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Detection of glycosaminoglycans as a copper (II) complex in capillary electrophoresis

Glycosaminoglycans including heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid were analyzed by reversed polarity capillary electrophoresis. Detection was achieved at 240 nm based on the formation of a copper (II) complex in copper sulfate solution at low pH. Glycosaminoglycans having a high ratio of iduronic acid to glucuronic acid, as well as ones having lower molecular weight, gave the highest detection sensitivity using this method. Detection of the copper (II)-heparin complex is extremely sensitive, permitting the analysis of as little as 10^{-9} g. This method was also successfully applied to the analysis of heparin oligosaccharides that lacked a chromophore prepared from heparin using controlled, low pH nitrous acid depolymerization.

1 Introduction

Glycosaminoglycans (GAGs) are a group of sulfated polysaccharides that display a variety of important biological roles [1, 2]. There are two major classes of glycosaminoglycans: (i) glucosaminoglycans, heparin, heparan sulfate, keratan sulfate, and hyaluronic acid; and (ii) galactosaminoglycans, chondroitin sulfates, and dermatan sulfates. These polysaccharides are polydisperse, having average molecular weights ranging from 10 000 to over 100 000 [3]. Their structural complexity is further compounded by their sequence heterogeneity, caused primarily by a variation of degree and position of sulfate groups [1, 2]. Hyaluronic acid, however, does show sequence homogeneity. With the advent of high-resolution separation methods, there has been an increased interest in the fractionation and analysis of these complex polymers. Gel and membrane supported electrophoresis have been widely used for GAG analysis [1]. Cellulose acetate was first used for resolving different GAGs and permits their visualization by Alcian blue staining [4]. Improvements in separations were afforded using high porosity agarose gels that decreased band diffusion [5]. Polyacrylamide gel electrophoresis (PAGE) affords greatly improved resolution and often results in discrete banding of structurally simple GAGs, such as hyaluronic acid [6–8]. While gel electrophoresis also relies on Alcian blue staining for visualization, PAGE gels stained with Alcian blue can be enhanced with silver staining to visualize nanogram quantities of GAG [9].

Capillary electrophoresis (CE) has been used in the analysis of GAG oligosaccharides prepared by polysaccharide lyase-catalyzed depolymerization [10–16]. Polysaccharide lyases degrade GAGs through an eliminase mechanism that affords unsaturated uronic acid residues at their nonreducing termini [17]. These unsaturated uronic acid residues absorb in the ultraviolet (UV) region at 232 nm, facilitating their detection in CE analysis. High-resolution CE has been used to fractionate lyase-derived GAG oligosaccharides that could be detected at attomole levels [10]. In general, intact, underivatized GAGs have not been successfully analyzed by CE because of the absence of a chromophore. The exception is hyaluronic acid from eye vitreous humor, which has been analyzed by direct UV detection [18]. Mukergee *et al.* [19] reported that copper (II)-GAG complexes gave optical properties that permitted their UV detection. Furthermore, Grant and co-workers showed that these complexes were sufficiently tight to be useful in separations [20]. Wiley [21] recently demonstrated the use of copper (II) complexes to visualize the CE separation of polycarboxylates. These papers suggested that it may be possible to use copper (II) complexes to visualize GAGs separated by CE. The current study explores this possibility.

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Nonstandard abbreviations: CE, capillary electrophoresis; **GAG,** glycosaminoglycan; **LMW,** low molecular weight; **MWavg,** average molecular weight; Ac, acetate; **PAGE,** polyacrylamide gel electrophoresis; **UV,** ultraviolet

Keywords: Glycosaminoglycans / Capillary electrophoresis / Copper (II) sulfate / Oligosaccharides

2 Materials and methods

2.1 Materials

Shark cartilage chondroitin 4-sulfate (average molecular weight, MWavg 50 000; Seikagaku America, Rockville, MD), porcine skin dermatan sulfate (MWavg 16 000; Seikagaku), human umbilical cord hyaluronic acid (MWavg 100 000; Seikagaku), porcine intestinal mucosa heparan sulfate (MWavg 14 800; Celsus, Cincinnati, OH), porcine intestinal mucosa heparin (MWavg 15 000; Celsus) and low molecular weight heparin (MWavg 4800; Celsus) were obtained from commercial sources. Copper (II) sulfate was from Mallinckrodt (Kentucky, KY). Sodium dodecyl sulfate, myristyl trimethylammonium bromide, Triton X-100 and bovine lung heparin were from Sigma (St. Louis, MO). All other chemicals and reagents were of the highest commercially available grade of purity.

