucosamine residue may be either N-acetylated or N-sulphated [1]. Moreover, the disaccharide units are O-sulphated to varying extents at C-6 and/or C-3 of the various glucosamine residues and at C-2 of the uronic acid residues [1]. Thus very heterogeneous polymers, both in molecular mass and structure, are formed. A final cause of heterogeneity is the occurrence of a unique D-glucosamine-N,6-disulphate-(α1–4)-L-iduronic acid-2-sulphate-(β1–4)-D-glucosamine-N,3,6-trisulphate-(α1–4)-D-glucuronic acid-(β1–4)-D-glucosamine-N,6-disulphate pentasaccharide sequence [2]. This pentasaccharide occurs in approximately one third of the heparin molecules and is reponsible for the well documented anticoagulant activity of heparin through a high-affinity binding to antithrombin III, resulting in a highly increased inhibition of factor II_a, X_a and XII_a activity [3–7].

For over 50 years the anti-blood-clotting activity of heparin, which is commercially prepared from porcine intestinal mucosa and bovine lung, has been exploited in the treatment of venous thrombosis. Until two decades ago the application was limited to therapeutic treatment of established thrombi. However, a second major use of heparin has evolved, namely the prevention of postsurgical thrombosis. After major surgery there is a greatly increased risk of thrombosis, especially for middle-aged and elderly patients [8]. A significant advantage of the prophylactic use of heparin is that lower

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dosages can be applied which prevents the persistent hypocoagulation associated with therapeutic use [9,10].

However, the vast structural heterogeneity of heparin renders the biological activity, in both qualitative and quantitative respects, undefined and presents a serious limitation of its value as a pharmaceutical drug. This concern is even greater as it is becoming increasingly clear that unfractionated heparin displays a wide variety of biological effects not related to anticoagulant or antithrombotic activity [11–18].

Therefore, during the past decade attempts have been made to produce better defined heparin preparations. This has been achieved in part by the generation of low-molecular-mass heparin fragments of more or less uniform mass by either fractional precipitation and gel filtration [19] or chemical [20] or enzymatic depolymerization [21]. It has been demonstrated that low-molecular-mass heparin fragments of relative molecular mass 4000–5000 can be effectively applied as prophylactic drugs after surgery [22]. As heparin preparations of $M_r <$ 5000 retain a high anti-factor X_a activity (for which the pentasaccharide sequence is responsible) but have a reduced or no anti-factor II_a (thrombin) activity, it has been suggested that these preparations may have a better benefit/risk ratio in terms of antithrombotic activity and risk of bleeding. Although the mechanism of action is still a matter of debate, data are accumulating that after subcutaneous injection, low-molecular-mass heparins cause less hypocoagulation, give rise to a longer lasting inhibition of factor X_a activity and are indeed less haemorrhagic than native heparin [22,23]. Moreover, it has been shown [23] that these preparations have a twofold prolonged half-live in circulation (which, in contrast to heparin, is not dose-dependent) and have a four- to ninefold higher bioavailability on subcutaneous injection. Finally, there are indications that the incidence of adverse side-effects (lipolysis, thrombocytopenia) associated with the administration of heparin is lower with low-molecular-weight heparins [23].

However, the low-molecular-mass preparations still show a considerable structural heterogeneity, thereby impeding predictions about bioactivity and pharmokinetics. Moreover, fractional precipitation and chemical depolymerization result in a relative enrichment of material without the unique pentasaccharide sequence and hence proportional loss of anticoagulant activity. Alternatively, enzymatic depolymerization leads to the formation of fragments with modified reducing end-groups which may raise concerns with respect to the potential immunogenic effects.

A more attractive way to produce a well defined heparin fragment with a high specific anticoagulant activity was found in the chemical synthesis of the unique pentasaccharide sequence [24–27]. These preparations have shown to be safe and effective anticoagulant drugs in *in vivo* studies [2,28] and at present clinical studies are in progress. Following the synthesis of the natural pentasaccharide, various derivatives have been synthesized [6,27,29–32], some of them displaying an increased AT-III affinity [29].

An important aspect of the production of natural and synthetic heparin fragments for pharmaceutical use is the availability of analytical procedures for the characterization of intermediates and final products. As the heparin fragments have an inherent high potential for microheterogeneity, the analytical procedures must meet stringent criteria. Described methods for the separation of mixtures of heparin fragments rely on high-performance size-exclusion [33], anion-exchange [34] and reversed-phase ion-pair chromatography [20,35] and polyacrylamide gel electrophoresis [36].

Recently, high-performance capillary electrophoresis (CE) was reported as a sensitive and high-resolution method for the determination of the disaccharide composition of several proteoglycans [37,38]. In these studies the disaccharides were fractionated as borate complexes at relatively high pH. Here we report the separation of complex mixtures of heparin fragments, having relative molecular masses up to 3500 and containing well over ten (negatively) charged groups, without the addition of borate, and we compare the performance of high-performance anion-exchange chromatography (HPAEC) with CE. It is demonstrated that CE is potentially a good method for the quality control of natural and synthetic heparin fragments.

EXPERIMENTAL

Materials

Colominic acid oligomers were prepared by partial hydrolysis of colominic acid (Sigma, St. Louis, MO, USA). Heparin disaccharide reference compounds were obtained from Grampian Enzymes (Aberdeen, UK). Natural heparin-derived fragments were obtained from Diosynth (Oss, Netherlands) and were prepared by treating mucosal porcine heparin with heparinase. Synthetic pentasaccharides were made at Organon International (Oss, Netherlands) in cooperation with Sanofi (Toulouse, France) and the structures were verified by ^{1}H and ^{13}C NMR spectroscopy [30–32] and fast atom bombardment mass spectrometry [39].

Preparation of colominic acid standards

A mixture of polyanionic oligosaccharides was prepared by controlled depolymerization of colominic acid [poly (α2–8)-N-acetylneuraminic acid, $(NeuAc)_n$, from *E. coli*; Sigma] as follows. A 10-mg amount of colominic acid was dissolved in 1 ml 0.1 *M* HCl (J. T. Baker, Deventer, Netherlands) and incubated in an air-tight reaction vial at 80°C for 45 min. The reaction products were desalted on a Bio-Gel P-2 (200–400 mesh) column (18 × 0.8 cm I.D.) (Bio-Rad, Veenendaal, Netherlands), lyophilized and stored at −20°C until used.

