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Multi-dimensional mapping of pyridylamine-labeled N-linked oligosaccharides by capillary electrophoresis

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Abstract

A simple, sensitive and reproducible multi-dimensional capillary electrophoresis (CE) oligosaccharide mapping method is reported. The structures of 20 identified N-linked oligosaccharides have been assigned mapping positions from which co-migrating unknown oligosaccharides can be characterized. The separation protocols developed have been demonstrated to separate both charged and neutral oligosaccharides. One dimension involves electroosmotic flow-assisted CE in a sodium acetate buffer, pH 4.0. A second dimension involves separation based on borate complexation electrophoresis in a polyethylene glycol-containing buffer. A third dimension developed specifically for neutral oligosaccharides, using a sodium phosphate buffer, pH 2.5, has been shown to resolve neutral species not able to be separated by the other two dimensions. Thus, a three-dimensional map was generated to facilitate structural characterization of these oligosaccharides.

1. Introduction

Interest in understanding the importance of the glycosylation of glycoproteins has led to the exploration of new techniques for the analysis of glycoprotein-derived oligosaccharides. Structure analysis by consecutive digestion of oligosaccharides with exoglycosidases followed by chromatographic or electrophoretic separation can be effective [1–6]. However, this approach is limited by the lack of availability of enzymes with sufficient specificity to characterize all structures found in nature. The collective application of

NMR, mass spectrometry, composition and linkage (methylation) analysis can generally determine most oligosaccharide structures, but requires extensive sample purification, a significant quantity of material, expensive instrumentation and significant expertise. Mapping of retention data obtained from high-performance liquid chromatography (HPLC) based on reversed- and normal-phase packings has been demonstrated to be an approach that can simultaneously identify multiple oligosaccharides on a picomolar level [7]. Such two-dimensional chromatographic mapping, however, depends on gradient elution and is subject to reproducibility problems in gradient formation, column support media and buffer formulations.

Recently, capillary electrophoresis (CE) has been used as an alternative to HPLC for map-

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ping of pyridylamine (PA) labeled N-linked asialo-oligosaccharides [8,9]. We have modified this approach to be inclusive of naturally charged oligosaccharides containing terminal sialic acid residues and/or pendant sulfate groups. The PA was used to attach a positive charged group to all oligosaccharides studied and to impart detectability through fluorescence.

2. Experimental

2.1. Preparation of oligosaccharides from glycoproteins

Glycoproteins (500 mg): bovine ribonuclease *b*, fetal calf fetuin, human transferrin and porcine thyroglobulin were reduced and carboxymethylated (RCM) via the protocol outlined by Carr and Roberts [10] and treated with 10 units of peptide-N-glycosidase F (PNGase F) at room temperature for 24 h in 10 ml 100 mM ammonium acetate buffer, pH 7.5, containing 10 mM EDTA. The released oligosaccharides were separated from the deglycosylated protein on a Bio-Gel P-6 column using 500 mM ammonium acetate as eluent. Further separation of the oligosaccharides was achieved using a combination of lectin affinity chromatography with concanavalin A (Con A)-agarose (Sigma) using hapten sugars, and anion-exchange chromatography using a Mono-Q column (Pharmacia) [11,12]. All oligosaccharide structures isolated and mapped in this study were characterized by composition analysis, matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) and ^1H NMR spectroscopy, as described below. In all cases, the oligosaccharides of the glycoproteins used to prepare our samples had been previously characterized as described in the literature for ribonuclease *b* [11], fetuin [13,14], transferrin [12] and thyroglobulin [15,16], such that our own composition analysis, mass spectrometry and NMR analysis was used for structure verification rather than original structure determination.

2.2. Composition analysis

The glycosyl residue compositions of the isolated oligosaccharides used in this study, excluding pendant sialic acids that are not determined by this method, were quantified by acid hydrolysis followed by 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization, and reversed-phase HPLC, as previously described [17]. Compositions thus determined were consistent with those indicated (data not shown).

2.3. MALDI-TOF-MS

Mass spectra were recorded on an Applied Biosystems prototype MALDI mass spectrometer, equipped with a linear TOF analyzer and a pulsed nitrogen laser (337 nm). The conditions and methods used for this work have been described elsewhere [11]. Table 1 lists the calculated and observed masses for the oligosaccharides used in this study.