2.2 Depolymerization of heparin

Nitrous acid depolymerization was carried out as described by Shively and Conrad [23]. Briefly, 0.1 mL of heparin stock solution (100 mg/mL) was treated with freshly prepared 0.2 mL of pH 1.5 nitrous acid (100 mg of sodium nitrite was dissolved in 1.0 mL of 1 N

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hydrochloric acid). After 10 min at room temperature the reaction was quenched by the addition of 60 μL of 2 M sodium bicarbonate, and then lyophilized. The dried sample was dissolved in 0.5 mL of water and desalted using a 2.8 ID × 34 cm Bio-Gel P-2 (Bio-Rad, Richmond, CA, USA) column and lyophilized. The partial digestion of heparin by heparin lyase I was performed for 24 h at 30°C by treating bovine lung heparin (200 μg) in 50 mM sodium phosphate buffer (pH 7.1) containing 200 mM sodium chloride (100 μL) with heparin lyase I (3 units) [9, 15, 24]. Aliquots were removed at various times and the reaction was terminated by addition of 30 mM hydrochloric acid, resulting in a solution at pH 3.0. To remove the enzyme, the reaction mixture was poured onto an SP-Sephadex (Pharmacia, Piscataway, NJ) microcolumn (100 μL total gel volume) preequilibrated with 30 mM hydrochloric acid at pH 3.0. The column was washed with 30 mM hydrochloric acid (2 mL, pH 3.0), and the eluent was adjusted to pH 7.0 with sodium hydroxide (0.01 N) and lyophilized.

2.3 CE

The experiments were performed on a Dionex capillary electrophoresis system (Sunnyvale, CA) equipped with a variable wavelength ultraviolet detector. System operation and data processing were fully controlled using version 3.1 A1–450 chromatography software on an IBM-compatible PC. The CE sytem was operated in the reverse polarity mode by applying the sample at the cathode and using 5 mM copper (II) sulfate adjusted to pH 4.5 with 0.1 M sulfuric acid. The capillary (75 μm ID, 375 μm OD, 68 cm long) was manually washed before use with 0.5 mL of 0.5 M sodium hydroxide followed by 0.5 mL distilled water, then 0.5 mL running solution. The polyimide external coating on the capillary was removed 48 mm from the inlet end to provide a detection window. Samples were applied using gravity injection (12 s) by hydrostatic pressure (45 mm) resulting in a sample volume of 9.2 nL. Each experiment was conducted at a constant 20 kV. Detection was by on-capillary UV absorbance measurements, at a wavelength of 240 nm. When using copper (II) sulfate as an electrolyte, the cathode platinum wire was plated by copper. While plating of the cathode had no effect on reproducibility or the baseline noise, in separations using copper (II) sulfate as electrolyte it did result in severe baseline noise on the subsequent use of other buffer systems with UV detection. Thus, it was important to clean the cathode after the use of copper (II) sulfate and prior to the use of a second buffer system. The cathode was cleaned by washing it with concentrated nitric acid followed by water.

3 Results and discussion

3.1 Copper (II)-GAG complex formation

GAGs are a family of structurally complex, polydisperse linear polysaccharides composed of the disaccharide repeating units shown in Fig. 1. All GAGs contain a hexosamine residue and (except keratan sulfate) a uronic acid residue. With the exception of hyaluronic