Preparation of heparin fragments

Mucosal heparin was dissolved in 0.25 *M* ammonium acetate solution to a concentration of 20 mg/ml and 5 U/ml of heparinase (from *Flavobacterium heparinum*, E.C. 4.2.2.7; Sigma) were added. The mixture was incubated for 60 h at 35°C. The degraded heparin was batch adsorbed on an anion exchanger and desorbed stepwise by elution with NaCl (Merck, Darmstadt, Germany) solutions of increasing molarity. The material eluting between 5 and 10% (w/v) NaCl was applied to a gel permeation chromatographic system, consisting of two columns in tandem, namely a TSK Fractogel HW 40 S column (70 × 2.6 cm I.D.) (Merck) and a Bio-Gel P-2 (400 mesh) column (100 × 2.5 cm I.D.) (Bio-Rad). Elution was carried out with 0.5 *M* ammonium acetate (Sigma), pH 5.0 at a flow-rate of 0.7 ml/min and the chromatogram was recorded with a refractive index detector (ERC 7510 Erma). Fractions of increasing molecular mass were combined into six pools, which on the basis of elution volume correspond to di-, tetra-, hexa-, octa-, deca- and dodecasaccharides. The pools containing the di- and dodecasaccharides were not used for further studies. The remaining pools were desalted on Bio-Gel P-2, lyophilized and stored at −20°C until used.

High-performance anion-exchange chromatography

The colominic acid hydrolysate, the disaccharide reference compounds, the heparin fragments obtained by heparinase treatment and gel permeation chromatography and the synthetic pentasaccharides were each dissolved in water purified with a Milli-Q-system (Millipore, Milford, MA, USA) to a concentration of 0.1–5 mg/ml and separated by anion-exchange chromatography on a Hewlett-Packard (Palo Alto, CA, USA) Model 1050 HPLC system equipped with an HR 55 Mono Q column (Pharmacia, Uppsala, Sweden). The injection volume was 25–200 μl and the column was eluted at a flow-rate of 1 ml/min using linear concentration gradients of 0–1, 0–2 or 0.88–1.60 *M* NaCl in Milli-Q-purified water. The elution profiles were recorded at 214 or 215 nm.

High-performance capillary electrophoresis

The colominic acid hydrolysate, the disaccharide reference compounds, the heparin fragments obtained by heparinase treatment and gel permeation chromatography and the synthetic pentasaccharides were each dissolved in Milli-Q-purified water to a concentration of 0.1–5 mg/ml and separated by high-performance capillary electrophoresis using a Beckman (Palo Alto, CA, USA) P/ACE 2100 CE system equipped with a UV absorbance detector and a fused-silica capillary tube (57 cm × 75 μm I.D., detector at 50 cm). System operation and data handling were fully controlled and integrated via the System Gold software (version 6, Beckman) running on an IBM 55 SX personal computer. The apparatus was operated in the reversed polarity mode, *i.e.*, the sample was introduced at the cathodic side of the capillary. Samples were loaded by applying pressurized nitrogen for 10 s, resulting in the injection of 25 nl of sample solution. Before introduction of the first sample, the capillary was rinsed for 15 min each with 0.1 *M* H_3PO_4, 0.5 *M* NaOH, 0.1 *M* NaOH (all from J. T. Baker), Milli-

Q-purified water and running buffer. Between runs only the last three wash steps were applied. Separations were carried out using 200 m*M* NaH_2PO_4 (J. T. Baker), adjusted to pH 2, 3 or 4 with concentrated H_3PO_4, as running buffer at a potential of 7.5 kV (131.5 V/cm) and 40°C. On-capillary detection was performed by UV absorbance measurement at 214 nm.

RESULTS AND DISCUSSION

The optimum conditions for the separation of sulphated glycosaminoglycans by CE were determined using colominic acid oligomers and heparin disaccharide reference compounds. It was established that at low pH the oligosaccharides can be effectively separated without complexation with borate. The apparatus is operated in the reversed polarity mode and hence the migration time is, in principle, inversely correlated with the negative charge of the oligosaccharide. Operation at low pH ensures that the electroosmotic flow is nearly eliminated and is smaller than the electrophoretic flow, affording migration of the solutes towards the detector. Colominic acid was partially hydrolysed to afford a mixture of mono- and di- to *n*-mers, having the general formula $(NeuAc)_n$, where *n* represents the degree of polymerization and the number of (charged) carboxyl groups. The oligomers were subjected to CE and HPAEC to compare the effect of (negative) charge on the migration and elution time, respectively. At pH 4, where the ionization of the carboxyl groups of the N-acetylneuraminic acid residues is nearly complete, CE resulted in baseline separation of the mono- to nonamers (Fig. 1A).

The identity of the peaks, as indicated in Fig. 1A, was established by injection of oligomers having a known degree of polymerization. The peaks migrating at 31.79 and *ca.* 31 min (inset in Fig. 1A) probably represent larger colominic acid polymers that remained after partial hydrolysis. As expected, the migration time increases as the degree of polymerization and hence the number of carboxyl groups decrease. The pH of the electrophoresis buffer appears to be very critical for a high resolution as neither at pH 3 nor at pH 5 was a satisfactory result obtained (results not shown). Probably at pH 3 the carboxyl groups of the colominic acid oligomers are not sufficiently ionized (p*K* 2.9) and at pH 5 the electroosmotic flow, which is in the direction of the injection site, is too high.

