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Indirect UV detection as a non-selective detection method in the qualitative and quantitative analysis of heparin fragments by high-performance capillary electrophoresis

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Abstract

The application of capillary electrophoresis (CE) in combination with indirect UV detection for the qualitative and quantitative analysis of synthetic low-molecular-mass heparin fragments, at low pH, is described. It is demonstrated that, in contrast to direct UV detection, with indirect UV detection the signal obtained for various synthetic heparin pentasaccharides is nearly independent of their molecular structure. Moreover, the sensitivity of indirect UV detection is at least one order of magnitude higher than that of direct UV detection. CE–indirect UV detection for the qualitative and quantitative analysis of low-molecular-mass glycosaminoglycans was achieved by using 5 m*M* 5-sulphosalisylic acid, pH 3 or 5 m*M* 1,2,4-tricarboxybenzoic acid, pH 3.5 as electrophoresis buffer and chromophore. The technique is exemplified by the analysis of three pharmaceutical preparations of synthetic heparin pentasaccharides. The method employing indirect UV detection was validated with respect to repeatability, limit of detection, limit of quantitation, linearity, accuracy and ruggedness. In the indirect detection mode, the limit of detection for synthetic pentasaccharides is below 5 fmol, whereas the limit of quantitation is about 25 fmol. The method shows excellent repeatability and is linear in the femtomole–picomole range. Finally, it is demonstrated that the method is suitable for the analysis of various types of glycosaminoglycans.

1. Introduction

Recently, high-performance capillary electrophoresis (CE) was reported as a sensitive, high-resolution method for the determination of the disaccharide composition of several proteoglycans [1,2]. In extension to these studies we described the separation of complex mixtures of natural and synthetic heparin fragments by CE [3]. It was demonstrated that the resolution obtained by CE for these mixtures was in general superior to that obtained by high-performance anion-exchange chromatography, while the amount of material needed for the analysis was about three orders of magnitude less. In the latter study the heparin fragments were detected on basis of their UV absorbance. However, the various heparin fragments may have different molar extinction coefficients, impeding a quantitative analysis by CE using UV detection. Especially in the case of synthetic pentasaccharide preparations the extinction coefficients

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of the individual sample components, viz. main product and side products, may strongly deviate.

For a reliable quantitative analysis the heparin fragments should produce an equal detection response. In principle, there are several options for non-selective detection of heparin fragments, e.g. detection after chromophoric, fluorescence or radioactive labelling, refractive index detection, or detection by mass spectrometry (MS). Furthermore, conductivity detection of uniformly charged analytes and amperometric detection may serve as (pseudo) non-selective detection modes for CE. However, in case of CE of synthetic pentasaccharides that are intended for pharmaceutical use, attachment of a chromophore or fluorescence label is not possible or suitable. CE in combination with conductivity or (indirect) amperometric detection has been reported for the quantitative analysis of carboxylic acids [4], amino acids [5] and carbohydrates [6,7]. However, at present these detectors are not yet commercially available for CE. On-column laser-based refractive index detection for CE of carbohydrates has been described by Bruno et al. [8], but also this application is not yet commercially available. Fast atom bombardment (FAB) [9,10] and electrospray ionization [11,12] MS detection and multi-channel Raman spectroscopic detection [13] are potentially powerful techniques for the on-line detection and characterization of analytes by CE, although the feasibility of these options for the analysis of glycosaminoglycans (GAGs) by CE still needs to be demonstrated. Garner and Yeung [14] reported indirect fluorescence detection as a universal detection method for CE of charged carbohydrates. Unfortunately, the high cost of laser equipment and the fact that it is not available in most commercial CE systems compromise the applicability of this method. With respect to cost and feasibility, indirect UV detection [15] seems a more straightforward approach. Indirect UV detection has already been applied successfully to the analysis by CE of inorganic [16] and organic [17] anions as well as of various monosaccharides [18]. In the latter study the monosaccharides were ionized and separated at high pH using 6 mM sorbic acid, pH 12.1 as electrophoresis buffer and chromophore. Ionization of neutral sugars by high pH is a prerequisite as indirect UV detection is dependent on charge displacement [18]. For the analysis of synthetic heparin fragments by CE–indirect UV detection, high pH-induced ionization is not necessary since these compounds contain multiple carboxylic acid and sulphate groups. Wang and Hartwick [19] recently documented the use of binary buffers in CE–indirect UV detection, allowing for a wide range of pH of the electrophoresis buffers and mobility of the analyte ions. We have shown previously [3] that a high resolution of complex mixtures of heparin fragments can be obtained applying a single buffer system at relatively low pH and using direct UV detection. Here we report the applicability of indirect UV detection for the non-selective detection of various heparin fragments after their separation by CE at low pH using 5-sulphosalisylic acid or 1,2,4-tricarboxybenzoic acid as electrophoresis buffer and chromophore. A key question then is whether the background electrolyte that is required in the indirect detection mode does not interfere with an efficient resolution. Furthermore, it is essential that the background electrolyte has a high molar extinction coefficient at the selected detection wavelength to warrant sufficient detection sensitivity, and an effective mobility similar to that of the analyte ions in order to prevent fronting or tailing of analyte peaks. In this study the application of CE–indirect UV detection as an analytical method for the determination of the purity of GAG preparations is validated and it is demonstrated that the technique enables the qualitative and quantitative analysis of low-molecular-mass heparin fragments.

2. Experimental

2.1. Materials

Synthetic pentasaccharides were prepared at Organon (Oss, Netherlands) in cooperation with Sanofi (Toulouse, France) and the structures

were verified by ^{1}H and ^{13}C NMR spectroscopy [20–23] and FAB-MS [23]. Heparin disaccharide reference compounds were obtained from Grampian enzymes (Aberdeen, UK). Dermatan sulphate di-, tetra- and hexasaccharides were synthesized by Organon.

