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Review

Separation of negatively charged carbohydrates by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) has recently emerged as a highly promising technique consuming an extremely small amount of sample and capable of the rapid, high-resolution separation, characterization, and quantitation of analytes. CE has been used for the separation of biopolymers, including acidic carbohydrates. Since CE is basically an analytical method for ions, acidic carbohydrates that give anions in weakly acid, neutral, or alkaline media are often the direct objects of this method. The scope of this review is limited to the use of CE for the analysis of carbohydrates containing carboxylate, sulfate, and phosphate groups as well as neutral carbohydrates that have been derivatized to incorporate strongly acidic functionality, such as sulfonate groups.

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1. Introduction

Capillary electrophoresis (CE) is a high-resolution analytical technique that has been applied to a wide variety of different types of

molecules. The potential value of this technique for the analysis of biopolymers, such as proteins, peptides, and nucleic acids, is widely recognized [1–3]. Recently, the application of CE for the analysis of carbohydrates has evoked increased interest [4–7]. Carbohydrates represent yet another major class of biopolymers and are

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particularly difficult to analyze due to their high level of structural complexity. Carbohydrates are classified as monosaccharides, oligosaccharides, and polysaccharides based on their number of monomer units, and can be further divided into acidic and neutral molecules.

Since CE is basically an analytical method for ions, acidic carbohydrates that give anions in weakly acidic, neutral, or alkaline media are often the direct objects of this method. The scope of this review is limited to the analysis of carbohydrates containing carboxylate, sulfate, and phosphate groups as well as neutral carbohydrates that have been derivatized to incorporate strongly acidic functionality, such as sulfonate groups. The analysis of neutral carbohydrates and weakly acidic carbohydrate complexes and derivatives has been described [8–10] and is reviewed elsewhere [6,7]. This special volume also contains such reviews.

2. Capillary electrophoresis: current methods and general principles

Capillary electrophoresis (CE) has recently emerged as a highly promising technique consuming an extremely small amount of sample and capable of the rapid, high-resolution separation, characterization, and quantitation of analytes. CE is rapidly becoming a valuable technique for the separation of biopolymers, including acidic carbohydrates [1–7]. There has been an increasing number of reports of CE being used to analyze attomole or zeptomole quantities of analyte [4–7]. In addition to its high sensitivity, CE has several advantages over a variety of other analytical methods, including an extremely high separation efficiency; on line detection; simple operation; short analysis time; automated and reproducible analysis; and very low consumption of sample and buffers.

CE separates analytes under the influence of an applied electrical field as they migrate in a narrow (50–100 μm I.D.) capillary column (0.5–1.5 m long) spanning two buffer reservoirs and filled with a conducting buffer solution. Jorgenson and Lukacs [11,12] first demonstrated the

excellent separation efficiency of analytes at high field strength in a narrow (<100 μm I.D.) capillary and offered a simple theory of analyte dispersion by CE. At a high applied voltage, a narrow capillary provides a flat flow profile due to electroosmotic flow. This electroosmotic flow effects the mobility of analytes (i.e., neutral, positive, and negative species) through the capillary. CE relies on simple instrumentation [2], which includes a high-voltage power supply (5–30 kV), buffer reservoirs, a narrow-diameter (50–100 μm I.D.) capillary, and a detector. Many commercial instruments also include an automated sampler/injector and a programmable computer that controls applied voltage and data acquisition and processing.

The mobility of analytes under an electric field depends on a number of factors, including: analyte charge (i.e., neutral, positive, or negative); charge to mass ratio of species; the buffer system (i.e., pH and ionic strength); the presence of buffer additives (i.e., surfactants, ion-pairing agents, complexation agents); the voltage applied; the temperature inside the capillary; and the length and the diameter of the capillary. The distinguishing feature of CE is the electroosmotic flow, which drives all species, positive, negative, or neutral, from the injector through the capillary and into the detector. The capillary column is made of fused-silica and invariably carries negative charge on its inner surface because of the presence of silanol groups, which are ionized above pH 3.0. Positive ions in the buffer electrolyte are attracted to the negatively charged inner surface of the capillary. Under an applied high voltage, the positive buffer ions migrate from the anode to the cathode, resulting in a bulk flow of electrolytes called “electroosmotic flow”. The profile of this flow is “plug like” having a flat velocity distribution across the capillary diameter. When a sample, containing both ionic and non-ionic species, is injected into the capillary at the anode, all species, independent of charge, migrate to the cathode under electroosmotic flow. The second, albeit weaker, force of electrophoresis also influences the separation of species by CE. Positively charged species are pulled towards the cathode located at

the detector side of an instrument operating under “normal polarity”, while anionic species are pulled back through electrophoresis towards the anode and the injector. Thus, positive species, pulled towards the cathode under both electroosmosis and electrophoresis elute first, followed by neutral species, and then finally by negative species. Lukacs and Jorgenson [13] first demonstrated that the flat electroosmotic flow profile acted as a pump effecting separation and showed that electroosmotic flow could be modified by altering the buffer composition or pH or by chemically derivatizing the capillary wall. Tsuda et al. [14] also described the effect of buffer pH and current on electroosmotic flow in a fused-silica capillary and demonstrated that the buffer pH and applied voltage are linearly related to the electroosmotic flow. The combination of high efficiency of separation and the short analysis time in CE results in extreme constraints on the sampling system. There are three very simple approaches for sampling that permit the timely introduction of a minimum amount of sample into a capillary column, required to preserve separation efficiency [2]. A sample can be injected into a capillary by electroelution (or electrokinetic injection). In electroelution, a sample is introduced into the capillary by placing the end of the capillary in buffer containing sample, and a potential (i.e., 30 kV) is applied for a brief, fixed time period (i.e., 1 s). This mode of injection has a major drawback, since the introduction of analytes depends primarily on the mobility of the sample components under electrophoresis, molecules that migrate most rapidly are artificially concentrated in the capillary, giving a biased analysis. A second sampling technique is pressure injection, in which pressure is applied to the buffer reservoir on the injector side of the capillary. A third injection technique is hydrodynamic loading (or hydrostatic injection), in which the sample is introduced into the capillary by the application of negative pressure. In automated CE systems the sample is siphoned using a hydrostatic pressure gradient created by simply elevating the buffer reservoir containing sample to a defined position for a fixed period of time. Using this method, sample injection time is