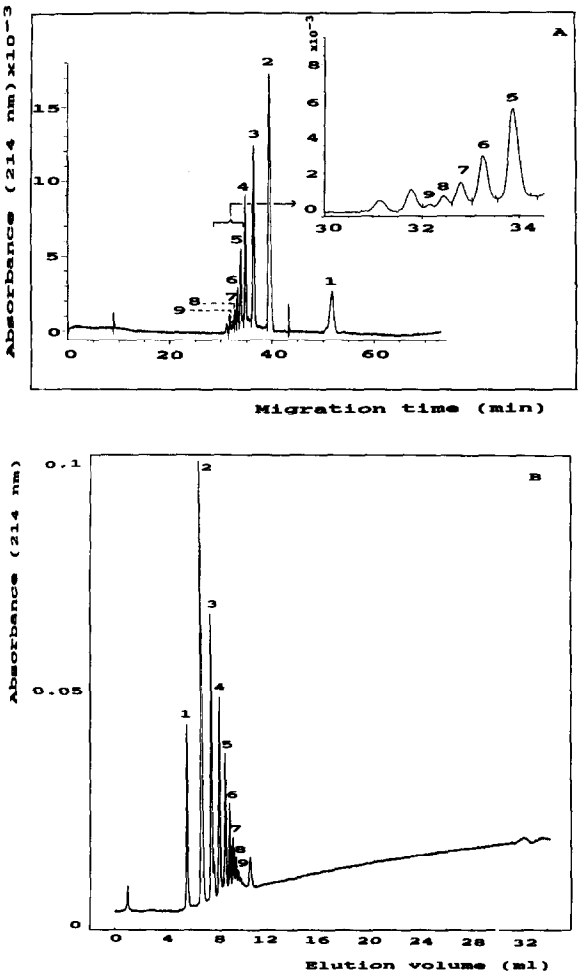

Fig. 1. (A) CE and (B) HPAEC of a partial hydrolysate of colominic acid. Prior to CE and HPAEC the hydrolysate was desalted by gel permeation chromatography on a Bio-Gel P-2 column. CE was carried out in 200 m*M* phosphate buffer (pH 4) at 40°C and 7.5 kV in the reversed polarity mode. Column: 57 cm (50 cm effective length) × 75 μm I.D. On-capillary detection was at 214 nm. Injection by pressurized nitrogen: 25 nl from a solution containing 5 mg/ml of the hydrolysate. The number of (negatively charged) N-acetylneuraminic acid residues of the oligomers is indicated. HPAEC was carried out on a Mono Q HR 5/5 column using a linear concentration gradient of 0–2 *M* NaCl in 30 ml of Milli-Q-purified water at a flow-rate of 1 ml/min. Detection at 214 nm. Injection volume, 25 μl from a sample solution containing 5 mg/ml of the hydrolysate.

In Fig. 1B, the chromatogram obtained for the same sample with HPAEC on Mono Q is depicted.

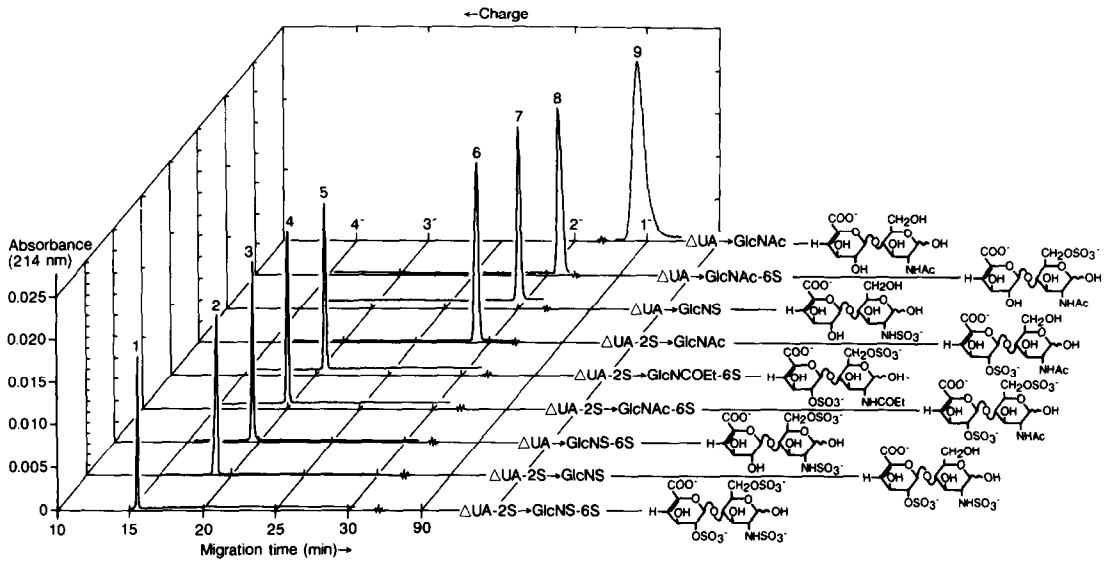

Fig. 2. CE of nine heparin disaccharides. The electropherograms are compiled in a three-dimensional plot. Electrophoresis was carried out as in Fig. 1A, except that the electrophoresis buffer was pH 3. Injection volume, 25 nl from a solution containing *ca.* 0.1 mg/ml of each disaccharide. The structures of disaccharides **1**–**9** are indicated. One part of the absorbance scale corresponds to 0.005 a.u.f.s.

Again, nine peaks, belonging to the mono- to nonamers, are visible with an additional peak at *ca.* 10.5 min which can be attributed to remaining polymeric material. As expected, the order of elution is reversed compared with CE. It should be noted that with HPAEC the separation is accomplished in a shorter time. However, much more material (125 μg *vs.* 125 ng) is needed for the analysis.

In Fig. 2, the capillary electropherograms obtained at pH 3 for nine heparin disaccharides (denoted **1**–**9**) are shown. $\Delta^{4,5}$-Uronic acid-2-sulphate-(β1–4)-glucosamine-N,6-disulphate (**1**, ΔUA-2S→GlcNS-6S), having four negative charges (three sulphate groups and one carboxyl group), migrates at 15.5 min.

$\Delta^{4,5}$-Uronic acid-2-sulphate-(β1–4)-glucosamine-N-sulphate (**2**, ΔUA-2S→GlcNS), $\Delta^{4,5}$-uronic acid-(β1–4)-glucosamine-N,6-disulphate (**3**, ΔUA→GlcNS-6S), $\Delta^{4,5}$-uronic acid-2-sulphate-(β1–4)-N-acetylglucosamine-6-sulphate (**4**, ΔUA-2S→ GlcNAc-6S) and $\Delta^{4,5}$-uronic acid-2-sulphate-(β1–4)-glucosamine-N-carboxyethyl-6-sulphate (**5**, ΔUA-2S→GlcNOEt-6S), all having three negative charges (two sulphate and one carboxyl group), migrate at 19.1, 19.8, 20.2 and 20.8 min, respectively.

$\Delta^{4,5}$-Uronic acid-2-sulphate-(β1–4)-N-acetylglucosamine (**6**, ΔUA-2S→GlcNAc), $\Delta^{4,5}$-uronic acid-(β1–4)-glucosamine-N-sulphate (**7**, ΔUA→GlcNS) and $\Delta^{4,5}$-uronic acid-(β1–4)-N-acetylglucosamine-6-sulphate (**8**, ΔUA GLcNAc-6S), each having two negative charges (one sulphate and one carboxyl group), migrate at 29.7, 29.9 and 30.8 min, respectively. Finally, $\Delta^{4,5}$-uronic acid-(β1–4)-N-acetylglucosamine (**9**, ΔUA→GlcNAc) with one negative charge migrates at 89.0 min. From this it is clear that the separation is based on (negative) charge, as already shown for the colominic acid oligomers, but also on structure (*i.e.*, charge distribution). Simultaneous injection of the nine disaccharides has no effect on the migration times, indicating that the migration properties do not influence each other (Fig. 3A). In this case ΔUA-2S→GlcNAc (**6**) and ΔUA→GlcNS (**7**), migrating at 29.7 and 29.9 min, respectively, show a large overlap.