2.4. ^1H NMR spectroscopy

^1H NMR spectra of the oligosaccharides used

Table 1
Calculated and observed masses of the oligosaccharides examined

Oligosaccharide	Calculated mass	Observed mass
Man 5	1235.2	1235.1
Man 6	1397.4	1396.9
Man 7	1559.5	1558.9
Man 8	1721.7	1721.0
Man 9	1883.8	1882.7
A/B-Gal	1479.3	1478.5
A/B	1641.5	1639.8
M/B-Gal	1770.6	1769.8
M/B	1932.7	1932.5
M/B + Fuc	2078.8	2079.3
(2,3) and (2,6) B/B	2224.2	2224.5
B/B + Fuc	2370.3	2370.6
M/T + Fuc + SO ₄	2540.3	2541.7
B/T + Fuc + SO ₄	2831.6	2832.9
(2,3) and (2,6) T/T	2880.9	2880.4
T/T + Fuc + SO ₄	3122.7	3121.6
(2,3) and (2,6) Te/T	3172.1	3171.4

Table 2
¹H chemical shifts of the significant structural reporter groups of the oligosaccharides used

Group	Residue	Anomer	Chemical shift (ppm)							
			Man 5	Man 6	Man 7	Man 8	Man 9	Biantennary	Triantennary	TetraTri
H-1	GlcNAc-1	α	5.191	5.187	5.190	5.187	5.191	5.189	5.185	5.187
		β	4.700	4.704	4.701	4.693	4.690	4.690	4.691	4.693
	GlcNAc-2	β	4.606	4.589	4.601	4.586	4.596	4.616	4.615	4.637
	Man-3	β	4.789	4.774	4.774	4.771	4.775	4.777	4.762	4.765
	Man-4	α	5.099	5.349	5.348	5.341	5.343	5.131	5.125	5.122
	Man-4'	α	4.875	4.870	4.874	4.870	4.869	4.949	4.908	4.901
	Man-A	α	5.093	5.090	5.091	5.085	5.414			
	Man-B	α	4.911	4.908	4.909	5.151	5.153			
	Man-C	α		5.050	5.310	5.309	5.317			
	Man-D1	α			5.057	5.042	5.047			
	Man-D2	α					5.047			
	Man-D3	α				5.042	5.047			
	GlcNAc-5	β						4.600	4.585	4.588
	GlcNAc-5'	β						4.570	4.571	4.598
	Gal-6	β						4.444	4.441	4.450
Gal-6'	β						4.547	4.441	4.445	
GlcNAc-7	β							4.545	4.569	
Gal-8	β							4.545	4.499	
H-2	Man-3	β	4.260	4.237	4.237	4.234	4.238	4.257	4.215	4.218
	Man-4	α	4.084	4.117	4.096	4.009	4.108	4.197	4.215	4.218
	Man-4'	α	4.152	4.149	4.147	4.149	4.161	4.118	4.116	4.108
	Man-A	α	4.074	4.066	4.071	4.069	4.108			
	Man-B	α	3.980	3.987	3.985	4.021	4.025			
	Man-C	α		4.066	4.100	4.099	4.108			
	Man-D1	α			4.071	4.069	4.074			
	Man-D2	α					4.074			
	Man-D3	α				4.069	4.074			
H-3ax	NeuAc (2,6)	α						1.721	1.721	1.725
	NeuAc (2,3)	α						1.800	1.801	1.803
	NeuAc + (2,6)	α								1.784
H-3eq	NeuAc (2,6)	α						2.667	2.668	2.670
	NeuAc (2,3)	α						2.756	2.755	2.731
	NeuAc + (2,6)	α								2.757
NAc	GlcNAc-1	α, β	2.044	2.041	2.043	2.037	2.042	2.040	2.036	2.057
	GlcNAc2	β	2.070	2.065	2.068	2.063	2.072	2.082	2.080	2.081
	GlcNAc-5	β						2.066	2.068	2.069
	GlcNAc-5'	β						2.066	2.044	2.046
	GlcNAc-7	β							2.076	2.069
	NeuAc (2,6)	α						2.028	2.036	2.028
	NeuAc (2,3)	α						2.028	2.036	2.028
	NeuAc + (2,6)	α								2.028
CH ₃	Fuc (1,6)	α						1.225		
H-5	Fuc (1,6)	α						4.128		

in this study were recorded at 300 MHz on a Varian Unity 300 NMR spectrometer at room temperature. Sample preparation and conditions were as previously described [11]. Table 2 presents the chemical shifts observed for the structural reporter groups of the compounds used in this study.