linearly related to peak area of analyte. Hydrostatic injection also can be used to selectively concentrate analyte present in dilute samples [15]. The second and third methods have the advantage that a truly representative sample is drawn into the capillary. The injection time is critical in all of these sampling methods, since large sample volumes markedly decrease separation efficiency [14].

Two different modes of CE are used to analyze both neutral and charged species. The first, normal polarity (or positive polarity), uses a basic or neutral buffer and requires sample application at the anode and detection at the cathode. Negatively charged species are prevented from migrating out of the capillary by the dominant force of electroosmotic flow. This has been the most common mode of separation by CE. The second mode, reversed polarity (or negative polarity), uses an acidic buffer and requires application of the sample at the cathode and detection at the anode. At very low pH (<3) the silanol residues on the capillary wall begin to lose their negative charge, as a result there is reduction in electroosmotic flow. The dominant force in the separation now becomes the mobility of ions under electrophoresis. In the absence of electroosmotic flow only anionic species migrate through the capillary towards the detector, making this method useful for the analysis of acid carbohydrates.

Commercial capillary electrophoresis instruments are equipped with either absorbance or fluorescence detectors. These detectors are based on a number of designs but are constrained to operate within the small dimensions of the capillary. New detector technologies are currently under development [2], and these are discussed in detail below.

3. Acidic carbohydrates: natural products and synthetic derivatives

Carbohydrates include simple monosaccharides, structurally complex linear and branched oligosaccharides, and polydisperse, microheterogeneous polysaccharides [16]. Carbohydrates

drates are found alone or as glycoconjugates covalently linked to other biopolymers including proteins and lipids [16]. Despite their high level of structural diversity, carbohydrates can be divided into acidic carbohydrates, having a negative charge at neutral pH, and neutral carbohydrates, remaining uncharged at pH < 12–13 (under very basic conditions the hydroxyl groups of the carbohydrate ionize).

A representative collection of naturally occurring acidic monosaccharides is shown in Fig. 1 and Table 1. The most common acidic functionalities found in carbohydrates are carboxylate

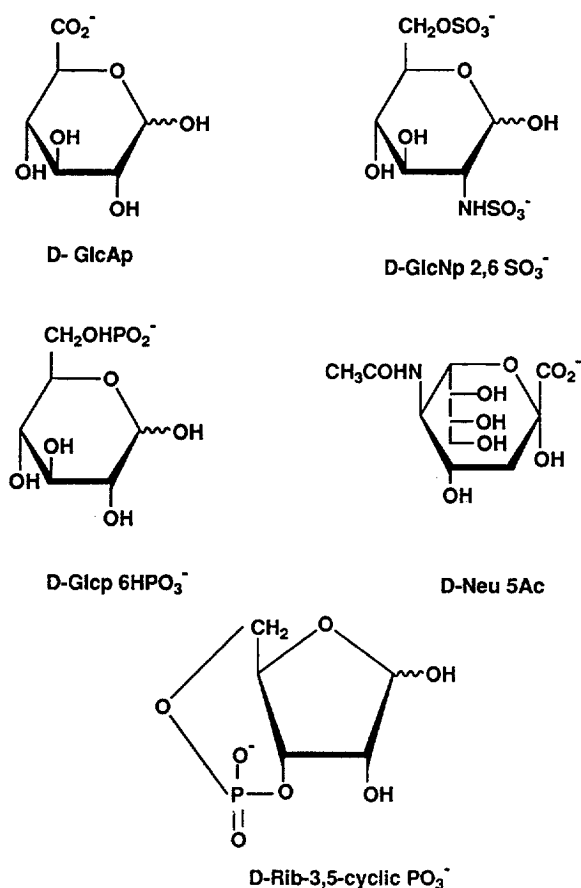


Fig. 1. Structures of selected acidic monosaccharides. The structures shown are D-glucuronic acid (D-GlcAp), D-glucosamine-2,6-disulfate (D-GlcNp2,6 SO_3^-), D-glucose-6-phosphate (D-Glcp6 HPO_3^-), sialic acid (D-Neu5Ac), and D-ribose-3,5-cyclic phosphate (D-Rib-3,5-Cyclic PO_3^-).

groups, monosulfate esters, monosulfate amides, and mono- and diphosphate esters. Some acidic monosaccharides, such as D-Glc6P, are found free in nature, serving important roles in biochemical energetics. Others, such as D-Rib5P, form the nucleotide bases of the informational molecule, RNA. Sialic acid is often found at the non-reducing terminus of the antennae of branched oligosaccharides of glycopeptides. Many different sialic acids are found in nature and are believed to have a wide variety of important biological roles [17]. Uronic acids are common to many acidic polysaccharides found in plants (i.e., pectin, alginate, etc.) and animals (i.e., glycosaminoglycan) [18]. Sulfated sugars are in the O-linked oligosaccharides of mucin glycoproteins and the polysaccharide (glycosaminoglycan) chains of proteoglycans. These molecules are often found on the surface of cells and are extremely important in cell–cell interaction [19].