2.2. Capillary electrophoresis

The synthetic pentasaccharides were each dissolved in Milli-Q water (Millipore, Milford, MA, USA) to a concentration of 1 mg/ml and separated by high-performance CE essentially as described [3], except that in this study the internal diameter of the capillary was 50 μm (unless stated otherwise). Furthermore, to allow indirect UV detection, the electrophoresis buffer was changed as outlined below. The eight commercially obtained heparin disaccharides were dissolved in Milli-Q water and mixed to give a final concentration of approximately 0.1 mg/ml of each compound. On-capillary detection was performed by UV absorbance at 210 or 230 nm, using 200 m*M* NaH_2PO_4 (J.T. Baker, Deventer, Netherlands), adjusted to pH 2.5 or 3.0 with concentrated H_3PO_4, as CE electrophoresis buffer. The potential across the capillary was 7.5 kV (131.5 V/cm) and the thermostatted capillary was kept at 40°C. Indirect UV detection by quenching of the UV signal at 214 nm was performed by using 5 m*M* 5-sulphosalicylic acid (pK_a carboxylic acid group 2.27), pH 3.0 or 5 m*M* 1,2,4-tricarboxybenzoic acid (pK_a values 2.28, 3.58 and 4.79), pH 3.5 as electrophoresis buffer at a potential of 5 kV (87.7 V/cm) and room temperature.

2.3. Validation of CE–indirect UV detection

The application of CE in combination with indirect UV detection as an analytical method for the determination of the purity of pentasaccharide preparations was validated by use of a "golden standard" preparation of 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,3,6-trisulphate (Org 31550). The purity of this preparation was determined to be at least 99.5% (mol/mol) by 360 MHz $^{13}C/^{1}H$ NMR spectroscopy as described [22]. The residual water content was 8.7% (w/w) ± 0.55 (standard error of the mean, S.E.M., $n = 3$) as determined by Karl Fischer titration [24] and chloride and (free) sulphate content were 0.5% (w/w) and 0% (w/w), respectively, as determined by isotachophoresis [25]. All CE validation experiments were carried out using 5 m*M* sulphosalicylic acid, pH 3.0 at a thermostatted temperature of 25°C, applying a potential of 5 kV across the capillary. The injection time was in each case 2 s resulting in injection of 1.8 nl of sample solution. These conditions are further referred to as standard conditions.

3. Results and discussion

All CE experiments were carried out using low pH ($\leqslant 3.5$) electrophoresis buffers to prevent dissociation of the silanol groups of the capillary inner wall, resulting in arheic or nearly-arheic separation conditions without the need for anticonvective gel filling or coating of the capillary inner wall [3,26].

Fig. 1A depicts the CE electropherogram obtained for a nearly equimolar mixture of eight heparin disaccharides (denoted 1–8, structures in Table 1) employing 200 m*M* NaH_2PO_4, pH 2.5 as electrophoresis buffer and using direct UV absorbance at 230 nm as detection method. Injection of ca. 5 pmol of each disaccharide yields a satisfactory signal-to-noise ratio, demonstrating the high mass sensitivity of CE. The identity of the peaks as compounds 1–8 (Table 1) was established in an earlier study [3]. Fig. 1B shows the CE pattern obtained for the same mixture applying 5 m*M* 1,2,4-tricarboxybenzoic acid, pH 3.5 as electrophoresis buffer and observing the quenching of the UV signal at 214 nm (further referred to as indirect UV detection). In this case only ca. 0.5 pmol of each disaccharide are injected. The disaccharides 2–4 are not completely separated under the conditions of indirect UV detection, but the three disaccharides denoted 6–8, all having two negative

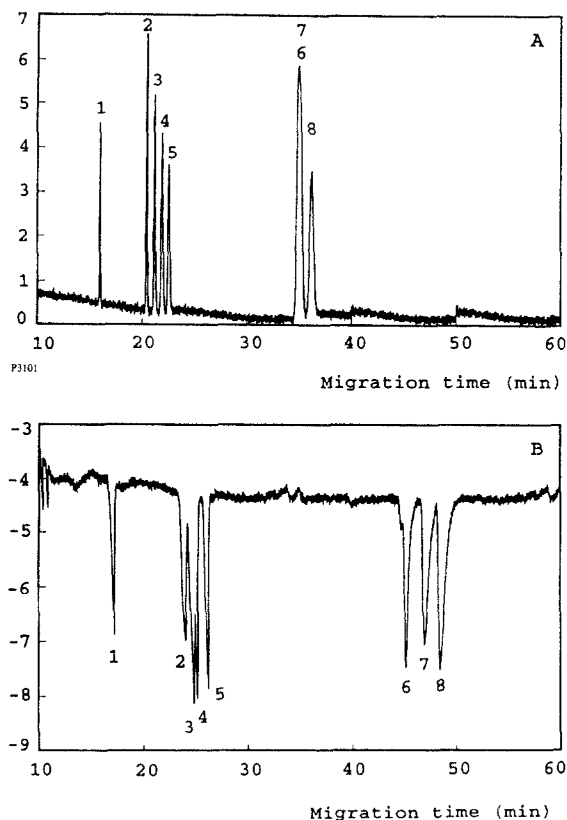

Fig. 1. CE of eight heparin disaccharides using direct (A) or indirect (B) UV detection. In case of direct UV detection the electrophoresis buffer was 200 mM Na_2HPO_4, pH 2.5 and the injection volume was 9 nl from a solution containing approximately 0.16 mg/ml of each disaccharide. The potential across the capillary was 7.5 kV (131.5 V/cm) and the thermostatted capillary was kept at 40°C. In case of indirect UV detection the electrophoresis buffer was 5 mM 1,2,4-tricarboxybenzoic acid, pH 3.5 and the injection volume was 1.8 nl from the same solution as in A, except that the concentration was approximately 0.1 mg/ml of each disaccharide. The potential across the capillary was 5 kV (87.7 V/cm) and the thermostatted capillary was kept at 25°C. The structures of disaccharides 1–8 are given in Table 1.

charges, are baseline separated which is not the case under the conditions used for direct detection. Unfortunately, the concentrations of the various disaccharides in the mixture were not exactly known, hampering a quantitative interpretation of the results.

The practical value of CE in combination with indirect UV detection for the determination of the purity of "real world" pentasaccharide preparations was demonstrated by the analysis of several synthetic pentasaccharide preparations.