2.5. Oligosaccharide derivatization using 2-aminopyridine

Pyridylamination of reducing oligosaccharides was carried out in the absence of water from the reaction mixture using a modification of the methods of Suzuki et al. [18] and Kondo et al. [19]. The absence of water from the reaction mixture largely, but not entirely, eliminates the loss of sialic acid. Under these conditions we observed desialylation of up to 3%. A dried sample containing 10–100 nmol of carbohydrate was dissolved in 70 μ l of labeling reagent [prepared by mixing 1.0 g of 2-aminopyridine (Aldrich) in 100 μ l of acetic acid and 900 μ l of methanol]. The reaction vial was sealed and heated at 90°C for 1 h. Excess reagents were removed by evaporation at 150 mmHg (1 mmHg = 133.322 Pa) at 60°C. A 100- μ l volume of reducing agent [borane–dimethylamine complex (Aldrich), 50 mg, in 25 μ l acetic acid and 225 μ l methanol], prepared just prior to use, was then added. The reaction vial was resealed and heated at 80°C for 1 h. The resulting product was dried at 60°C at 150 mmHg. Once dried, 150 μ l of methanol were added and the sample was re-dried in vacuo at 60°C. This was repeated using toluene in place of methanol. These sequential methanol/toluene additions with drying were repeated a second time prior to analysis by CE.

2.6. Capillary electrophoresis

Separations were achieved using an Applied Biosystems Model 270A CE system modified for fluorescence detection [20]. The electroosmotic flow-assisted separations were achieved using a 200 mM sodium acetate buffer, pH 4.5,

with a 122 cm \times (100 cm to detector) 50 μ m fused-silica capillary. The applied field strength was 123 V/cm (7 μ A current) at 30°C. Detection was accomplished using a 230 nm excitation wavelength with a 370 nm emission filter (illuminating with a Xe arc lamp).

The borate complexation electrophoretic separations were achieved using a 500 mM sodium borate buffer, pH 8.5, containing 1% polyethylene glycol (PEG) (M_r 10 000), with a 122 cm \times (100 cm to detector) 50 μ m fused-silica coated capillary. Charge reversal coating of the capillary was achieved by flushing the capillary with 10 column volumes of the MicroCoat reagent supplied by Perkin-Elmer–Applied Biosystems Division (Foster City, CA, USA) prior to filling capillary with running buffer. Separations were then carried out using an applied field strength of –123 V/Cm (20 μ A current) at 30°C.

Third-dimension separations were achieved using a 50 mM sodium phosphate buffer, pH 2.5 with a 122 cm \times (100 cm to detector) 50 μ m fused-silica capillary. The applied field strength was 170 V/cm (25 μ A current) at 30°C.

Each new fused-silica capillary, prior to use, was pretreated with two column volumes of 1 M NaOH and washed with three column volumes of deionized water prior to filling it with its respective separation carrier.

3. Results and discussion

The mapping protocol developed by Suzuki et al. [8] used an acidic phosphate buffer system paired with an alkaline borate buffer system. This set of CE separation systems yielded a two-dimensional plot that distributed the three types of PA oligosaccharides examined (high mannose, hybrid and complex) into separate domains. The phosphate buffer system was well suited for direct zone electrophoresis, in the absence of electroosmotic flow, of the cationic ammonium ions of the PA-labeled oligosaccharides that do not contain negatively charged groups. In this system, neutral oligo-

saccharides (those that contain no sialic acid or sulfate), when labeled with PA, carry a net positive charge resulting in electrophoresis towards the cathodic end of the capillary. In contrast, if one or more sialic acid residues is/are present on a PA-labeled oligosaccharide, the net charge on the oligosaccharide is negative, as the PA group imparts only a partial positive charge at the pH used for this separation. Thus, the electrophoretic mobility of sialic acid-containing species would be directed toward the anodic end of the capillary. Therefore, using this buffer system one can analyze only those PA-labeled oligosaccharides that do not contain sialic acid residues or pendant sulfate groups.

In contrast to the sample limitations presented by the direct electrophoresis methodology discussed above, the borate buffer system described in that same paper [8] was suitable for indirect electrophoresis of the anionic borate complexes formed with PA-labeled oligosaccharides. Indirect CE as borate complexes has been shown to achieve separations based on structural variations among the oligosaccharides [8,21]. However, this system was not always able to resolve similar species within a particular mapping domain. Thus, to be able to potentially map all naturally occurring N-linked oligosaccharides derived from glycoproteins and labeled with PA, it was necessary to develop a different combination of buffer systems. This combination had to be capable of electrophoresis of PA-labeled oligosaccharides that contain one or more terminal sialic acid residues and/or sulfate groups. Additionally, it was desired that this new set of separation systems should collectively be able to resolve samples containing very similar structures. To accomplish this we combined three different buffer systems, each of which exhibited separation mechanisms that exploited different features of the derivatized oligosaccharides being mapped.