Neutral carbohydrates can be transformed to acidic oligosaccharide derivatives through a number of chemical methods. Conversion of sugar hydroxyl groups into anionic groups can be accomplished by carboxymethylation, phosphorylation, or sulfation [20]. Unfortunately, this approach often leads to a complex product mixture because of the multiplicity of carbohydrate hydroxyl groups and the low selectivity of such derivatization reactions. Reducing sugars contain an aldehyde, or masked aldehyde in the form of a hemiacetal, at their reducing terminus. This aldehyde can be selectively derivatized by reaction with an amine to form a Schiff base, which can then be reduced under mild conditions to form an aminated sugar derivative. Lee and co-workers [21,22] introduced this chemistry to incorporate a negatively charged fluorescent label into a neutral oligosaccharide (Fig. 2). This chemistry is used in our laboratory for neutral oligosaccharide sequencing and in enzyme assays [21,23–25]. This approach has also recently been exploited in a commercial system for carbohydrate analysis known as FACE. The power of this approach is that a simple, high-yield chemical step affords a carbohydrate derivative that is anionic and intensely fluorescent, facilitating both its separation and detection.

Table 1
Common, naturally occurring carboxylated, sulfated, and phosphorylated sugars found as monosaccharides or in oligosaccharides and polysaccharides

Carboxylated sugars	Sulfated sugars	Phosphated sugars
<i>Simple uronic acids:</i>	<i>Monosulfated sugars:</i>	<i>Phosphate monoesters:</i>
D-Glucuronic	3- or 6-O-Sulfo-D-galactopyranose	6-O-Phospho-D-fructofuranose
L-Iduronic	2-O-Sulfo-L-idopyranose	6-O-Phospho-D-mannopyranose
D-Galacturonic	2- or 6-O-Sulfo-D-glucopyranose	6-O-Phospho-D-glucopyranose
D-Mannuronic	2-O-Sulfo-L-rhamnopyranose	1-O-Phospho-D-ribofuranose
D-Guluronic	2-O-Sulfo-L-xylopyranose	5-O-Phospho-D-ribofuranose
L-Ascorbic	2-Deoxy-2-N-acetyl-6-O-sulfo-glucopyranose	2-Deoxy-2-phospho-D-ribofuranose
D-Arabinic	2-Deoxy-2-N-acetyl-4-O-sulfo-D-galactopyranose	2-O-Phospho-D-glycerol
<i>Sulfated uronic acids:</i>	2-Deoxy-2-N-acetyl-6-O-sulfo-galactopyranose	1-O-Phospho-D-galactopyranose
2-O-Sulfo-D-glucuronic	2-Deoxy-2-N-acetyl-6-O-sulfo-galactopyranose	6-O-Phospho-2-deoxy-2-N-acetyl-glucopyranose
2-O-Sulfo-L-iduronic	<i>Disulfated sugars:</i>	5-O-Phospho-2-deoxy-D-ribofuranose
<i>Sialic acids:</i>	4,6-O-Disulfo-2-deoxy-2-N-acetyl-D-galactopyranose	9-O-Phospho-5-acetylneuraminic acid
N-Acetylneuraminic acids [17]	6-O-Sulfo-2-deoxy-N-sulfo-D-glucopyranose	5-O-Phospho-L-xylose
D-Glycero-D-galacto-2-nonulosonic acid (KDN)	<i>Trisulfated sugars:</i>	1-O-Phospho-D-glycerol
<i>Other sugars carboxylates:</i>	3,6-O-Disulfo-2-deoxy-N-sulfo-D-glucopyranose	5-O-Phospho-D-xylopyranose
3-Deoxy-D-manno-2-octalulosonic acid (KDO)		2-O-Phospho-D-xylopyranose
2-Amino-3-O-(1-carboxylethyl)-2-deoxy-D-glucopyranose		<i>Phosphate diesters:</i>
2-Deoxy-2-N-acetyl-3-O-(1-carboxylethyl)-D-glucopyranose (NAM)		1,5-Diphospho-D-ribulose
D-Gluconic acid		1,5-Diphospho-D-ribofuranose
D-Galactonic acid		1,5-Diphospho-2-deoxy-D-ribofuranose
		1,5-Diphospho-D-fructofuranose