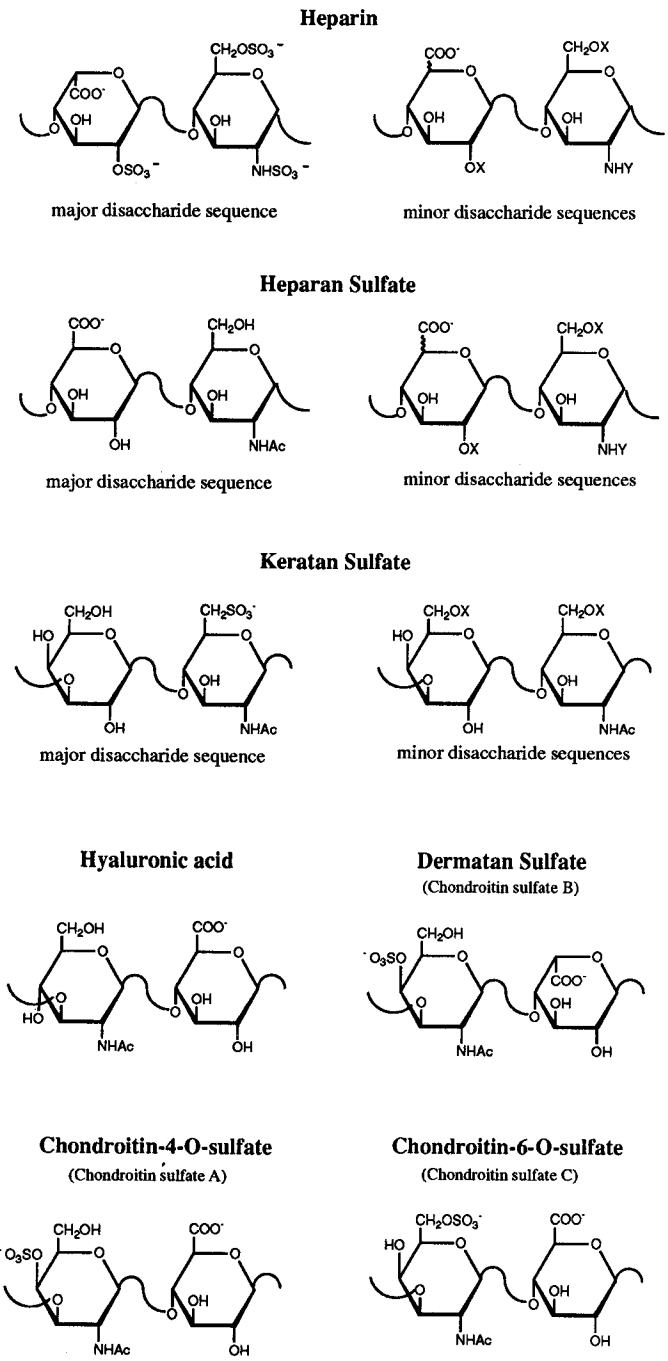

Figure 1. The structure of the major disaccharide repeating unit found in each GAG. In heparin, heparan sulfate and keratan sulfate, having diverse sequences, X = SO_3^- or H and Y = SO_3^-, Ac or H.

acid, all GAGs contain varying degrees of *O* and/or *N*-sulfate groups. Mukherjee *et al.* [19] demonstrated that all GAGs except for keratan sulfate were capable of binding copper (II), affording a complex that had an absorbance maxima near 237 nm. They suggested that this absorbance was the result of a charge transfer complex between copper (II) ion and the carboxylate group in the GAG (keratan sulfate has no carboxylate containing uronic acid and hence shows no absorbance). Mukherjee *et al.* [19] also showed that this absorbance was highest at pH 4.5 and considerably reduced at pH 3.0. Recently, Wiley [21] used a similar copper (II) com-

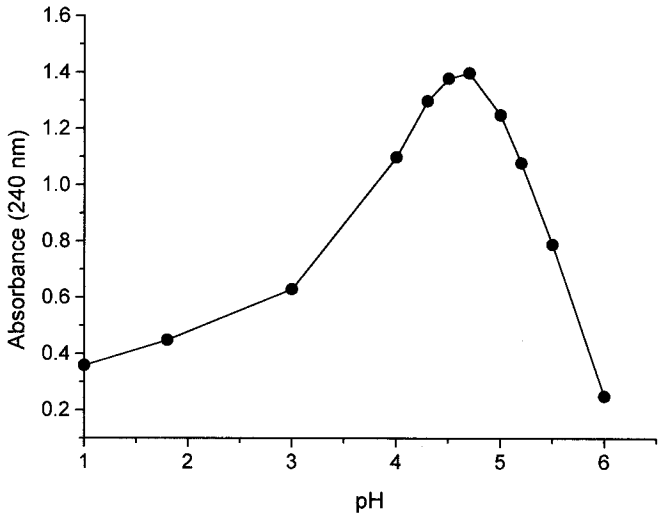

Figure 2. Absorbance of the copper (II) – heparin complex at 240 nm as a function of pH. The λmax shifts slightly with changing pH. These values are 235 nm at pH 1.0, nm, 237 nm at pH 3, 239 nm at pH 4, 240 nm at pH 4.7, 237 nm at pH 5.5, and 230 nm at pH 6.0.