In Fig. 2 the CE electropherograms obtained for Org 31540, batch E using direct (A) and indirect (B) UV detection are depicted. The synthetic pentasaccharide Org 31540 (structure in Table 2) corresponds to the unique natural pentasaccharide sequence that confers the anti-coagulant activity to heparin. Prior to CE, the organic purity of Org 31540-E was established by

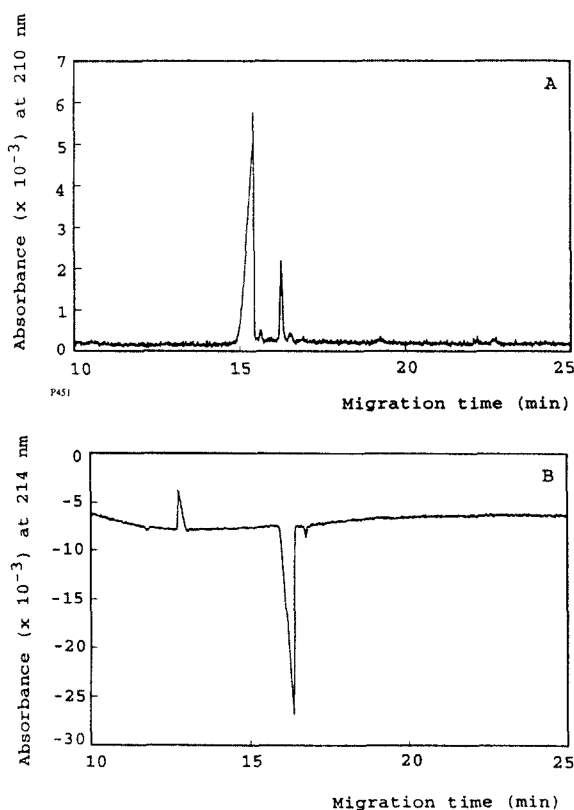

Fig. 2. Analysis of pentasaccharide preparation Org 31540-E by CE using direct (A) and indirect (B) UV detection. Direct UV detection was carried out using 200 mM Na_2HPO_4, pH 3.0 as electrophoresis buffer. The potential across the capillary (57 cm × 75 μm) was 7.5 kV (131.5 V/cm) and the thermostatted capillary was kept at 40°C. Indirect UV detection was performed applying 5 mM 5-sulphosalicylic acid, pH 3.0 as electrophoresis buffer. The potential across the capillary (57 cm × 50 μm) was 5 kV (87.7 V/cm) and the thermostatted capillary was kept at 25°C. Concentration of Org 31540 in the sample solution is 1 mg/ml, injection volume 50 nl (A) or 1.8 nl (B). The structure of Org 31540 is outlined in Table 2.

Table 1
Structures of eight heparin disaccharide reference compounds and their migration times in CE using direct or indirect UV detection

Disaccharide	Structure	Charge	Migration time (min)	
			Direct UV detection	Indirect UV detection
(1) δUA2S→GlcNS6S		4^-	16.1	17.3
(2) δUA2S→GlcNS		3^-	20.4	24.1
(3) δUA1→GlcNS6S		3^-	21.2	24.9
(4) δUA2S→GlcNAc6S		3^-	21.8	25.2
(5) δUA2S→GlcNCOEt6S		3^-	22.5	26.3
(6) δUA2S→GlcNAc		2^-	34.9	45.2
(7) δUA→GlcNS		2^-	34.9	46.9
(8) δUA→GlcNAc6S		2^-	35.9	48.4

Electropherograms and electrophoresis conditions are reported in Fig. 1.

Table 2
Structures of Org 31540, 31550, 33232, 34275, 34276 and 34277

GAG	Structure
Org 31540	10 Na^+
Org 31550	11 Na^+
Org 33232	11 Na^+
Org 34277	4 Na^+
Org 34276	7 Na^+
Org 34275	10 Na^+

Org 33271 and Org 33263 are close derivatives of Org 33232 having twelve and eleven negative charges, respectively.

NMR spectroscopy to be 98% (mol/mol). CE in combination with direct UV detection gives rise to a major peak at 15.4 min (79% of total peak area) representing Org 31540 and three minor peaks at 15.6, 16.2 and 16.5 min stemming from contaminants. In the indirect detection mode the main peak stemming from Org 31540 migrates at 16.6 min and forms 98% of the total peak area which exactly agrees with the NMR data. The peak belonging to the main contaminant is observed at 16.9 min and accounts for 1.8% of the total peak area. This peak probably corresponds to the peak at 15.6 min in the direct detection mode. Remarkably, the contaminant that yields a major signal at 16.2 min in the direct detection mode is not observed in the

indirect detection mode, implying that it represents less than 0.5% (w/w) relative to Org 31540-E (see below). It should be noted that, apart from yielding representative peak areas enabling quantitative analysis, the sensitivity of indirect UV detection is by far superior to that of direct detection. In the latter experiment 28 pmol (50 ng) and 1 pmol (1.8 ng) of Org 31540 were injected in case of direct and indirect UV detection, respectively, yielding still a better signal-to-noise ratio for the indirect detection mode.

A second example of the practical value of CE in combination with indirect UV detection for the determination of the purity of "real world" pentasaccharide preparations is furnished by the analysis of HH2174. Sample HH2174 represents a batch of raw material of Org 31550 (structure in Table 2) that was deliberately kept from further purification. Org 31550 is a derivative of the unique natural pentasaccharide sequence responsible for the anticoagulant activity of heparin. Relative to the natural sequence, Org 31550 contains one extra 3-O-sulphate group in the first glucosamine-N,6-disulphate residue (denoted number 6 in [22]) and it was synthesized by Organon in collaboration with Sanofi via a multistep procedure. As reported earlier [3], NMR spectroscopic analysis proves that HH2174 consists for approximately 85% (mol/mol) of Org 31550. When HH2174 is subjected to CE using direct UV detection at least nine peaks are discernable in the electropherogram (Fig. 3A). From injection of pure Org 31550 it is known that the peak at 14.7 min can be attributed to Org 31550. The additional peaks, accounting for 75% of the total peak area, belong to minor contaminants. This clearly demonstrates the limitations of direct UV detection for the quantitative analysis of these type of preparations by CE. The contaminants most probably represent synthetic precursors of Org 31550 that contain strong UV absorbing groups, which is the main reason for the overestimation of the amounts present. In contrast, when HH2174 is analyzed by CE applying the indirect detection mode a pattern is obtained displaying proportionality between peak areas and the amount of the components present (Fig. 3B). The main peak at