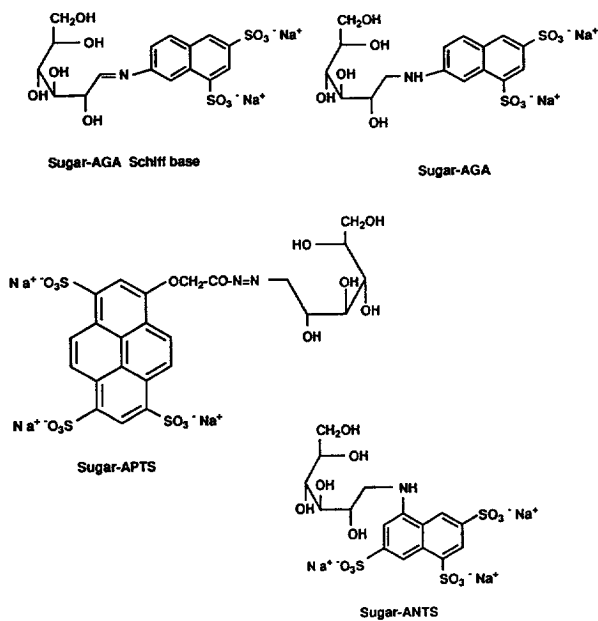


Fig. 2. Structures of selected acidic neutral carbohydrate derivatives used in CE analysis. The structures shown are the Schiff-base and reduced conjugate of D-Glcp and 7-amino-1,3-naphthalenedisulfonate (AGA), the hydrazide derivative of D-Glcp and aminopyrenetrisulfonate (APTS), and the reduced Schiff-base conjugate of D-Glcp and 8-amino-1,3,6-naphthalenetrisulfonate (ANTS).

4. CE for the analysis of glycosaminoglycans and related acidic polysaccharides

Glycosaminoglycans are linear, highly sulfated polysaccharides and are the subject of intensive study in our laboratory [23,26,27] because of their importance in a number of biological processes [28–30]. Glycosaminoglycans include heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid. With the exception of hyaluronic acid (a copolymer of glucuronic acid and N-acetylglucosamine), all the glycosaminoglycans are sulfated [18,31]. Because of their large size (average molecular mass 10 000–100 000) and structural complexity, glycosaminoglycans and related acidic polysaccharides are often first broken-down to oligosaccharides before being subjected to analysis and structure determination [18,31,32]. Glycosaminoglycans and related acidic polysaccharides that contain uronic acid

residues can be depolymerized under mild conditions using highly specific polysaccharide lyases [18]. A detailed method for the use of these enzymes has been reported [31]. These enzymes afford mixtures of oligosaccharide products, which have Δ 4,5-unsaturated uronic acid residues at their reducing termini (Fig. 3). Most of the CE methods developed for analyzing acidic oligosaccharides have focused on glycosaminoglycans, because of their mammalian origin and their importance to medicine as therapeutic agents [28–30]. It is important to note, however, that similar methods can be applied to acidic oligosaccharides derived from plants, including pectin, and alginic acid [18].

The first application of CE to acidic oligosaccharides, involving the analysis of disaccharides derived from chondroitin sulfate, dermatan sulfate, and hyaluronic acid, was simultaneously reported in 1991 by Al-Hakim and Linhardt [33] and Carney and Osborne [34]. Both research groups used a normal polarity in a basic borate buffer to separate a group of eight chondroitin sulfate and dermatan sulfate disaccharides (Figs. 3 and 4). This separation method was later extended to successfully resolve the eight disaccharides prepared from heparin and heparan sulfate (Figs. 3 and 4) [35]. The addition of sodium dodecylsulfate (SDS) improved resolution, possibly the result of introducing a mixed mode of capillary zone and micellar electropho-

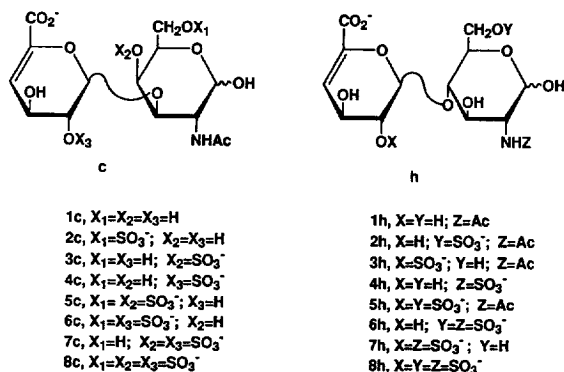


Fig. 3. Structures of chondroitin sulfate and heparin disaccharides. The structures labeled c are prepared from chondroitin sulfate and dermatan sulfate, and the structures labeled h are prepared from heparin and heparan sulfate.

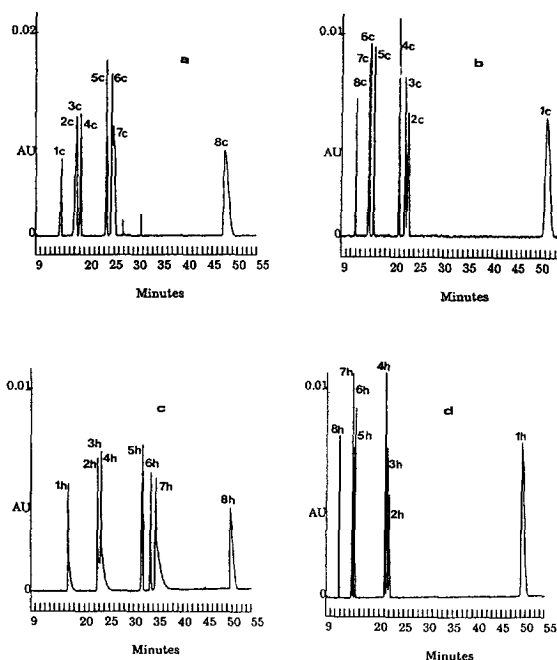


Fig. 4. Normal- and reversed-polarity CE of chondroitin sulfate and heparin disaccharides. The peaks in electropherograms a and b are labeled corresponding to structure c (Fig. 3) and the peaks in electropherograms c and d are labeled corresponding to structure h (Fig. 3). CE was performed using normal polarity in 10 mM sodium borate and 50 mM SDS, pH 8.80, at 12 kV for a and c. CE was performed using reversed polarity in 20 mM phosphoric acid buffer, pH 3.48, and 12 kV for b and d. In all separations identical capillaries (75 μ m I.D. \times 375 μ m O.D., 78 cm long) were used, and the absorbance full-scale unit was 0.01 at 232 nm.