For comparison, the nine disaccharide reference compounds were also analysed by HPAEC on Mono Q (Fig. 3B). As with CE, eight baseline-separated peaks are obtained. Compounds **4** (ΔUA-2S→GLcNAc-6S) and **5** (ΔUA-2S→GlcNOEt-6S), which were completely resolved by CE, give rise to overlapping peaks. On the other hand compounds **6**

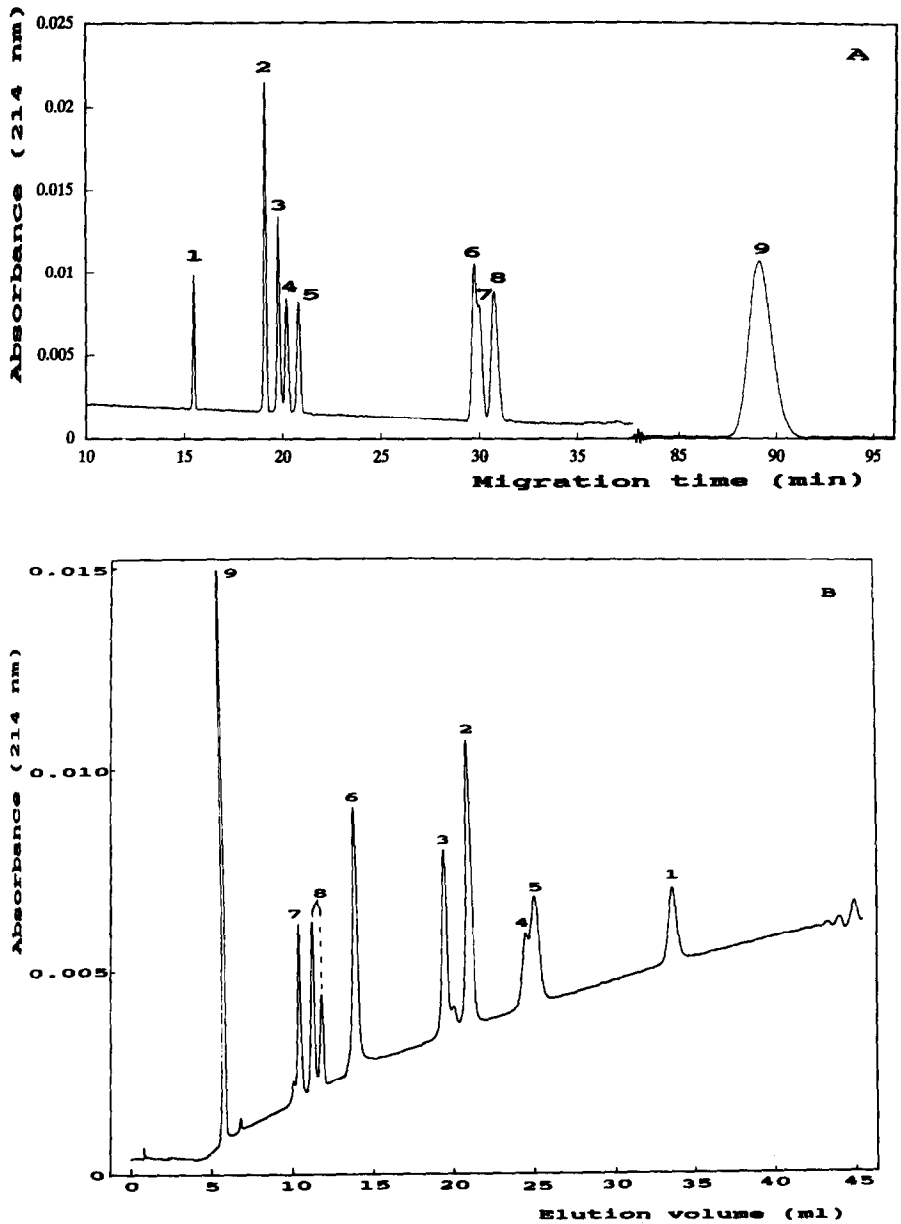

Fig. 3. (A) CE and (B) HPAEC of a mixture of nine heparin disaccharides. Conditions for CE and HPAEC as in Fig. 2 and 1B, respectively, except that for HPAEC a linear concentration gradient of 0–1 *M* NaCl in 45 ml of Milli-Q-purified water was used. Injection volume, 25 nl (CE) or 25 μl (HPAEC) from a sample solution containing *ca.* 0.1 mg/ml of each disaccharide (for the structures of disaccharides **1–9**, see Fig. 2).

(ΔUA-2S→GlcNAc) and **7** (ΔUA→GlcNS), only partly resolved by CE, are baseline separated. ΔUA→GlcNAc-6S (**8**) yields two peaks. Probably the major peak represents the disaccharide, while the minor peak stems from a negatively charged non-carbohydrate (because it is not visible in CE) contaminent. The identity of the peaks as disaccharides **1–9** was established by injection of the individ-

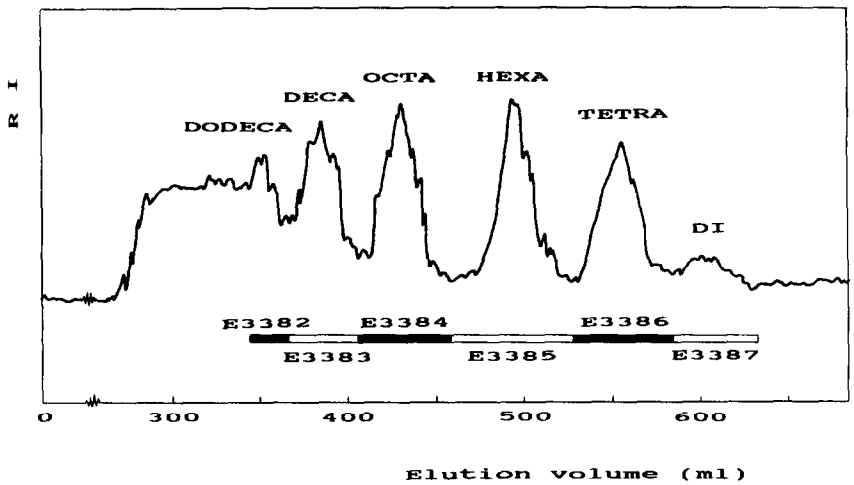

Fig. 4. Fractionation of heparinase-treated heparin by gel permeation chromatography. Heparin was partially degraded by heparinase and batch-adsorbed on an anion-exchanger. The material that desorbed from the anion-exchange column between 5 and 10% (w/v) NaCl was fractionated by a gel permeation chromatographic system, consisting of two columns in tandem, namely a TSK Fractogel HW 40 S column (70 × 2.6 cm I.D.) and a Bio-Gel P-2 (400 mesh) column (100 × 2.5 cm I.D.). The elution pattern was recorded by refractive index (RI) detection. Six fractions of increasing molecular mass were collected as indicated.