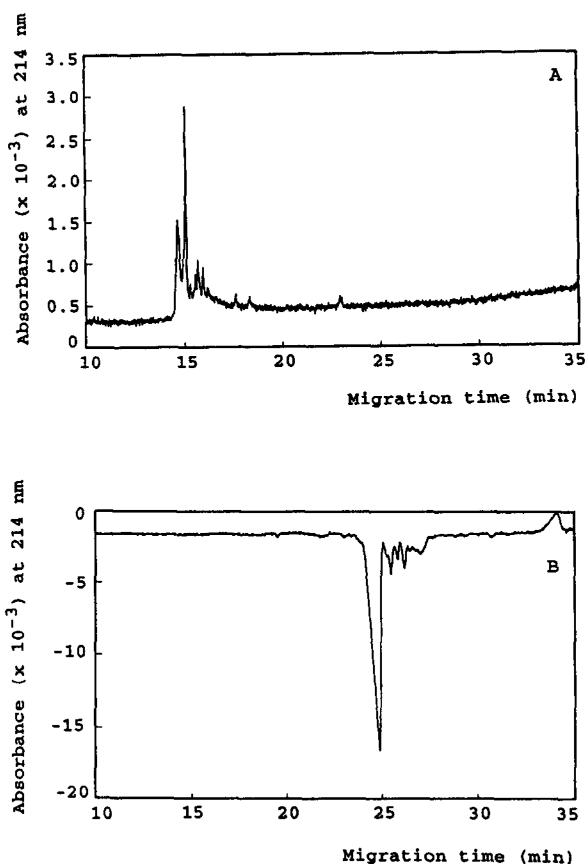

Fig. 3. Analysis of pentasaccharide preparation HH2174 by CE using direct (A) and indirect (B) UV detection. The electrophoresis conditions for direct and indirect UV detection are reported in Fig. 2, except that for indirect detection the pH of the electrophoresis buffer is 2.5. HH2174 represents a sample of Org 31550 (structure indicated in Table 2) that was deliberately kept from purification. The concentration of the pentasaccharide in the sample solution is 1 mg/ml, injection volume 50 nl (A) or 1.8 nl (B).

24.9 min corresponds with Org 31550 and constitutes 86% of the total peak area, which corresponds with the NMR data. Four minor peaks and three barely discernable peaks, all belonging to contaminants, are visible and account for 14% of the total peak area. Like in the previous example, the contaminant that gives rise to a major peak in the direct detection mode yields only a minor signal in the indirect detection mode. Note that in the latter experiment the pH of the electrophoresis buffer was 2.5 which

explains why the migration times obtained for the analytes in the indirect mode are higher than those obtained using the direct mode.

A final example of the applicability of CE–indirect UV detection for the determination of the purity of a glycosaminoglycan preparation for pharmaceutical use is presented by the analysis of Org 33263. Org 33263 is a derivative of Org 31550 in which the free hydroxyl functions have been methylated and the N-sulphate groups have been replaced by O-sulphate groups (structure of Org 31550 in Table 2). As is clear from Fig. 4 also these type of compounds are amenable to analysis by CE. Using indirect UV detection this preparation yields a major peak at 23.9 min, accounting for 95% of the total peak area which is corroborated by the NMR data.

The applicability of indirect UV absorption as a *quantitative* detection method for CE was further investigated by using known amounts of a highly purified batch of the synthetic pentasaccharide Org 31550 [>99.5% (mol/mol) pure by NMR spectroscopy, water content 8.7% ± 0.55% (S.E.M., $n = 3$) by Karl Fischer, chloride content 0.5% (w/w) and (free) sulphate content 0% (w/w) by isotachophoresis]. This compound was used in a series of validation experiments addressing the linearity and sensitivity of indirect UV detection and the overall repeatability of the method. Also the influence of the presence of sodium chloride, sodium sulphate and sodium phosphate in the electrophoresis buffer on the performance of the method and the effect of the migration time and the type of analyte on the detector response were examined.

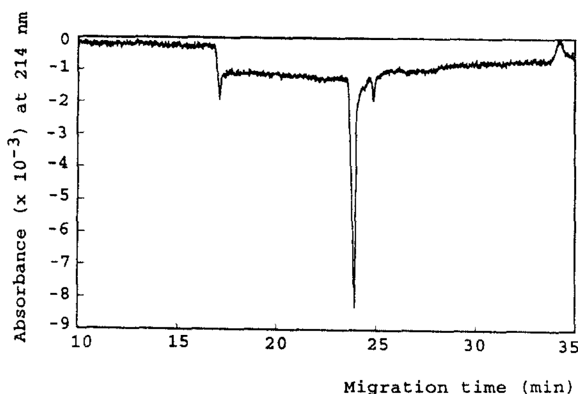

Fig. 4. Analysis of pentasaccharide preparation Org 33263 by CE–indirect UV detection. Electrophoresis conditions as in Fig. 2B, except that the pH of the electrophoresis buffer is 2.5. The concentration of Org 33263 in the sample solution is 1 mg/ml, injection volume 1.8 nl.

As a first step in the validation procedure, the repeatability of the method was investigated by six successive injections of 1 pmol Org 31550, employing the standard conditions as described in the Experimental section. It turns out that the method shows excellent repeatability as the average migration time of Org 31550 is 17.9 min ± 0.03 (S.E.M., $n = 6$), whereas the average integrated peak area is 17.4 ± 0.43 (S.E.M., $n = 6$) The day-to-day reproducibility of the migration time is somewhat less favourable. The average migration time found for Org 31550, analyzed on ten different days in a time interval of four weeks, keeping all analysis conditions as much as possible identical, is 17.0 ± 0.81 (S.E.M., $n = 10$), whereas the average peak area is 15.6 ± 2.44 (S.E.M., $n = 10$).