To address both neutral and charged oligosaccharides at acidic pH, electrophoresis was carried out using a sodium acetate buffer, pH 4.5. Electrophoresis was assisted by electroosmotic flow and thus allowed for the inclusion

of sialic acid-containing species, that otherwise would be carried in the opposite direction from PA-oligosaccharides not containing charged functionalities. In this system, the electroosmotic component propels all PA-oligosaccharides toward the cathode. However, the net negative charge of sialic acid or sulfate groups, when present on an oligosaccharide, more than offsets the label-induced partial positive charge on the species, thus changing the electrophoretic mobility of these oligosaccharides from cathodic to anodic. Therefore, while the net movement of all species is cathodic, migration is increasingly retarded with increasing degree of sialylation or sulfation of a given general structure type (e.g. biantennary structure with zero, one or two sialic acid residues pendant).

Using the system described above, separations of the various biantennary oligosaccharides released from transferrin are illustrated in Fig. 1. The predominant separation mechanism was based on charge differences between the variously sialylated and neutral oligosaccharide derivatives. While migration order was based primarily on the charge states of the oligosaccharides, this did not alone dictate migration characteristics. When sialic acid-containing species were separated, the corresponding structure containing only one galactosyl residue was not resolved from the monosialyl-biantennary structure containing two galactosyl residues (Fig. 1b). However, the neutral counterparts of these structures were at least partially resolved (Fig. 1a).

Indirect electrophoresis of PA-oligosaccharides as borate complexes, as described in the literature [8], was not able to resolve structurally similar oligosaccharides using only a fused-silica capillary and borate buffer. To achieve higher-resolution electrophoresis of borate complexes, we applied a charge-reversal coating to a fused-silica capillary. In addition, the borate buffer was supplemented with 1% (w/v) PEG 10 000 in order to reduce endosmotic flow. Borate complexation allows for separations to occur facilitated by the formation of negatively charged complexes with carbohydrates [22,23]. Complex formation is dependent on oligosaccharide

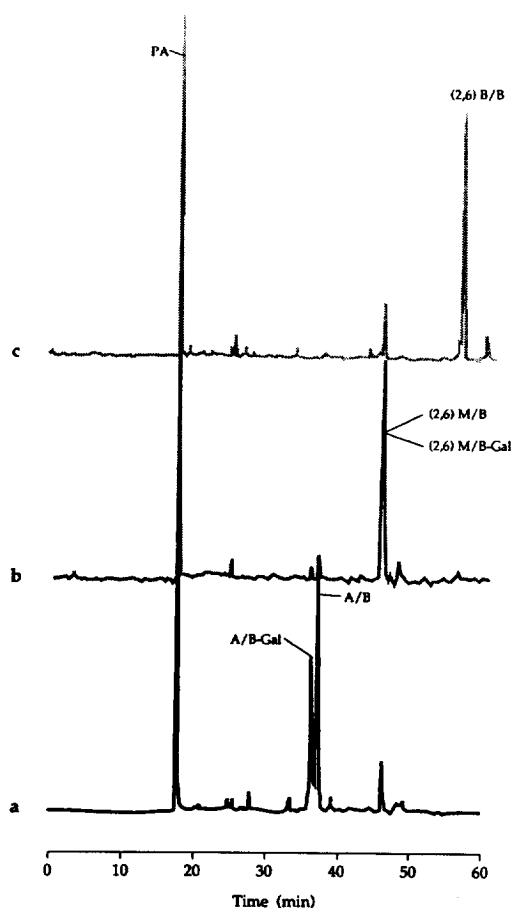


Fig. 1. CE separation of PA-labeled sialylated biantennary oligosaccharides in sodium acetate buffer. Highly enriched oligosaccharide fractions derived from human serotransferrin were separated individually by CE. The samples examined were: (a) asialyl-biantennary oligosaccharides containing one or two galactosyl residues (A/B and A/B-Gal; 1.3 and 2.0 ng, respectively); (b) monosialyl-biantennary oligosaccharides containing one or two galactosyl residues (M/B and M/B-Gal; 1.3 and 2.0 ng, respectively); and (c) a bisialyl-biantennary (B/B; 2.2 ng) structure. All structures are illustrated in Fig. 4. The endosmotic flow-assisted electrophoretic conditions are described under Experimental.