ual compounds (results not shown). The order of elution of the differently charged molecules is reversed as compared with CE, as was already shown for the colominic acid oligomers. However, the reversal of the migration/elution order is not maintained within a series of identically (negatively) charged compounds. This is demonstrated, for instance, for the disaccharides containing three negative charges, having an elution order **2→3→4→5** in CE *vs.* **3→2→4→5** in HPAEC. This indicates that while charge exerts an opposite effect on the migration and elution time in CE and HPAEC, respectively, the influence of charge distribution is unpredictable in this respect.

On the basis of the CE conditions established for the reference compounds, more complicated mixtures of heparin fragments were analysed by CE. Also in this instance the CE performance was compared with the performance of HPAEC. Heparin was treated with heparinase and batch-absorbed on an anion exchanger. The pool of heparin fragments that desorbed from the anion-exchange column between 5 and 10% NaCl was fractionated by gel permeation chromatography. The fractions were collected as indicated in Fig. 4 and the relative molecular mass of each pooled fraction, which still contains many carbohydrate structures, was calculated on basis of elution position to be 4200 (E3382), 3500 (E3383), 3100 (E3384), 2500 (E3385), 1600 (E3386) and 600 (E3387). According to their apparent molecular masses, the fractions should represent mixtures of dodeca-, deca-, octa-, hexa-, tetra- and dimers, respectively; however, no attempts were made to resolve the structures. The deca- to tetramer mixtures were each subfractionated by either CE or HPAEC.

In Fig. 5A, the patterns obtained by CE are collected. The separation was carried out at pH 2 which nearly eliminates the electroosmotic flow and results in relatively short retention times. It should be noted that in this instance an optimum separation is achieved at pH 2, which is lower than that for the separation of heparin disaccharides (having a smaller molecular mass and fewer sulphate groups) and colominic acid oligomers (having carboxyl instead of sulphate groups as charged constituents). It is clear that with increasing molecular mass the structural heterogeneity of the heparin fragments increases and the resolution obtained decreases. Interestingly, the migration times of all heparin preparations are virtually in the same range (16–24 min). With increasing molecular mass the negative charge increases accordingly, which means that the separation is based not only on charge and charge distri-

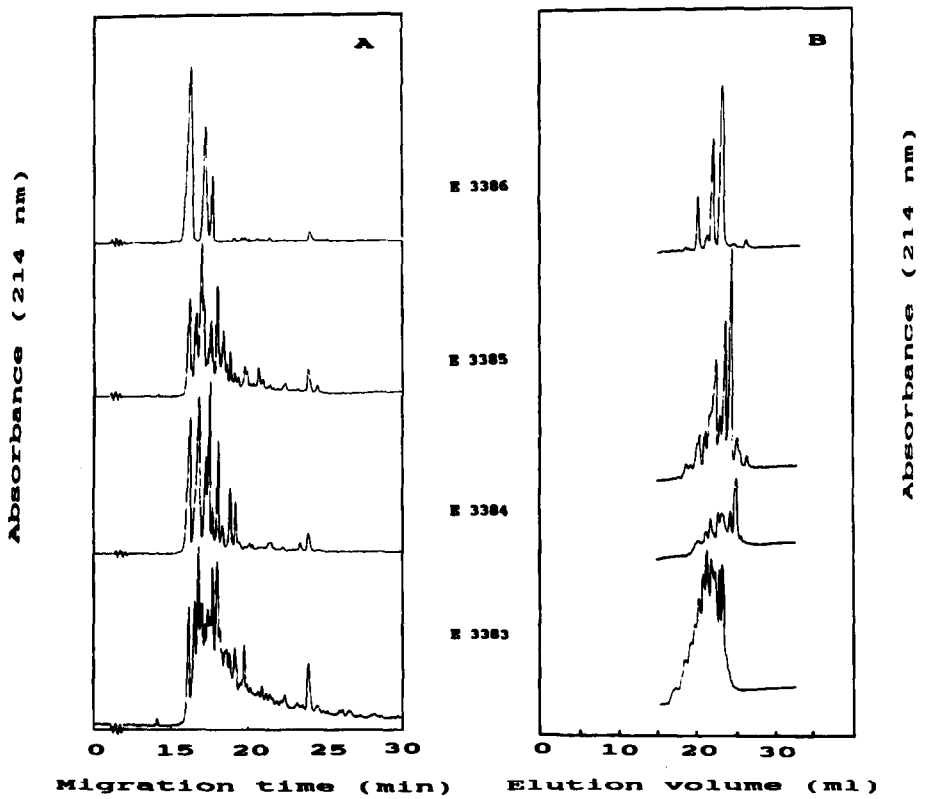

Fig. 5. (A) CE and (B) HPAEC of four heparin oligosaccharide pools. The oligosaccharide pools were obtained by anion-exchange chromatography and gel permeation chromatography of heparinase-treated heparin (Fig. 4). Each oligosaccharide pool was fractionated by CE and HPAEC using the conditions described in Fig. 1A and B, respectively, except that the total concentration of the oligosaccharides in the sample solutions was 4 mg/ml and the phosphate buffer for CE was of pH 2.

bution, as already discussed, but also on molecular mass, which apparently exerts an opposite effect on the migration time. This result is in agreement with the Debye–Hückel–Henry theory on electrophoretic mobility. According to this theory, the electrophoretic mobility is, to a good approximation, proportional to the charge-to-mass (radius) ratio of the analyte [40]. The opposing effect of the molecular mass might also explain why colominic acid oligomers, differing in negative charge and molecular mass from each other, migrate relatively close together (Fig. 1A) while heparin disaccharides, differing only in negative charge, migrate far apart (Fig. 2).