For determination of the limit of detection (LOD), limit of quantitation (LOQ) and linearity, the stock solution of Org 31550 (1 mg/ml, 546 nmol/ml) was diluted with Milli-Q water to 75, 50, 25, 2.5, 1 and 0.5% (v/v). In addition, more concentrated solutions, containing 1.25, 1.50 and 2.00 mg/ml of Org 31550, were prepared (corresponding to 125, 150 and 200% of the stock solution). From each solution 1.8 nl were injected and analyzed applying the standard conditions. The obtained peak areas (electropherograms not shown) are compiled in Table 3. Injection of 5 fmol Org 31550 (1.8 nl from the 0.5% solution) still gives a signal-to-noise ratio of 3. This means that the LOD for synthetic pentasaccharides is about 5 fmol. In Fig. 5 the peak areas vs. the injected amounts are plotted. Taking into account all data points, except that for the highest injected amount (1960 fmol), a regression line $y = 0.015x + 0.487$ and a correlation coefficient $R = 0.996$ is obtained. When all data points are taken into account, the regression line is $y = 0.018x - 0.581$ with $R = 0.981$. It should be mentioned however, that the data points found for the three lowest concentrations

Table 3
LOD, LOQ and linearity of CE–indirect UV detection

Stock solution (%)	Injected amount		Peak area	
	pg	fmol	area	area%
200	3600	1960	39.43	265
150	2700	1480	21.42	144
125	2250	1230	19.93	134
100	1800	980	14.86	100
75	1350	740	12.00	81
50	900	490	8.63	58
25	450	245	4.90	33
2.5	45	25	0.33	2.2
1.0	18	10	0.26	1.7
0.5	9	5	0.07	0.5

Four stock solutions containing 1.00 (100%), 1.25 (125%), 1.50 (150%) and 2.00 (200%) mg Org 31550 per ml Milli-Q water were prepared. The 100% solution was diluted to the concentrations indicated in the table. From each solution 1.8 nl were analyzed by CE–indirect UV detection applying the standard conditions as indicated in Fig. 2B. The injected amount of Org 31550 is given in pg and fmol. The resulting peak areas are in arbitrary units (area) and are expressed relative to the peak area obtained for injection from the 100% solution (area%).

(injected amounts 5, 10 and 25 fmol pentasaccharide) are close together and might be less reproducible. Taken together, it can be stated that the LOQ is better than 25 fmol pentasaccharide and that the method is linear at least from 25 to 1480 fmol injected pentasaccharide. Consequently, for accurate quantitative analysis, the concentration of the pentasaccharide(s) in the sample solution should be between 0.025 and 1.50 mg/ml. More concentrated solutions may be diluted prior to analysis, whereas sample solutions containing less than 0.25 mg pentasaccharide per ml should be concentrated or, alternatively, the injection volume is to be increased. Furthermore, it can be deduced from the above data that for quantitative analysis of pentasaccharides in a multicomponent mixture, e.g. main component plus contaminants, the ratio between the compounds should preferably be about 1:60 (w/w) or smaller. This means that the LOQ for contaminants is about 1.7% (w/w) relative to the main component. This issue is more accurately addressed in the experiment described below.

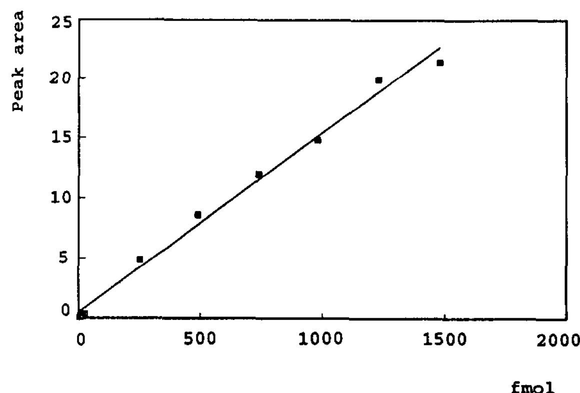

Fig. 5. Peak areas vs. injected amounts, obtained for various amounts of Org 31550 by CE–indirect UV detection. Peak areas are given in arbitrary units. Experimental details in Table 3.

For determination of the accuracy of the method, Org 31540 was spiked with decreasing amounts of Org 31550. Prior to CE, the purity and the residual water content of Org 31540 were established to be >98% (mol/mol) by 1H NMR spectroscopy and 19.2% (w/w) ± 0.28 (S.E.M., $n = 3$) by Karl Fischer titration, respectively. Ten mixtures of Org 31550 and Org 31540 were prepared, the total concentration of all mixtures being 1 mg pentasaccharide per ml (defined as 100%, Table 4). Analysis of a mixture containing 50% Org 31540 and 50% Org 31550 (i.e., both compounds 0.5 mg/ml) yields

Table 4
Accuracy of CE–indirect UV detection

Org 31540			Org 31550		
% Org 31540	Peak area	Area%	% Org 31550	Peak area	Area%
0	0	0	100	16.83	100
50	8.85	51.1	50	8.49	48.9
60	11.45	60.3	40	7.53	39.7
80	15.10	79.9	20	3.81	20.1
90	16.89	88.6	10	2.17	11.4
95	22.14	93.9	5	1.43	6.1
96	18.03	95.9	4	0.77	4.1
97	20.73	96.0	3	0.86	4.0
98	16.80	99.9	2	0.02	0.1
99	16.03	99.8	1	0.03	0.2
99.5	16.03	99.8	0.5	0.03	0.2
100	16.46	100	0	0	0

Ten mixtures of Org 31540 and Org 31550 having an increasing Org 31540/Org 31550 ratio were analyzed by CE–indirect UV detection applying standard conditions. In each mixture the total pentasaccharide concentration (Org 31540 + Org 31550) was 1 mg/ml (defined as 100%). Peak areas were corrected for residual water content (details in text). % Org 31540 = Percentage (w/w) of Org 31540 in the Org 31540/Org 31550 mixture; Peak area = observed peak area (in arbitrary units) in CE–indirect UV analysis; Area% = observed peak area for Org 31540 or Org 31550 in CE–indirect UV analysis of a mixture of both Org compounds. The total peak area obtained for Org 31540 + Org 31550 is taken as 100%.