composition and structure. Thus, separations in borate are based partially on oligosaccharide structure and not purely on the mass-to-charge ratios calculable for the corresponding oligosaccharides not complexed by borate ion. The selectivity of the borate system used was therefore different from that of the sodium acetate buffer system. In the borate system, electroen-

dosmosis and electrophoresis work in concert with each other for all oligosaccharides; the electroosmotic component of the charge-reversal system propels the oligosaccharides, as their negatively charged oligosaccharide–borate complexes, toward the anode, while electrophoresis drives this migration toward the anode as well. To reduce the effect of electroosmotic flow in this system, 1% PEG was added to reduce, but not eliminate, electroosmosis. Thus all complexes and neutral species present in the sample mixture, including the PA, will eventually reach the anodically located detector. In this system the species with the highest charge-to-mass ratios will be detected first. Neutral species such as PA,

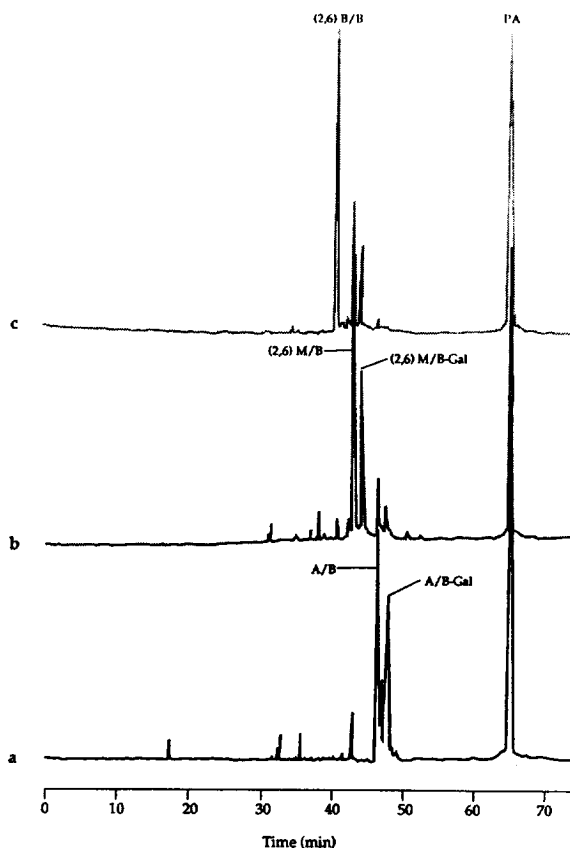


Fig. 2. CE separation of PA-labeled sialylated biantennary oligosaccharides in sodium borate buffer containing 1% PEG. The samples described in Fig. 1 were separated using a second CE dimension orthogonal to the sodium acetate system. The borate-complexation electrophoretic conditions used are described under Experimental.

however, will eventually reach the detector, due solely to the effect of endosmotic flow. The additional charge imparted on a borate-complexed oligosaccharide by the presence of sialic acid residues or sulfate groups further increases the electrophoretic component, thus increasing the rate of migration toward the detector (anodic end). The borate buffer system allowed separation of both sialylated and neutral oligosaccharides. Fig. 2 illustrates the separation of biantennary oligosaccharides from transferrin with varying degrees of sialylation. This system was, however, inefficient in resolving closely related high mannose structures (see boxed section in Fig. 3).

The relative migration values for all the confirmed oligosaccharide structures examined in this study are listed in Table 3. The values are expressed as a simple ratio of t_R (migration time of sample) to t_S (migration time of PA standard), to compensate for subtle differences in absolute migration times between runs. To estimate reproducibility for the technique, each individual sample was examined five different times. These runs were accomplished using five different combinations of three individual instruments, three individual buffer preparations and three individual capillaries. Thus, we were better able to assess the reproducibility of the methodology. The mean and relative standard deviation of the