resis [35]. CE has been applied to the disaccharide compositional analysis of glycosaminoglycans [23,26], resulting in a method comparable to the amino acid analysis of proteins. A detailed method for the disaccharide analysis of glycosaminoglycans by CE has been reported [4]. Improvements in disaccharide compositional analysis have been made, using a normal polarity separation, by applying micellar electrokinetic capillary electrophoresis in basic borate buffer containing a positively charged ion-pairing agent, cetyltrimethylammonium bromide [36]. Disaccharide analysis can also be performed in acidic buffer under reversed polarity (Figs. 3 and 4) to afford greatly improved resolution of glycosaminoglycan-derived disaccharides [37,38].

Larger oligosaccharides prepared from glycosaminoglycans can also be analyzed by CE [38,39]. The analysis of oligosaccharide mixtures prepared from enzyme treatment of glycosaminoglycans affords an oligosaccharide map and is comparable to the peptide mapping of proteins [23,39]. Normal-polarity separations in basic borate buffer containing SDS appear to give the best separation of mixtures of higher oligosaccharides [38]. While organic solvent additives, such as formamide, can improve these separations, such additives have an adverse impact on the sensitivity of detection [39].

CE analysis of glycosaminoglycan-derived oligosaccharides relies on ultraviolet absorbance detection. The unsaturated uronic acid residue present at the non-reducing termini of these molecules has molar absorptivity at 232 nm of approximately 5 000 [40]. This results in a limit of detection of a femtomole of oligosaccharide on commercially available absorbance detectors. Recently, chondroitin sulfate-derived oligosaccharides have been labeled with 1-phenyl-3-methyl-5-pyrazolone and analyzed in basic borate buffer under normal polarity using fluorescence detection [41]. The increased sensitivity of this method permitted the disaccharide analysis of urinary glycosaminoglycans.

5. Sialic acid containing oligosaccharides

Glycoproteins, common to all eukaryotic organisms, contain a wide variety of N-linked and O-linked oligosaccharides [16]. The N-linked oligosaccharides of glycoproteins have been extensively studied and have been shown to contain common structural motifs originating from their biosynthesis [42] that markedly influence their biology [21]. N-linked oligosaccharides are commonly branched, and these branches (or antennae) are often terminated with sialic acid residues [17]. The presence and type [17] of sialic acid at the non-reducing terminus of each branch can influence glycoprotein biology, including: targeting to a particular tissue; clearance; resistance to proteolysis; and biological activity [21]. Thus, the study of oligosaccharide sialylation is

important in understanding glycoprotein biology and in the application of glycoproteins as therapeutic agents. To measure sialylation, oligosaccharides are first removed from a purified glycoprotein or glycopeptide fragment of a glycoprotein by hydrazinolysis or through the use of PNGase F [43]. The resulting oligosaccharides are then often analyzed or purified by high-performance anion-exchange chromatography using pulsed amperometric detection [43].

Sialic acids are carboxysugars (Fig. 1) and most commonly Neu5Ac, which is α 2,3 or α 2,6 linked to D-Galp [17]. In a tetraantennary structure (one containing four branches) it is possible to have 0 to 4 residues of Neu5Ac. There are a large number of permutations for undersialylated structures (i.e., oligosaccharides with 0–3 residues of Neu5Ac) since the four antennae of a tetraantennary oligosaccharide are structurally inequivalent and may carry α -2,3- and α -2,6-linked Neu5Ac at the various branches. An understanding of the actual structures present in an oligosaccharide at a particular site in a glycoprotein might lead to important insights into biology of endogenous glycoproteins and the improved utilization of glycoprotein-based therapeutics.

Hermentin et al. [43,44] have proposed an oligosaccharide mapping technique of sialylated oligosaccharides based in part on CE. Sialic acids are negatively charged, in neutral and basic solutions of sodium phosphate buffer, permitting their separation using normal-polarity electrophoresis. Sialylated oligosaccharides from α ₁-acid glycoprotein migrate in distinct populations (Fig. 5) based on the number of Neu5Ac residues (Fig. 1) present. Neutral, non-sialylated species migrate fastest under electroosmotic flow (Fig. 5). These are followed by clusters of peaks corresponding to monosialylated, disialylated, trisialylated, and tetrasialylated (migrating slowest) species. Similar elution profiles have been reported in the analysis of oligosaccharides from recombinant tissue plasminogen activator under normal polarity in basic borate buffer containing putrescine as an additive [45,46]. CE analysis gives a highly repeatable oligosaccharide map that is useful in characterizing the glycosylation

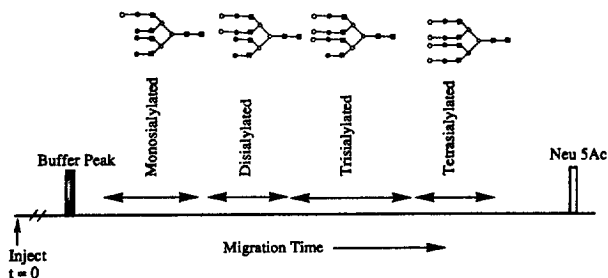


Fig. 5. Separation of sialic acid containing complex type oligosaccharides prepared from α ₁-acid glycoprotein by CE. Following the migration of a buffer peak and prior to the migration of D-Neu5Ac standard, mono-, di-, tri- and tetrasialylated oligosaccharides migrate in the discrete regions of the model electrophorogram shown. The monosaccharide residues are D-GlcNpAc (■), D-Manp (◇), D-Galp (●) and Neu5Ac (○). Figure adapted from data presented in Hermentin et al. [43].

and sialylation of a particular glycoprotein and may lead to a better understanding of the biological activities of those glycoproteins [44]. The low sample consumption of CE makes it possible to use sufficient oligosaccharide concentrations for direct absorbance detection at 190 nm, even though the molar absorptivity of sugars is low and the interferences are significant. Detailed methods for the direct analysis for sialylated oligosaccharides have been reported [4].