two baseline-separated peaks at 15.57 and 16.09 min, followed by a minor peak at 16.47 min (Fig. 6). From injection of Org 31550 and Org 31540 separately (not shown), it is known that the signal at 15.57 min stems from Org 31550, whereas the signals at 16.09 and 16.47 min are derived from Org 31540 and an impurity in Org 31540, respectively. The peak areas found for Org 31540 (impurity not included) and Org 31550 are 8.49 and 7.83, respectively (Table 4). However, since the water contents of Org 31550 and Org 31540 are 8.7 and 19.2%, respectively, a correction factor of 1.13 for the area found for Org 31540 must be applied, determining the corrected peak area of Org 31540 as 8.85. Nine additional mixtures were made, gradually increasing the ratio Org 31540/Org 31550. In fact, in this experiment Org 31550 can be regarded as a contaminant in the Org 31540 preparation. As is clear from Fig. 6 and Table 4, the theoretical and observed ratios are in accordance with each other, indicating on the one hand that, using indirect UV detection, a nearly identical detector response is obtained for Org 31540 and Org 31550, and that on the other hand the presence of a small amount of Org 31550 (e.g., 9 pg or 4.9 fmol) can be detected in the presence of a large excess of Org 31540 (e.g., 1791 pg or 1036 fmol, Table 4). There is, however, a discrepancy between the limit of detection and the limit of quantitation of Org 31550 in the presence of excess Org 31540. Fig. 6 demonstrates that the observed signal for Org 31550 continuously decreases, relative to the signal obtained for Org 31540, as the ratio Org 31540/Org 31550 increases. Therefore, the Org 31550/Org 31540 ratio (w/w) can be assessed in a qualitative way at least up to a ratio of 0.5/99.5, i.e. the limit of detection of Org 31550 in the presence of an excess Org 31540 is about 0.5% (w/w). However, from Table 4 it will be clear that the limit of quantitation for Org 31550 in a mixture with Org 31540 is about 2% (w/w). This difference with the limit of detection is mainly due to the limited accuracy of the integration software, as it is evident from Fig. 6 that the detection signal does reflect the actual ratio. Taken together it can be concluded that CE coupled with indirect

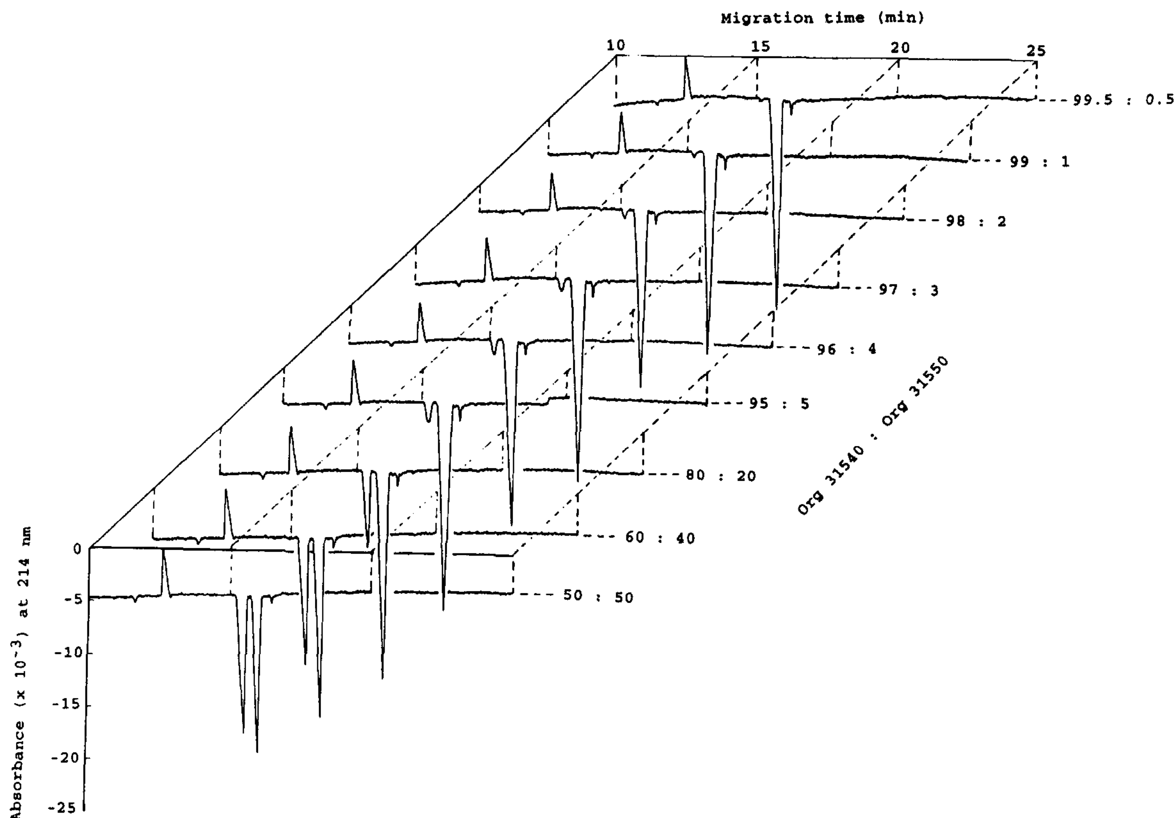

Fig. 6. CE–indirect UV detection of nine mixtures of Org 31540 and Org 31550, containing a decreasing amount of Org 31550. The electropherograms are compiled in a three-dimensional plot. The Org 31540/Org 31550 ratio (w/w) is indicated on the *Z*-axis. Experimental details in Table 4.

UV detection enables the non-selective detection of different types of pentasaccharides and allows the quantitative detection of small amounts (>2%, w/w) of pentasaccharide contaminants.