Fig. 5B shows the corresponding patterns obtained with HPAEC on Mono Q. Also with HPAEC the elution times of all heparin fragments are in the same range. When the capillary electropherograms are compared with the HPAEC traces it is evident that the general pattern remains although, as expected, the order of elution is reversed, as is especially clear for fraction E3386. The resolution obtained for sample E3386 with CE and HPAEC is comparable but for the more complex samples E3385, E3384 and E3383 CE appears to be superior. It should be noted that for analysis by CE only small amounts of sample are needed. In this instance 25 nl of sample solution, corresponding to 0.1 μg of carbohydrate material, was injected (*vs.* 0.1 mg for HPAEC). Concentration of the sample solution, allowing reduction of the sample volume, should yield an even better resolution.

Based on these results, the applicability of CE for assessment of the quality of synthetic heparin pentasaccharide preparations was investigated. The

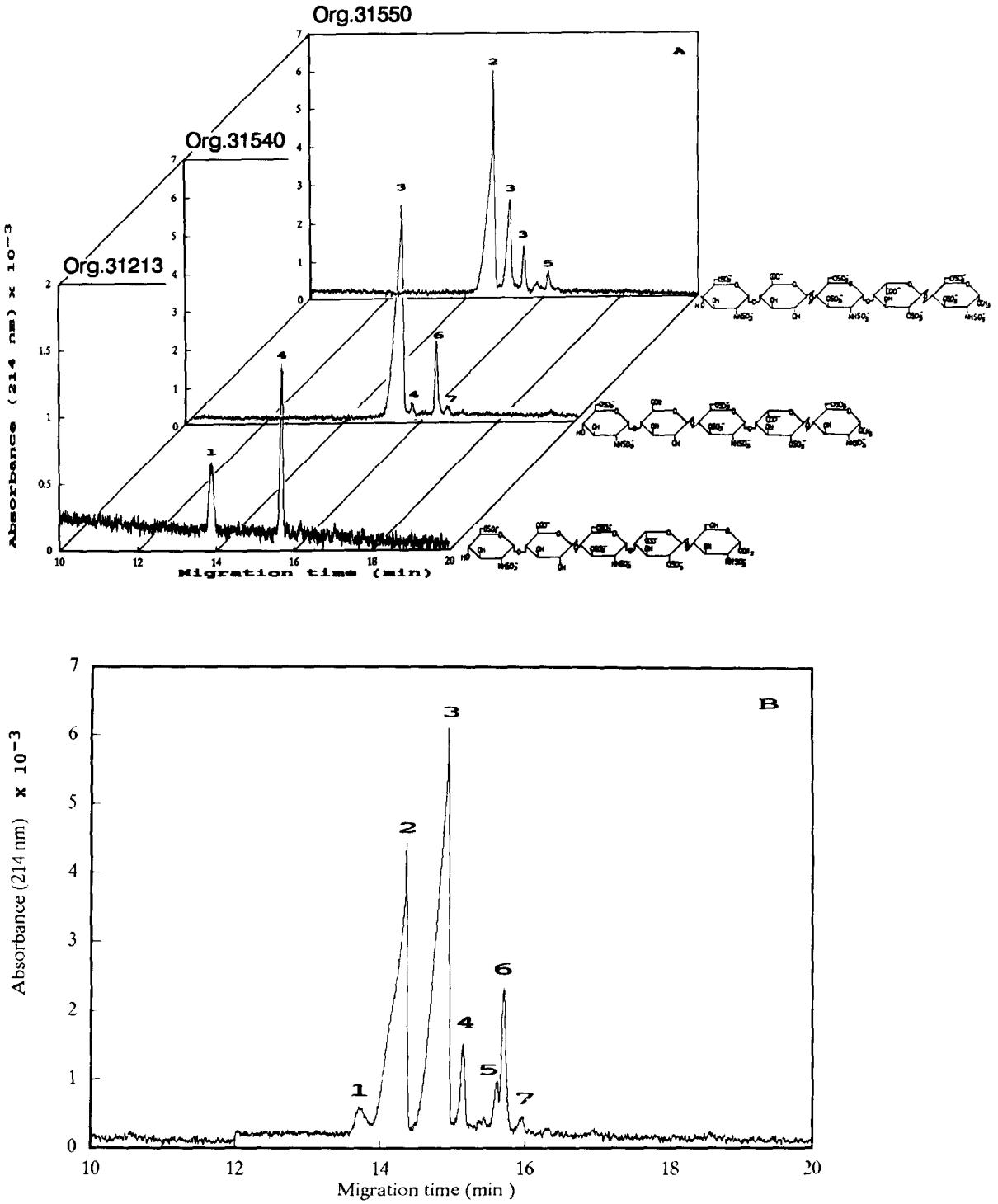

Fig. 6. CE of Org 31213, Org 31540 and Org 31550, injected (A) separately or (B) as a mixture. Conditions as in Fig. 1A, except that the pH of the electrophoresis buffer was 3 and the concentration of the Org compounds was 0.2–1 mg/ml.

pentasaccharides are identical with, or are derivatives of, the unique sequence responsible for the anticoagulant activity of heparin and were synthesized by Organon in collaboration with Sanofi via a multi-step procedure. In Fig. 6A the capillary electropherograms obtained for three synthetic pentasaccharides, denoted Org 31213, Org 31540 and Org 31550, are compiled. The three compounds have an identical pentasaccharide backbone (structures in Fig. 6A) and differ from each other only with re-