Sialylated oligosaccharides can also be covalently derivatized with a label such as 2-amino pyridine [47] or 3-(4-carboxybenzoyl)-2-quinolin-carboxaldehyde (CBQCA, originally developed for detection with laser-induced fluorescence [48]) and detected by fluorescence [3]. Hence, while the separation is comparable to that obtained on the underivatized sialylated oligosaccharides, the sensitivity of fluorescence detection is substantially better and much less sample is required [5]. Enhanced absorbance detection of sialylated oligosaccharides is also possible by the conjugation of sialylated oligosaccharide to either ethyl-4-aminobenzoate or 4-aminobenzoic acid through reductive amination [6,49]. While derivatization offers distinct advantages of improved detection sensitivity, it also poses certain difficulties. It is difficult to quantitatively derivatize carbohydrates, particularly when they are at

very low concentrations or are in samples, such as biological fluids, containing interfering substances. In the derivatization of carbohydrates with excess uncharged hydrophobic tag (i.e. 2-aminopyridine) it is also often difficult to remove the unreacted tag [4,50], which often interferes with CE analysis [4]. Clearly, additional work is required to circumvent these difficulties to achieve improved detection sensitivity of sialylated oligosaccharides.

6. Conversion of neutral oligosaccharides to acidic oligosaccharide derivatives

The concept of converting neutral oligosaccharides to charged oligosaccharides for analysis and sequencing, using electrophoresis, was introduced by Lee and co-workers [21,22,51,52]. The chemistry initially developed involved the reductive amination (using sodium cyanoborohydride) of a reducing sugar with a charged fluorescent tag, such as an aminonaphthalene sulfonate or an aminopyrene sulfonate [22]. This affords an intensely visibly fluorescent acidic oligosaccharide derivative (Fig. 2) that can be separated using capillary electrophoresis (Fig. 6) [51,52]. There are significant advantages of this approach over many previously described. The labeling chemistry is simple and the use of a hydrophilic sulfonated tag eliminates some problems with removal of excess reagent [4]. Aminonaphthalene sulfonates and aminopyrene sulfonates are highly charged hydrophilic tags, so that excess reagent can be conveniently removed using a desalting column eluted with water [53], by strong-anion-exchange high-performance liquid chromatography [21], or by preparative gel electrophoresis [21]. Uncharged hydrophobic tags (i.e., 2-aminopyridine) require multiple extractions with organic solvent, for the removal of excess reagent, followed by gel permeation chromatography in a volatile buffer and repeated lyophilization [50]. Oligosaccharides labeled with aminonaphthalene sulfonates or aminopyrene sulfonates are visibly fluorescent and can be easily observed during sample handling or purification by using a transilluminator or a hand-held

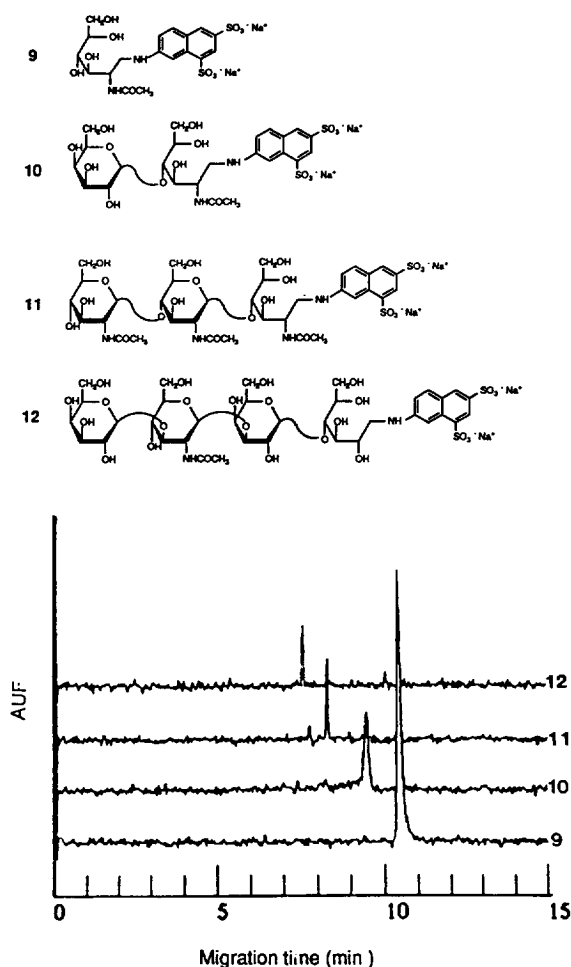


Fig. 6. CE analysis of oligosaccharide-AGA conjugates 9–12. Eclipsed overlay of four electropherograms is shown. CE was performed using normal polarity in 10 mM sodium borate and 50 mM boric acid, pH 8.8, at 20 kV. The capillary was 75 mM I.D. \times 375 μ m O.D. and 68 cm in length. Fluorescence detection was at 420 nm (excited at 250 nm).