The former experiment shows that closely related pentasaccharides yield a (nearly) identical detector response in the indirect detection mode. Moreover, determination of the purity of Org 31540-E and HH2174 (see above) demonstrates that also pentasaccharides which carry additional chromophoric substituents are registered in proportion to their relative (gravimetric) abundance. Notwithstanding these observations, it is of importance to ascertain that equal amounts of GAGs widely differing in molecular mass, charge and migration time are detected with equal sensitivity, since these parameters could influence the detector response. The influence of the molecular mass and charge of the GAG on the detector response was investigated by CE–indirect UV analysis of equal amounts of Org 31550 and δUA2S→GlcNS6S (structure in Table 1). The possible influence of the migration time is more appropriately dealt with in a separate experiment (see below). Injection of 1.8 nl from a solution containing 0.5 mg/ml Org 31550 (injected amount 0.9 ng or 0.49 pmol) and 0.5 mg/ml δUA2S→GlcNS6S (injected amount 0.9 ng or 1.35 pmol) yields two peaks (Fig. 7) at 17.2 min, corresponding to Org 31550 and 20.1 min, corresponding to δUA2S→GlcNS6S having nearly identical peak areas, namely 12.48 (48.2%) and 13.43 (51.8%), respectively. This convincingly demonstrates that injection of equal

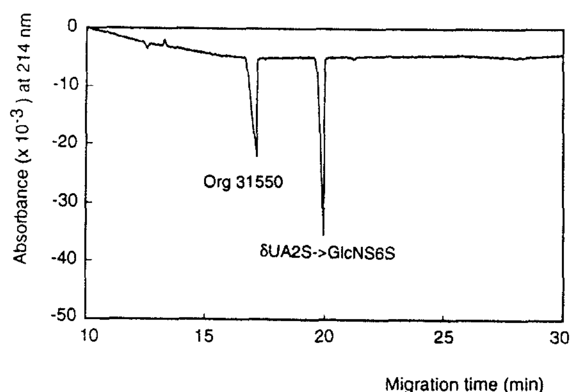

Fig. 7. CE–indirect UV detection of a mixture containing 1 mg/ml of Org 31550 and 1 mg/ml of δUA2S→GlcNS6S. The injection volume is 1.8 nl. Experimental conditions as in Fig. 2B.

mass amounts of different types of GAGs yield an equal detector response, enabling their quantitative analysis by CE–indirect UV detection. It is of importance to note that the detector response correlates with the gravimetric amount of the saccharide and not with the molar amount.

With respect to the ruggedness of the method two parameters were investigated, namely the possible influence of the migration time on the peak area and the influence of the presence of salts in the sample solution on the overall performance of the method.

As mentioned earlier, indirect UV detection offers a possibility to detect analytes indiscriminative of their chromophoric constituents and molecular structure. However, it cannot be excluded that the sensitivity of detection of an analyte is influenced by its migration time. This was studied by CE analysis of Org 31550 applying different voltages across the capillary. In this way the migration time of a certain analyte species can be varied, keeping the experimental conditions constant (except for the applied voltage). The migration times and peak areas obtained for Org 31550 at 2.5, 5.0, 7.5, 10.0 and 12.5 kV are compiled in Table 5. A shift in migration time from 35.8 min (2.5 kV) to 7.3 min (12.5 kV) gives rise to an increment of the peak area of Org 31550 from 17.8 to 18.2. Apparently, the migration time has little effect

Table 5
The influence of migration time on peak area

Voltage	Migration time (min)	Peak area
2.5	35.8 ± 0.10	17.8 ± 0.03
5.0	18.9 ± 0	16.1 ± 0.04
7.5	12.0 ± 0	16.7 ± 0.01
10.0	9.1 ± 0	17.6 ± 0.18
12.5	7.3 ± 0	18.2 ± 0.09

CE–indirect UV detection of Org 31550 using standard conditions, except that the voltage over the capillary was varied. In each case 1 pmol of Org 31550 was injected. Data are the mean value ± S.E.M. of two experiments.

on the detector response. However, when the data obtained at 2.5 kV are neglected the tendency for peak areas to increase as the migration times decrease is more pronounced (Table 5).

Since the synthetic pentasaccharide samples may contain traces of residual salts, it was relevant to test the influence of the presence of salts in the sample solution on the performance of CE–indirect UV detection. The maximal tolerated salt content of pentasaccharide preparations for pharmaceutical use is 10% (w/w). Typically, injections are made from a sample solution containing 1 mg pentasaccharide per ml, and consequently the salt concentration should be below 0.1 mg salt per ml. In Fig. 8 the electropherogram obtained for a mixture containing 1 mg Org 31550 per ml and 0.1 mg NaCl per ml is shown. Clearly, the presence of NaCl is detected by the chloride peak at 12.81 min. The minor peak, migrating at 13.35 min, belongs to sulphate ions (see below). Apparently, the NaCl used in this experiment was contaminated with Na_2SO_4. It is of note that in an earlier study applying direct UV detection [3] the presence of up to 1 *M* NaCl is not noticed. Similarly, the presence of 0.1 mg Na_2SO_4 or 0.1 mg NaH_2PO_4 per ml sample solution gives rise to peaks migrating at 13.72 and 33.52 min, respectively (Fig. 8). For Na_2SO_4 an additional peak at 12.48 min is noticed probably belonging to traces of NaCl that are present in the Na_2SO_4 used. These observations demonstrate that the presence of salts may compromise the interpretation of the electropherogram. Therefore it seems advisable,

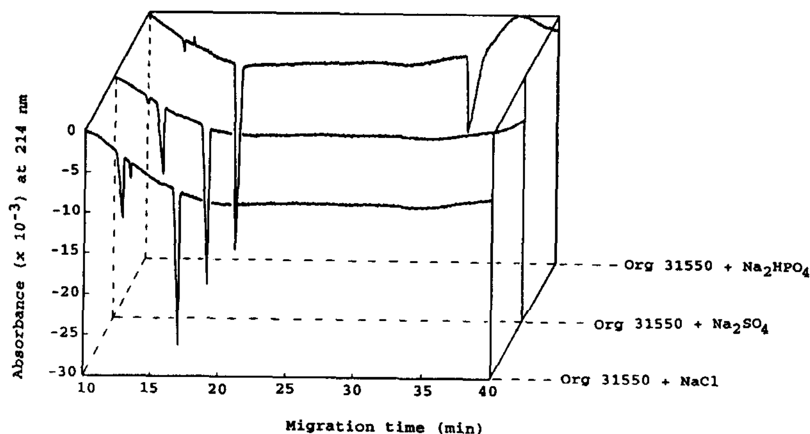

Fig. 8. Influence of the presence of NaCl, Na_2SO_4 or Na_3PO_4 in the sample solution (each at a concentration of 0.1 mg/ml) on CE–indirect UV detection of Org 31550. Experimental conditions as in Fig. 2B. The concentration of Org 31550 in the sample solution is 1 mg/ml.

especially in case of ambiguities in the interpretation of the electropherogram, to desalt the pentasaccharide sample prior to analysis by CE–indirect UV detection.