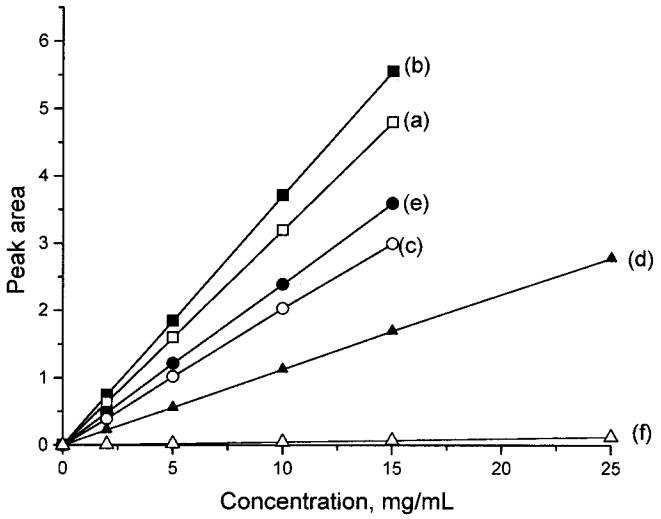

Figure 3. Absorbance (240 nm) as a function of GAG concentration. (a) Heparin, (b) LMW-heparin, (c) heparan sulfate, (d) chondroitin sulfate A, (e) dermatan sulfate, and (f) hyaluronic acid.

plex to detect polycarboxylic at 254 nm that had been separated by CE at approximately pH 5. Our laboratory [15] had recently demonstrated that it was possible to separate heparin, heparan sulfate, chondroitin sulfate and dermatan sulfate (see Fig. 1) derived oligosaccharides (containing a chromophoric unsaturated uronic acid residue) by CE at pH 3.5. Based on these data we undertook to examine whether the copper (II)-GAG complex could be used for detection following separation by CE.

Copper (II)-heparin complexes were prepared and their absorbance spectra were obtained at a variety of pH values. An absorbance maxima of 240 nm was observed at each pH with optimum sensitivity obtained at pH 4.7. The other GAGs (Fig. 1), including low molecular weight (LMW) heparin (heparin has an MWavg of 15 000 while LMW heparin is approximately 5000 [1]), showed the same absorbance maxima at 240 nm. The detection sensitivity was greatest for heparin and LMW heparin and lowest for hyaluronic acid (Fig. 3). As previously reported [19], keratan sulfate gave no detectable enhancement of UV absorbance in the presence of copper (II).

A close examination of the structure of GAGs is important for understanding GAG structural features that effect the sensitivity of copper (II)-based detection. Keratan sulfate, although a sulfated polyanion, contains no carboxylate group and thus can not be detected as a copper (II) complex. However, hyaluronic acid, while having the same number of carboxylate groups as the remaining GAGs, can only be detected as the copper (II) complex at very high concentrations (Fig. 3). Hyaluronic acid is an unsulfated, glucuronic acid containing GAG of high molecular weight (MWavg 100 000). This suggests that while carboxylate groups are essential for copper (II) complex formation, other factors such as molecular weight, sulfation level or type of uronic acid residue (either glucoronic or iduronic acid) influence detection sensitivity. Chondroitin sulfate, dermatan sulfate and heparan sulfate each have similar sulfation levels (approximately one sulfate group per disaccharide repeating unit), yet dermatan and heparan sulfates can be detected with approximately twice the sensitivity of chondroitin sulfate. While the uronic acid residue in chondroitin sulfate is exclusively glucuronic acid, both dermatan and heparan sulfates contain approximately equal proportions of iduronic and glucuronic acids. In addition, chondroitin sulfate (MWavg 50 000) has a higher molecular weight that either dermatan sulfate (MWavg 16 000) or heparan sulfate (MWavg 14 800). This suggests that the structure of the uronic acid and/or the molecular weight of the GAG influences the sensitivity of detection of the copper (II) – GAG complex. Heparin and LMW heparin, detected with the highest sensitivity, contain the highest sulfation (approximately 2.7 sulfates/disaccharide) and the highest percentage of iduronic acid residues (approximately 90% of the total uronic acid) [1]. LMW heparin gives a slightly higher response than heparin as the copper (II) complex (Fig. 3). While LMW heparin (MWavg 4800) is substantially smaller than heparin (MWavg 15 000), it is also slightly enriched in iduronic acid because of the selective destruction of glucuronic acid residues in the depolymerization process [25]. Thus, the issue of whether increased uronic acid content or reduced molecular weight is the most important factor in the sensitivity of detection of the copper (II) complex remains unsettled. Note that even the small, iduronic acid containing oligosaccharides, afforded on nitrous acid depolymerization of heparin, can be detected as the copper (II) complex. Additional studies with structurally defined oligosaccharides are required to better understand all the factors that influence detection sensitivity.