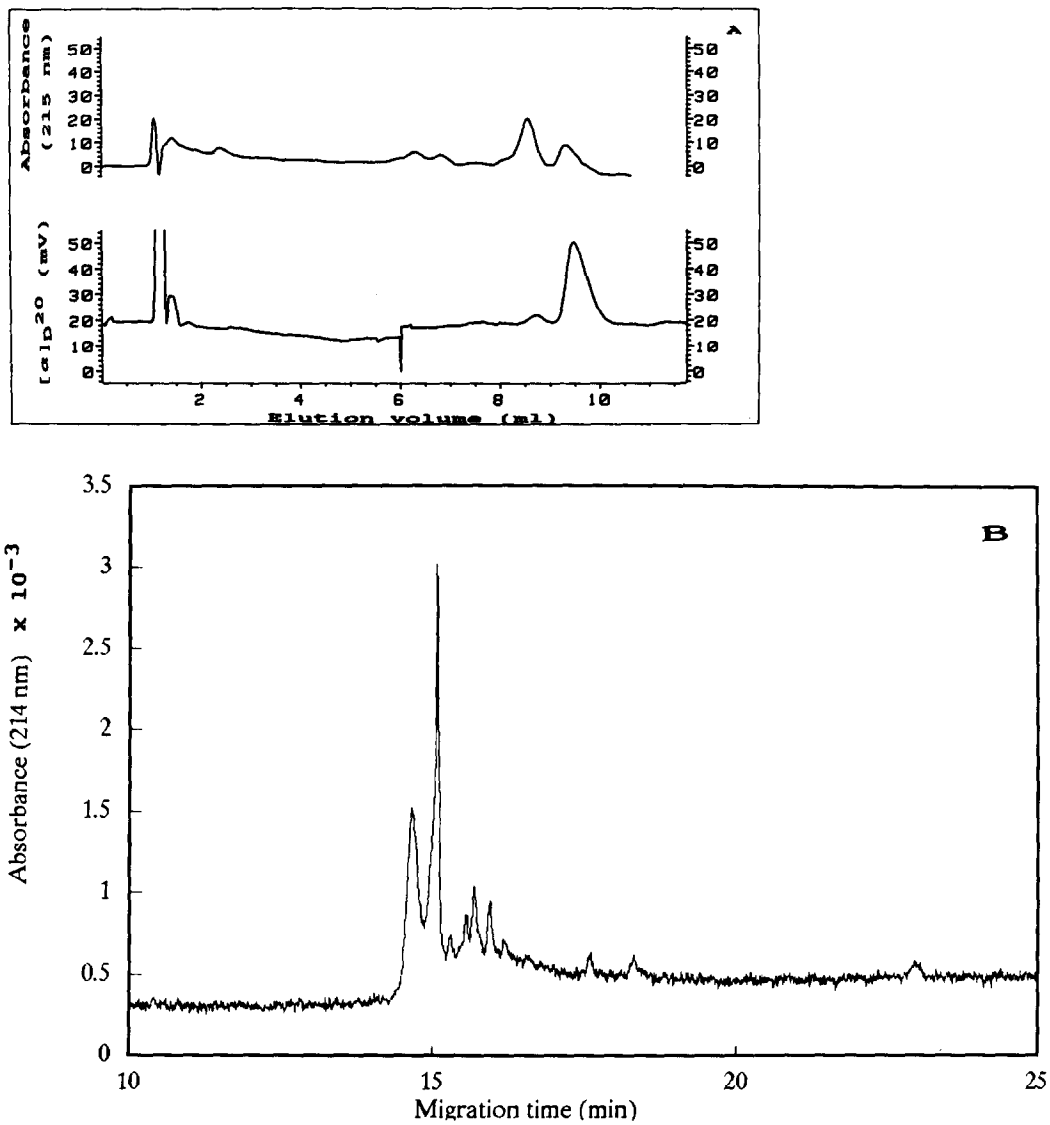

Fig. 7. (A) HPAEC and (B) CE of a batch of raw material of Org 31550 (1 mg/ml). HPAEC was carried out on a Mono Q HR 5/5 column at a flow-rate of 1 ml/min. The column was eluted with a mixture of eluents A and B, starting with 45% (v/v) A–55% (v/v) B, followed by a linear increase of B to 100% in 11 min, where eluent A is Milli-Q-purified water containing 0.1% (v/v) of dimethyl sulphoxide and eluent B is 1.6 *M* NaCl. Injection volume, 0.2 ml. Detection was based on UV absorbance at 215 nm (upper part) or on measurement of optical activity by a polarized laser [41] (Chiramonitor) (lower part). In both instances the detector signal is expressed in arbitrary units. CE conditions as in Fig. 1, except that the pH of the electrophoresis buffer was 3 and the concentration of Org 31550 in the sample solution was *ca.* 1 mg/ml.

spect to the number of sulphate substituents, which can be seven (Org 31213), eight (Org 31540) or nine (Org 31550). The purity of the preparations has been determined previously by 360- and 500-MHz ^{1}H NMR spectroscopy and is at least 99% (mol/mol) [30–32]. Using the CE conditions established for the heparin disaccharides, the Org 31213 sample gives rise to two peaks at 13.9 (numbered 1 in Fig. 6A and B) and 15.7 min (4), respectively. Based on its migration time the main peak at 15.7 min is assigned to Org 31213. Similarly, CE of the Org 31540 preparation yields two peaks at 15.3 (3) and 16.2 min (6), respectively. According to its migration time, the main peak at 15.3 min is assigned to Org 31540. In addition, two minor peaks at 15.7 (4) and 16.5 min (7) are visible. Although the minor peak at 15.7 min has the same migation time as Org 31213, it is unlikely (on basis of the synthesis route) that the Org 31540 preparation could contain Org 31213. The Org 31550 sample yields four peaks, namely a main peak at 14.7 min (2), which is assigned to Org 31550, and three smaller peaks at 15.2 (3), 15.5 (3) and 16.1 min (5). Apparently, the various pentasaccharide structures can be separated efficiently from each other by CE. This is confirmed by co-injection of the three pentasaccharide samples (Fig. 6B). In this instance, however, the compounds migrating at 15.2, 15.3 and 15.5 min, which are separately visible in Fig. 6A, give rise to a multicomponent peak (3). The contaminants in the pentasaccharide preparations most likely represent synthetic precursors, although at present their identity is not known. It should be realized that, in general, detection based on UV absorption is much more sensitive for the synthetic precursors, containing strong UV absorbing substituents, than for the end products, which leads to a major overestimation of the amount of contaminants. This is confirmed by the NMR data on the purity of these preparations.

The value of CE as a qualitative assay for the purity of pentasaccharide preparations is also demonstrated by the analysis of sample HH2174 by CE and HPAEC. HH2174 represents a batch of raw material of Org 31550 that was deliberately kept from further purification. NMR spectroscopic analysis had previously shown that HH2174 consists of *ca.* 85% (mol/mol) or Org 3155. HPAEC of HH2174 and detection at 215 nm give rise to two broad peaks eluting at 8.5 and 9.3 ml (Fig. 7A). Additionally, two minor, partly overlapping peaks are discernible at 6.3 and 6.8 ml. From the injection of pure Org 31550 it is known that the peak at 9.3 ml represents Org 31550. Again, it is clear that detection of contaminants is far more sensitive than detection of the main product. A better estimate of the molar ratio of the compounds present in HH2174 is obtained when the detection is carried out with a Chiramonitor (Applied Chromatography Systems, Macclesfield, UK), where detection is based on optical activity [41]. In this instance the two minor peaks are no longer visible and the areas of the two main peaks are in accordance with the expected values. When HH2174 is subjected to CE at least nine peaks are visible in the electropherogram (Fig. 7B), demonstrating the high resolution achieved with CE. Based on the migration time (compare Fig. 6A) and relative peak area (compare Fig. 7A), the peak at 14.7 min can be attributed to Org 31550. Most likely the peak at 15.1 min represents the major contaminant that in HPAEC elutes at 8.5 min (Fig. 7A). At least six additional peaks belonging to minor contaminants are discernible. It should be noted that the order of migration of the various peaks is, as expected, reversed compared with HPAEC.