UV light. The sulfonate group is very strongly acidic so that it remains anionic at pH > 1, facilitating its use in both normal- and reversed-polarity modes of separation. These clear advantages have led to the development of a commercial technology, FACE, utilizing polyacrylamide gel electrophoresis based on 8-amino-1,3,6-naphthalenetrisulfonate. A recent study [54] has demonstrated that neutral sugars labeled with this fluorescent tag give excellent separations using both normal- and reversed-polarity CE. The

mobility of labeled malto-oligosaccharides under reversed-polarity CE, for example, shows nearly ideal behavior when plotted as a function of molecular mass.

Methods of labeling neutral oligosaccharide with anionic fluorescent tags have been recently developed that have additional advantages. A hydrazidopyrenesulfonate (Fig. 2) can be used to give a stable acidic oligosaccharide derivative without the use of a reducing agent, such as sodium cyanoborohydride. Similarly, the Schiff-base form of 7-amino-1,3-naphthalenedisulfonate oligosaccharide conjugate (Fig. 2) is stable under neutral and basic conditions, permitting its fractionation [55]. At pH 3.5 this derivative is sufficiently stable to survive a 1 h separation at room temperature [55]. This Schiff-base conjugate can then be decomposed by mild treatment with acid (i.e., 0.1 M hydrochloric acid, 5°C, 12 h [55]) to regenerate the original neutral oligosaccharide, thus providing the first truly reversible label for oligosaccharide analysis.

7. CE to study acidic oligosaccharides and acidic oligosaccharide derivatives as enzyme substrates

The application of CE in enzymology involving acidic oligosaccharide and polysaccharide substrates began as a simple outgrowth from oligosaccharide mapping experiments on glycosaminoglycans [23,39]. Heparin oligosaccharides were treated with various heparin lyases, and their breakdown was followed by capillary electrophoresis to study the specificity of each heparin lyase [56] or to confirm the structure of the oligosaccharide starting material based on the known specificity of a heparin lyase [27]. CE was next used to study the specificity of sulfoesterases acting on chondroitin sulfate and heparin-derived disaccharides [25]. The presence of small amounts of sulfoesterase contamination in commercial samples of polysaccharide lyases could also be assessed using CE [25].

More sophisticated use of CE in enzymology has involved the use of acidic derivatives of neutral oligosaccharides as enzyme substrates.

Neutral oligosaccharides were conjugated to 7-amino-1,3-naphthalenedisulfonate through reductive amination, and these conjugates were easily purified (Fig. 2) [21,22]. These oligosaccharide conjugates can be used simply as substrates to determine glycosidase activity [21,24] or the presence of minor contaminating glycosidases in commercial enzymes [21]. Alternatively, these acidic oligosaccharide derivatives can be used in conjunction with CE as glycosyltransferase substrates to follow their purification or to measure glycosyltransferase activity [24]. The limits of substrate and product detection in such measurements are approximately 50 fmol [24].

Fluorescent derivatives of chito-oligosaccharides have been used to study the action pattern of chitinases using CE [57]. In this sophisticated use of CE, a chito-hexaose was fluorescently labeled with 7-amino-1,3-naphthalenedisulfonate, and its decomposition on chitinase treatment was monitored. The disappearance of derivatized chito-hexaose was observed at the same time as smaller chito-oligosaccharides (i.e., chito-tetraose, -triose, -biose) appeared. Some of the products formed appeared only transiently (i.e., chito-tetraose and -triose), eventually giving rise to a group of stable final products (i.e., chito-biose). The reaction followed by CE provided kinetic data valuable in the detailed analysis of the action pattern of chitinase. For the future it is anticipated that similar, sophisticated use of CE will improve our understanding of enzymes acting on acidic oligosaccharides and acidic oligosaccharide derivatives.

8. CE to follow the chemical synthesis of acidic oligosaccharides

One of the most difficult problems confounding synthetic chemists working with acidic oligosaccharides is their rapid analysis during the routine development of a synthetic reaction [20]. Typically, such analysis relies heavily on thin-layer chromatography. Acidic carbohydrates, however, behave poorly on thin-layer chromatography. Chemical modification of heparin-derived disaccharides can be conveniently followed

using CE (Fig. 7). Analyses times range from 10–60 min, and minimum sample preparation is required, permitting the sampling of an ongoing reaction [20]. This approach has also been applied to monitoring the sulfation of neutral carbohydrates such as sucrose [20]. In the absence of a UV-active chromophore, indirect ultraviolet detection in basic buffer containing sodium benzoate or by fluorescence with added sodium salicylate is possible [2–7]. As carbohydrate chemists begin to target more acidic oligo-

saccharides for synthesis, it is likely that CE will become a routine method to follow these reactions.

9. Future prospects of CE for the analysis of acidic carbohydrates

Three areas of advancement are likely in the analysis of acidic carbohydrates by CE: 1. identification of new analytical targets; 2. development of new CE-based separation methods; and 3. development and improvement of detection methods.