Finally, to establish the scope of the method, additional types of GAGs, comprising alkylated and O-sulphated synthetic pentasaccharides, synthetic dermatan sulphate di-, tetra- and hexasaccharides and a natural heparin disaccharide (structures indicated in Tables 1 and 2), were analyzed by CE–indirect UV detection in a single run. Fig. 9 depicts the electropherogram obtained for a mixture containing approximately 1 mg/ml of each of the mentioned GAGs, using standard conditions. The identity of the peaks was determined by injection of the pure compounds (results not shown). Evidently, all GAGs can be analyzed in a single run which demonstrates the general applicability of CE–indirect UV detection for the analysis of GAG fragments. In Table 6 the charge and migration time of the various GAG fragments are compiled. The O-sulphated, alkylated pentasaccharide Org 33271, possessing 12 negative charges migrates at 18.2 min. The N,O-sulphated pentasaccharides Org 31550 and Org 31540, having 11 and 10 negative charges, respectively, show slightly higher migration times (18.7 and 19.1 min, respectively) in line with their reduced negative charge compared to Org 33271. The relationship between the molecular mass and charge of carbohydrate molecules and their electrophoretic mobility was recently studied in detail by Chiesa and Horváth [26] using maltooligosaccharides derivatized with 8-aminonaphthalene-1,3,6-tri-

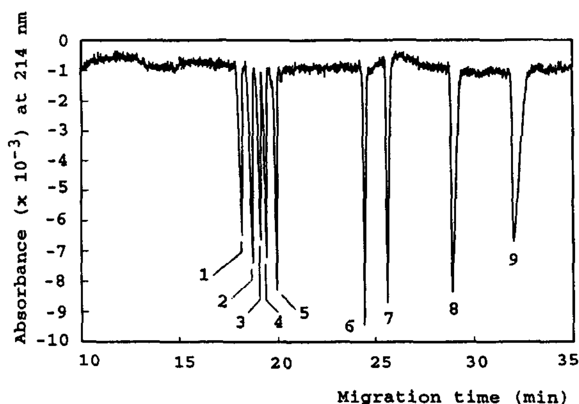

Fig. 9. CE–indirect UV detection of a mixture of various glycosaminoglycans, comprising different synthetic pentasaccharides (1 = Org 31550; 2 = Org 31540; 3 = Org 33271; 4 = Org 33263; 5 = Org 33232), synthetic dermatan sulphate hexa-, tetra- and disaccharides (6 = Org 34275; 7 = Org 34276; 8 = Org 34277, respectively) and a natural heparin disaccharide (9 = δUA2S→GlcNAc6S). The concentration of each GAG in the mixture is approximately 1 mg/ml and the injection volume is 1.8 nl. The structures of the GAGs are compiled in Table 2. Org 33271 and Org 33263 are close derivatives of Org 33232 having twelve and eleven negative charges, respectively. Electrophoresis conditions as in Fig. 2B.

Table 6
Migration time of GAGs in CE–indirect UV detection

GAG	Type	Charge	Migration time (min)
Org 33271 (1)	Synthetic pentasaccharide	12	18.2
Org 31550 (2)	Synthetic pentasaccharide	11	18.7
Org 31540 (3)	Synthetic pentasaccharide	10	19.1
Org 33263 (4)	Synthetic pentasaccharide	11	19.4
Org 33232 (5)	Synthetic pentasaccharide	11	19.9
Org 34275 (6)	Dermatan sulphate hexasaccharide	10	24.4
Org 34276 (7)	Dermatan sulphate tetrasaccharide	7	25.6
Org 34277 (8)	Dermatan sulphate disaccharide	3	28.9
δUA2S→GlcNAc6S (9)	Heparin disaccharide	3	32.0

Experimental details in Fig 9. Org 33271, Org 33263 and Org 33232 differ from Org 31550 and Org 31540 in that they contain exclusively O-sulphated groups and methylated hydroxyl functions, whereas Org 31550 and Org 31540 possess N- and/or O-sulphated and free hydroxyl functions. The numbers in brackets refer to the peaks in Fig. 9.

sulphonic acid as model compounds. In the latter study it was shown that the electrophoretic mobility can be expressed as $\mu_{ep} = CqM_r^{-2/3}$ where μ_{ep} represents the electrophoretic mobility, C is a constant, q is the electrical charge of the analyte and M_r is the molecular mass of the analyte. Although the results presented in this study are in line with the relationship set forth by Chiesa and Horváth, for a correct interpretation of the results it should be noted that also the charge distribution of the analyte may influence the migration time [3]. The alkylated pentasaccharides Org 33263 and Org 33232, both having 11 negative charges, migrate at 19.4 and 19.9 min, respectively. The fact that the latter two compounds have higher migration times than Org 31540, in spite of containing one extra negative charge, must be ascribed to their higher molecular mass (Org 31540: 1727; Org 33263: 1917; Org 33232: 1917) and/or differences in charge distribution. Since Org 33263 and Org 33232 have an identical molecular mass and charge, the difference in migration time must be ascribed to the unequal charge distribution. The dermatan sulphate hexasaccharide, bearing ten negative charges, migrates at 24.4 min. The higher migration time of the dermatan sulphate hexasaccharide compared to the heparin pentasaccharides can be ascribed to its reduced charge and/or higher molecular mass (2108) The dermatan sulphate tetra- and disaccharides, possessing 7 and 3 negative charges, respectively, migrate at 25.6 and 28.9 min, respectively. Finally, the heparin disaccharide δUA2S→GlcNAc6S, having 3 negative charges, migrates at 32.0 min.

In summary, we conclude that CE in combination with indirect UV detection enables a reliable qualitative and quantitative analysis of the purity of sub-picomole levels of low-molecular-mass heparin preparations by CE.

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