3.2 CE of copper (II) – GAG complexes

Reversed polarity CE has been successful in the low pH analysis of a variety of GAG-derived oligosaccharides [15]. CE analysis of the copper (II) complexes of heparin, LMW-heparin, heparan sulfate, chondroitin sulfate A, dermatan sulfate and hyaluronic acid are presented in

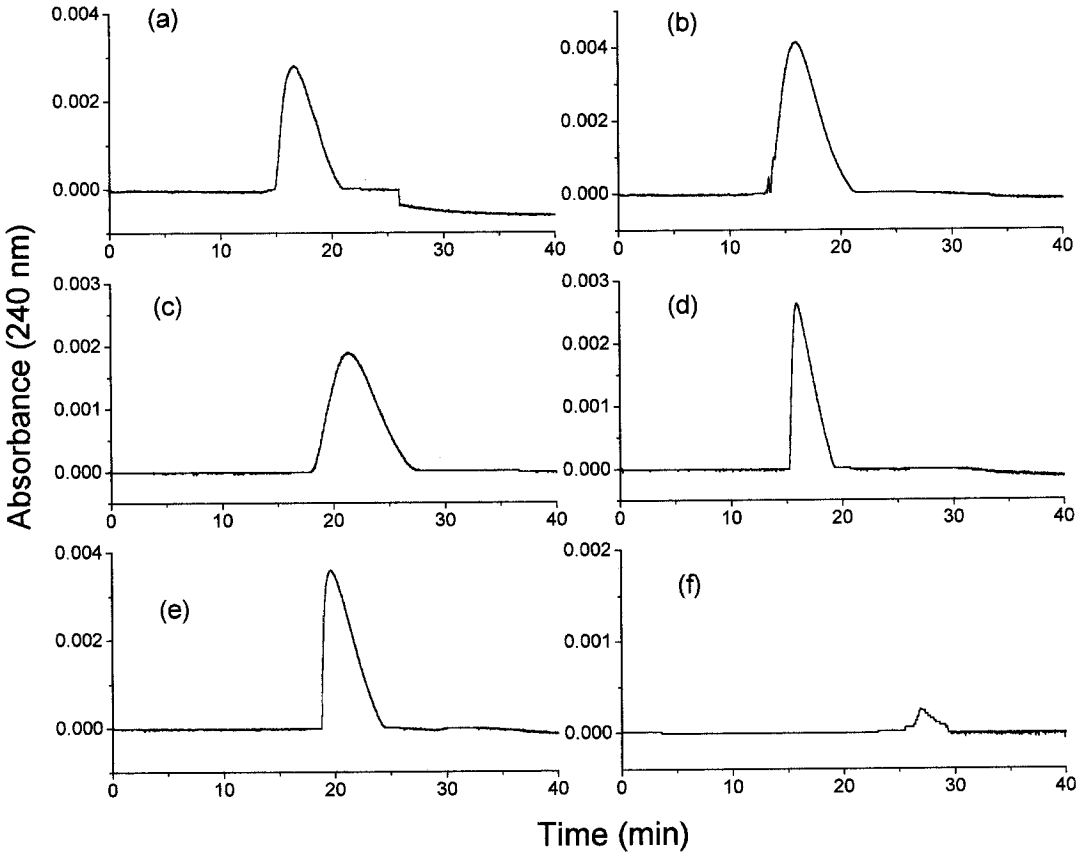

Figure 4. CE analysis of copper (II) – GAG complexes. (a) Heparin, (b) LMW-heparin, (c) heparan sulfate, (d) chondroitin sulfate A, (e) dermatan sulfate and (f) hyaluronic acid. Sample concentration was 10 mg/mL and detection was at 240 nm and separation was in 5 mM copper (II) sulfate at pH 4.5 and 20 kV.