New targets for CE analysis might include very large acidic oligosaccharides such as a heparin-derived tetradecasaccharide ($M_r = 4655$, with a net charge of -28) [38] or acidic polysaccharides such as pectin [5]. Clearly, as the size of these molecules becomes larger, new methods, such as CE under pulsed-field conditions, may offer improved resolution [58]. It also may be possible to determine the molecular-mass distribution of polydisperse polysaccharides using CE in the same way as synthetic polymers have been analyzed [59]. Chiral separations of acidic sugars or acidic sugar derivatives by CE might be possible in the presence of chiral buffer additives. This approach would be analogous to the chiral separation of small organic molecules by CE using carbohydrate carriers, such as cyclodextrins [60]. Affinity CE [61] is also possible using lectins that interact with acidic carbohydrates or acidic carbohydrate derivatives to facilitate separations [62]. Such an approach has been successfully used in one-dimensional [63] and two-dimensional [23,53] gel electrophoresis formats to separate acidic oligosaccharide derivatives based on their differential interaction with lectins. It might even be possible to use CE in a micropreparative mode [64] to purify enough oligosaccharide for structural characterization.

The separation methods currently applied to acidic oligosaccharides include normal- and reversed-polarity CE [38], micellar electrokinetic CE [36], and, most recently, the use of coated (with linear polyacrylamide) capillaries [58,65]. Future applications will likely include use of a

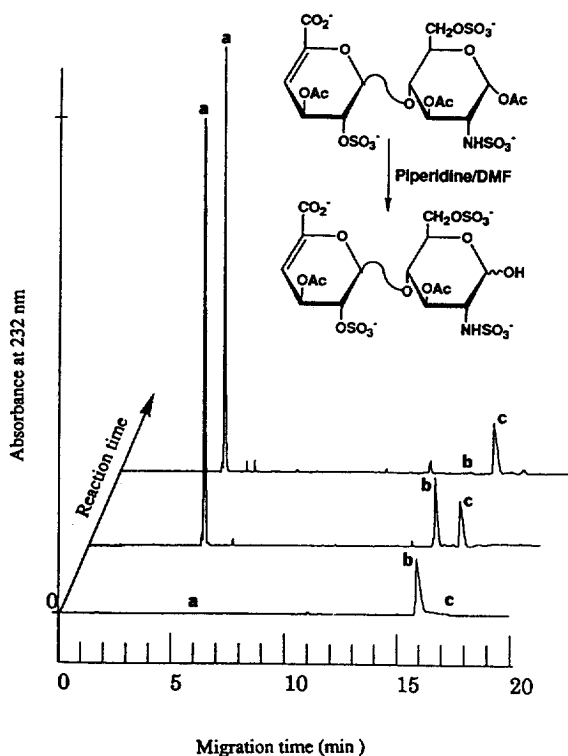


Fig. 7. Use of CE to monitor the chemical deprotection of the 1-position of a peracetylated heparin-derived disaccharide (**8h**, Fig. 1). At reaction time $t = 0$ (lower electropherogram), peracetylated **8h** migrates as a single peak (b) at 16 min. On addition of piperidine (center electropherogram) in dimethylformamide (DMF, peak a, at 6 min) a new product peak (c), corresponding to loss of acetate from the 1-position of peracetylated **8h**, is observed with a concomitant decrease in starting material (peak b). At reaction completion (upper electropherogram) starting material (peak b) has disappeared and only solvent (peak a) and product (peak c) are observed. The conditions used in CE analysis are the normal-polarity conditions described in Fig. 4.

variety of different capillary coatings, including ones designed specifically to interact with carbohydrates. The application of entangled polymer matrices and gel-filled capillaries also promises improved resolution, particularly in the separation of large oligosaccharides and polysaccharides. Gel-filled capillaries containing lectins might be useful in the high-resolution affinity fractionation of acidic oligosaccharides and acidic oligosaccharide derivatives. The investigation of new buffer systems, including the use of buffer additives, also holds considerable promise. Chiral additives, surfactants, and ion-pairing reagents might further improve these separations.

New detection technology as well as new derivatization strategies will significantly impact all areas of CE, including the analysis of acidic oligosaccharides. Currently, only absorbance and fluorescence detectors are available on commercial instrumentation. Laser-induced fluorescence, particularly using custom-designed fluorescent labels [48], offering exquisite detection sensitivity, are currently under intensive investigation. A conductivity detector for CE has been developed and should become commercially available in the near future. Such detectors have been extremely useful in the analysis of acidic carbohydrates separated chromatographically [40]. Pulsed amperometric detection of carbohydrates separated by CE [2] is possible by sampling the separation through a break (after separation has taken place but before the cathode buffer reservoir) in the capillary column. Pulsed amperometric detection has been used extensively to analyze carbohydrates separated chromatographically [43]. Electrochemical detection by conductivity or amperometry may be possible at the end of the capillary column using alternating current. Radiochemical and mass spectrometric detectors for CE are also in development. Mass detection by methods such as refractometry might also be applied to CE. Finally, each new detector suggests many additional derivatization methods. Specific, quantitative, covalent derivatization of low concentrations of acidic oligosaccharides, requiring only a stoichiometric amount of derivatization reagent, would markedly improve

analysis. Indirect detection using buffer additives might also make CE applicable to a wider variety of acidic carbohydrates [66,67].

In conclusion, the prospects for the application of CE to the analysis of acidic carbohydrates are excellent. The presence of a negatively charged group on these molecules ensures their migration under a variety of CE conditions. The small sample requirements of CE are particularly suited for the analysis of such precious biological samples. Finally, the impressive initial successes in the analysis of these molecules using a limited number of columns, derivatization chemistries, and detectors suggest that, as technology improves, many additional advances should result.

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