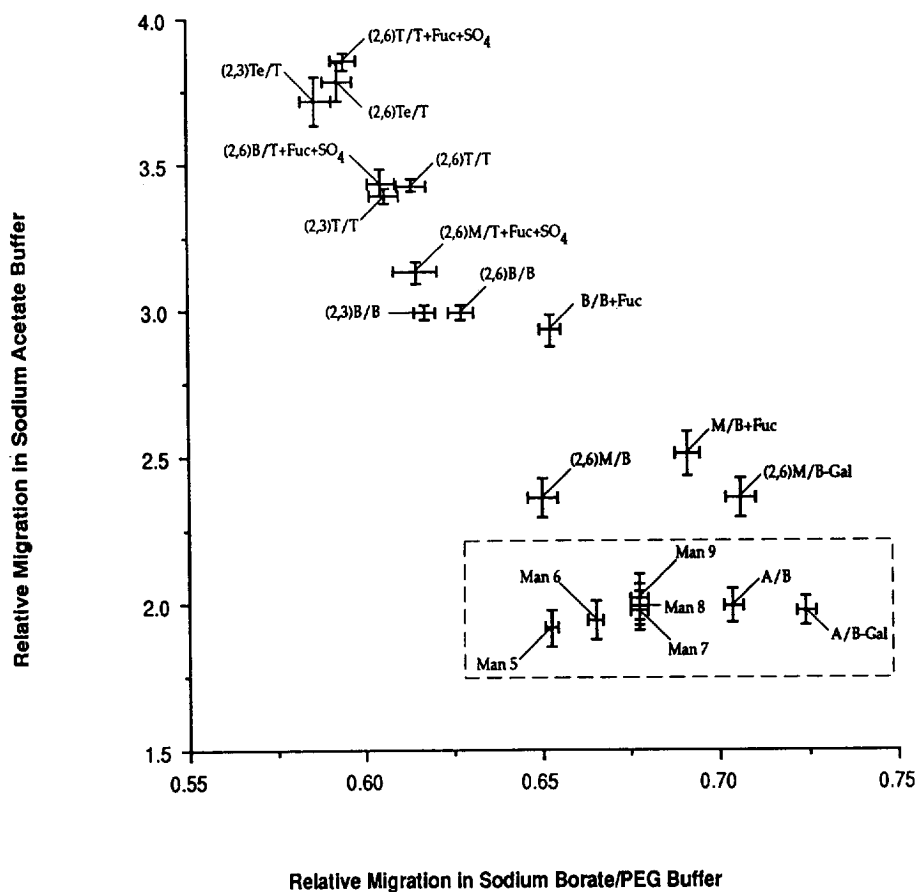


Fig. 3. Two-dimensional mapping of PA-labeled oligosaccharides by CE. The relative mobilities of the PA-oligosaccharides determined using the two separation systems illustrated in Figs. 1 and 2 are shown plotted against each other, resulting in a two-dimensional map. Means and standard deviations for each sample in each separation system were calculated as described in the text and are represented by error bars.

Table 3
Migration data for the PA-labeled oligosaccharides analyzed by CE

Sample ^a	Migration relative to PA (PEG–borate)		Migration relative to PA (sodium acetate)		Migration relative to PA (sodium phosphate)	
	Mean	R.S.D. (%)	Mean	R.S.D. (%)	Mean	R.S.D. (%)
Man 5	0.6524	0.0018	1.9146	0.0633	3.8468	0.0129
Man 6	0.6650	0.0022	1.9432	0.0678	4.0570	0.0150
Man 7	0.6772	0.0023	1.9741	0.0680	4.2673	0.0156
Man 8	0.6772	0.0023	1.9957	0.0684	4.4318	0.0174
Man 9	0.6772	0.0023	2.0205	0.0762	4.5565	0.0183
A/B-Gal	0.7240	0.0027	1.9761	0.0514	4.2695	0.0097
A/B	0.7036	0.0027	1.9914	0.0559	4.4438	0.0109
(2,6)M/B-Gal	0.7058	0.0043	2.3577	0.0683		
(2,6)M/B	0.6504	0.0040	2.3577	0.0683		
(2,3)B/B	0.6175	0.0031	2.9936	0.0245		
(2,6)B/B	0.6277	0.0036	2.9936	0.0245		
(2,3)T/T	0.6062	0.0040	3.3939	0.0253		
(2,6)T/T	0.6138	0.0040	3.4291	0.0242		
(2,3)Te/T	0.5867	0.0043	3.7159	0.0803		
(2/6)Te/T	0.5929	0.0041	3.7830	0.0646		
M/B + Fuc	0.6909	0.0036	2.5049	0.0754		
B/B + Fuc	0.6525	0.0029	2.9314	0.0555		
(2,6)M/T + Fuc + SO ₄	0.6149	0.0062	3.1300	0.0397		
(2,6)B/T + Fuc + SO ₄	0.6052	0.0039	3.4359	0.0448		
(2,6)T/T + Fuc + SO ₄	0.5942	0.0034	3.7884	0.0288		

^a Abbreviations are for structures indicated in Figure 4.

five runs were calculated for each oligosaccharide listed. Fig. 3 shows a two-dimensional map combining the data from the endosmotic flow-assisted CE using sodium acetate buffer and charge-reversal borate complexation electrophoresis. The structures of the oligosaccharides studied are shown in Fig. 4. These structures include neutral and charged species containing not only sialic acid, but pendant sulfate groups as well.