Bearing in mind, the potential suitability of CE as a quality control for heparin fragments, it was important to obtain a better insight into the ruggedness of the method. In this respect the effects of the presence of salts in the sample solution or a change of pH of the electrophoresis buffer are relevant. As a first approach, samples of E3384 were dissolved in Milli-Q-purified water, 0.5 *M* NaCl and 1 *M* NaCl, and subjected to CE (Fig. 8A). Obviously, the NaCl concentrations tested have virtually no effect on the CE performance. The slight increase in migration times that is noted is probably caused by the increased conductivity and concomitantly decreased electrical field in the sample zone. Because injection volumes are in general very small (typically 25 nl), the disturbance of the electical field is marginal. However, the pH of the electrophoresis buffer has a major influence on the CE performance. This is exemplified in Fig. 8B where CE of E3384 at pH 3 and 2 are compared. Evidently, lowering the pH of the operating buffer, leading to a decrease in the electroosmotic flow and net charge of the heparin fragments, gives rise to an enhancement of the resolu-

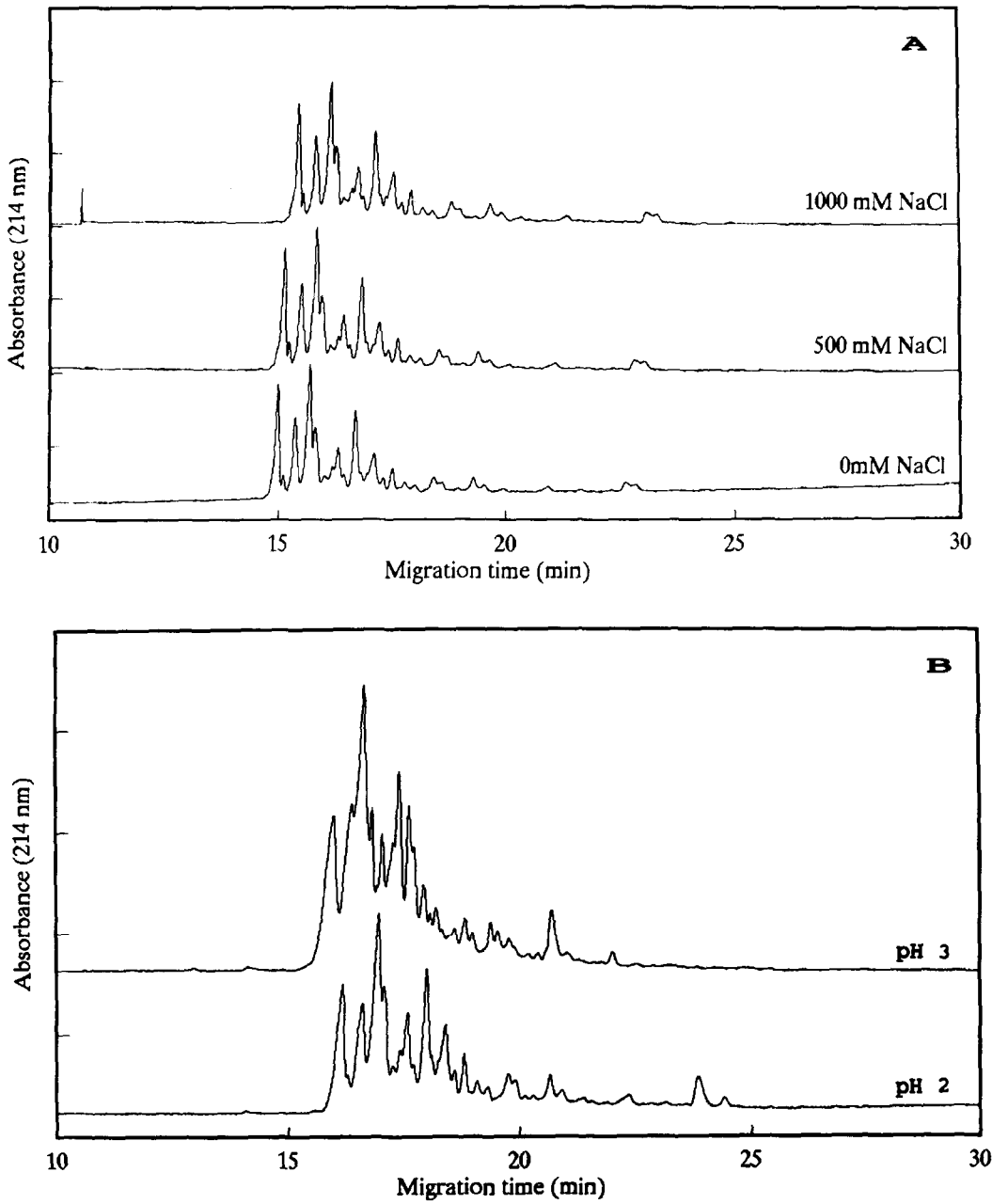

Fig. 8. Effect of (A) NaCl concentration in the sample solution and (B) pH of the electrophoresis buffer on CE of heparin oligosaccharide pool E3384. The oligosaccharide pool, denoted E3384, was obtained as described in Fig. 4. Desalted and lyophilized E3384 was dissolved in Milli-Q-purified water to a concentration of 5 mg/ml. Subsequently the effect of the presence of NaCl (0, 0.5 and 1 *M*) in the sample solution, using a 200 m*M* phosphate electrophoresis buffer (pH 2), or the influence of the pH of the electrophoresis buffer (pH 3 and 2) was examined. Injection volume, 25 nl. The absorbance at 214 nm is expressed in arbitrary units. For further details, see Fig. 1.

tion. This was also the case for the other heparinase-derived heparin fragments.

In summary, we conclude that CE may open the way to a new, sensitive and easy to use analytical procedure for the assessment of the purity of low-molecular-mass heparin preparations.

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