Fig. 4. Under optimized conditions (see Fig. 4 legend and Section 2.3), each GAG showed a broad, featureless peak having a distinctive migration time. While the migration times for each GAG were different (except for heparin and LMW heparin), the broad peaks observed made it impossible to completely separate mixtures of two different GAGS. Interestingly, LMW-heparin and dermatan sulfate afforded narrower peaks, making it possible to resolve a mixture of these two species (data not shown). The contamination of heparin and LMW-heparin preparations with dermatan sulfate has been reported and is of some concern to the pharmaceutical industry [22]. While it might be possible to use CE to assess such a contamination, the detection sensitivity of dermatan sulfate as the copper (II) complex is less than that of LMW-heparin (Fig. 3).

A variety of approaches was examined to improve the resolution shown in Fig. 4. The concentration of copper (II) sulfate solution was varied between 1.25 and 10 mM. Dectection sensitivity increased from 1.25 to 5 mM where it was optimum. Above 5 mM, baseline noise reduced detection sensitivity. The effect of solution pH was examined between 1 and 6. The sensitivity increased with increasing pH, reaching a maximum at pH 4.7, after which the sensitivity decreased. We examined detergents such as sodium dodecyl sulfate (SDS), myristyltrimethylammonium bromide (reportedly useful in improving the CE separation of polycarboxylic acids [21]), and Triton X-100. Cationic and neutral detergents reduced the sensitivity and resulted in peak broadening, while the peaks completely disappeared with added SDS. The voltage used in the separation was varied between 15 and 22.5 kV. As the voltage was increased the peaks sharpened but the resolution decreased, affording an optimum voltage of 20 kV. Optimal separation and resolution was achieved in 5 mM copper (II) sulfate at pH 4.5 in the absence of detergents at 20 kV. A detection sensitivity of heparin of 10^{-9} g was determined under these separation conditions by decreasing the concentration of analyte until no peak could be detected above the baseline noise. The peak breadth observed in Fig. 4 is believed to be primarily ascribable to the polydispersity and/or sequence heterogeneity of the GAGs being analyzed.

3.3 Separation and detection of heparin oligosaccharides as their copper (II) complexes

A mixture of heparin oligosaccharides was first prepared by the controlled partial depolymerization (30% of reaction completion) of bovine lung heparin using heparin lyase I [9, 15, 24]. Because this enzyme is an eliminase each oligosaccharide formed through its action contains an unsaturated uronic acid residue at its nonreducing terminus that absorbs in the UV at 232 nm [17]. Analysis of this mixture showed the presence of separate, discrete peaks, presumably associated with specific oligosaccharides (Fig. 5a). Somewhat lower resolution is obtained in the absence of copper (II) sulfate, in sodium phosphate at pH 3.5 using detection at 232 nm based on the unsatu-