As noted above, some closely related structures were not completely resolved by either the acetate or borate buffer systems (boxed section in Fig. 3). These unresolved PA-oligosaccharides are neutral and could be separated using a third dimension orthogonal to those previously detailed. A low-pH direct electrophoresis system [8] proved well suited for such a separation. As shown in Fig. 5, the PA-labeled high-mannose

structures isolated from ribonuclease *b* were effectively separated using a sodium phosphate buffer, pH 2.5, with an uncoated fused-silica capillary. In such a system, electrophoresis occurs with minimal electroosmotic flow. This electrophoretic system complements the endosmotic flow-assisted electrophoretic system which allowed for separations dominated by charge state differentials. It also differs markedly from the borate system which resolved carbohydrates on the basis of their ability to form borate complexes. This third system comes closest to achieving separations strictly on the basis of molecular size [8].

The map shown in Fig. 6 plots the relative mobilities of the previously poorly resolved PA-oligosaccharides in the sodium phosphate system against mobilities of these molecules in the sodium acetate system. The mean and standard

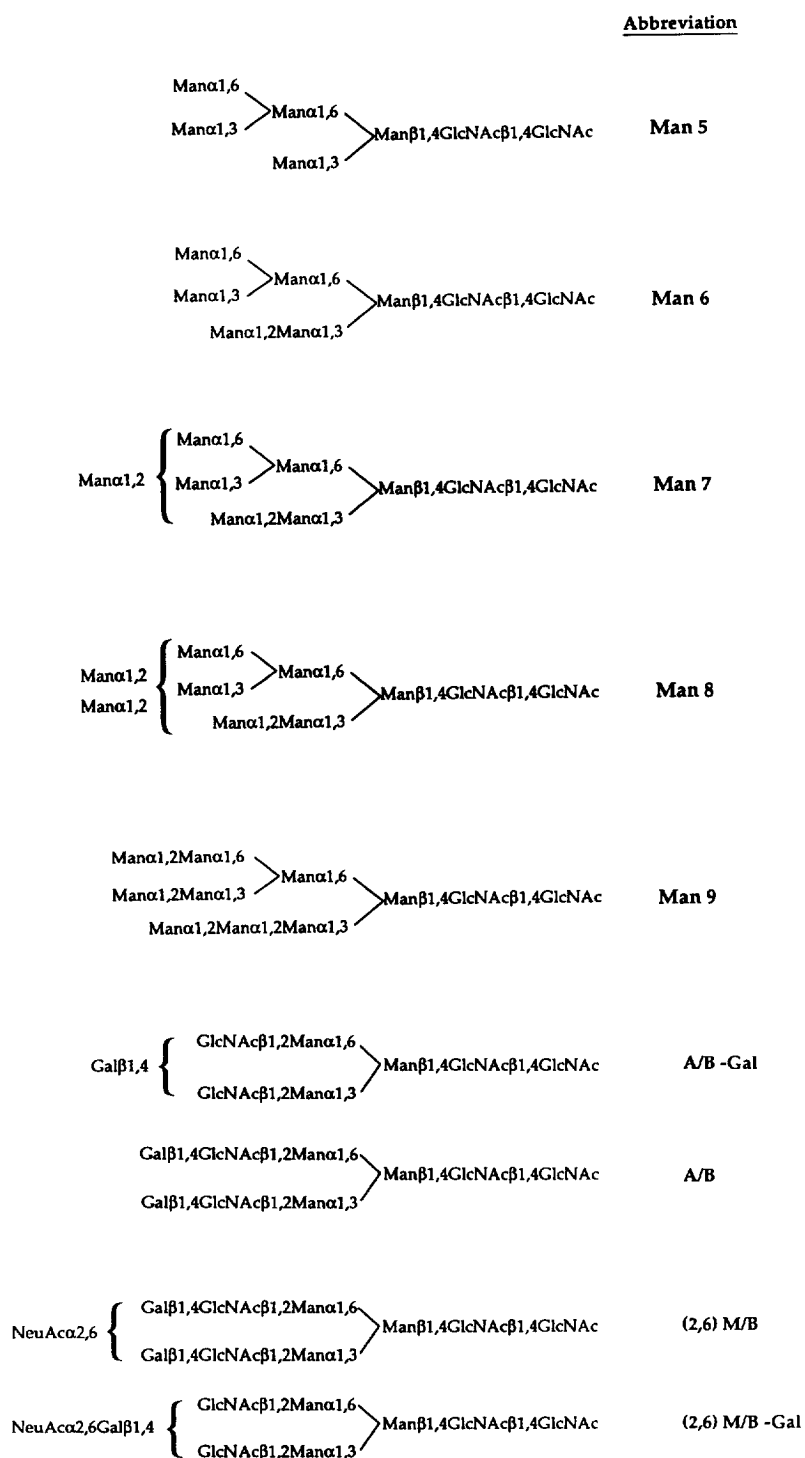


Fig. 4 (continued on p. 404).