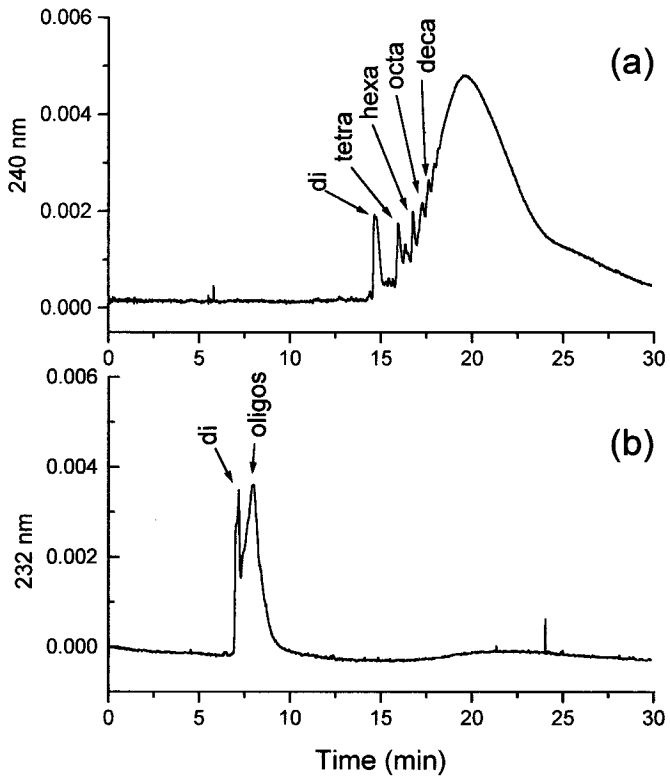

Figure 5. CE of an enzymatically prepared heparin oligosaccharide mixture. (a) Prepared using a 30% digestion of heparin with heparin lyase I using copper (II) sulfate under conditions described in the legend of Fig. 4 and (b) in the absence of copper (II) sulfate in pH 3.5, 20 mM sodium phosphate as previously described [15]. Assignments of peaks labeled disaccharide (di) through decasaccharide (deca) were made based on their similar retention times when compared to oligosaccharide standards [24].

rated uronic acid residues in each oligosaccharide (Fig. 5b). UV detection in the presence of copper (II) sulfate at 240 nm showed similar sensitivity to direct detection in the absence of copper (II) sulfate at 232 nm. Since the 240 and 232 nm chromophores are very close it is not possible to distinguish which is most responsible for the detection of complex. From this experiment it is only possible to conclude that the copper (II) complex affords similar or slightly improved resolution under reverse polarity CE separation when compared to the uncomplexed oligosaccharide mixture.

Controlled partial depolymerization of heparin was accomplished using low pH nitrous acid [23] to afford a mixture of heparin oligosaccharides having minimal UV absorbance > 210 nm. Such heparin oligosaccharides have always posed on-line detection problems when analyzed by HPLC [26] and can only be detected by indirect detection when analyzed by CE [27]. The electropherogram (Fig. 6) shows that a complex pattern of peaks (migration time 10–40 min), representing an oligosaccharide map [28] is obtained by CE when using copper (II) based detection of these oligosaccharides. This electropherogram can be compared directly to the intact heparin polymer showing a single broad peak (migration time 15–20 min) when analyzed under identical conditions (Fig. 4a). Thus, CE might be useful in monitoring nitrous acid depolymerization reactions or in following

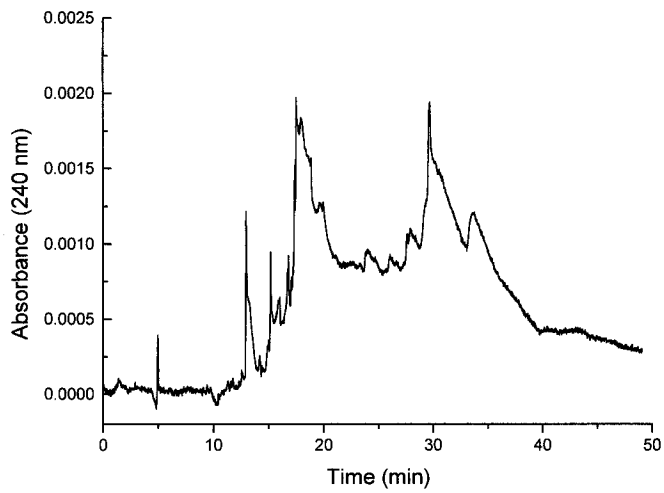

Figure 6. CE of heparin oligosaccharide mixture prepared by controlled nitrous acid depolymerization. See Fig. 4a for the electropherogram of intact heparin and Fig. 4 legend for conditions used.

the purification of oligosaccharide intermediates afforded in this reaction to assist in their structural characterization.

4 Concluding remarks

In conclusion, CE at low pH in the presence of copper (II) sulfate can be used to analyze GAGs and GAG-derived oligosaccharides in the absence of any additional chromophore. The separations obtained were equal to those obtained in the absence of copper (II) sulfate and the detection sensitivity was remarkably high (as low as 10^{-9} g for heparin and LMW heparin).

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