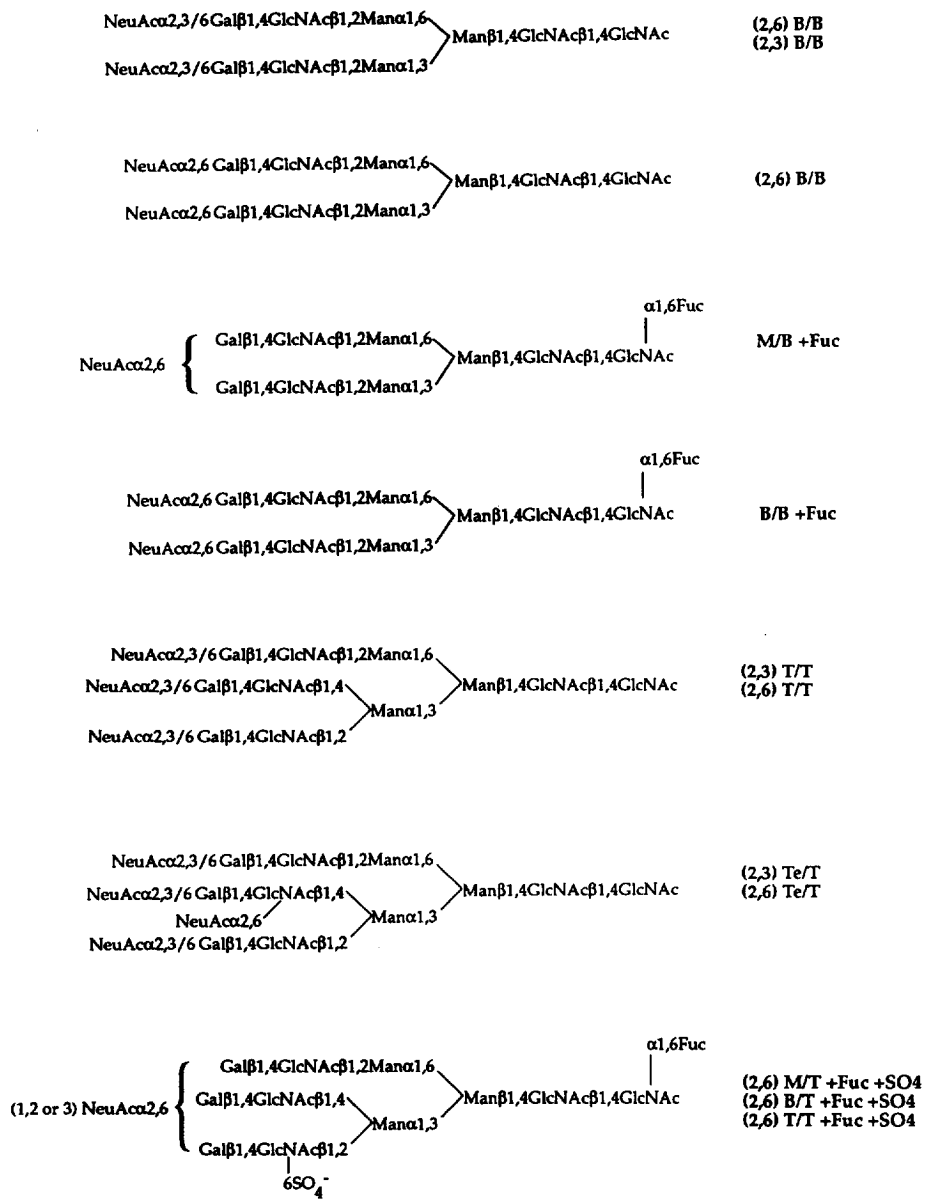


Fig. 4. Oligosaccharide structures. The structures shown (with their respective abbreviations) were used throughout this investigation. Their identity has been verified using composition analysis, MALDI-TOF-MS and ¹H NMR, as described.

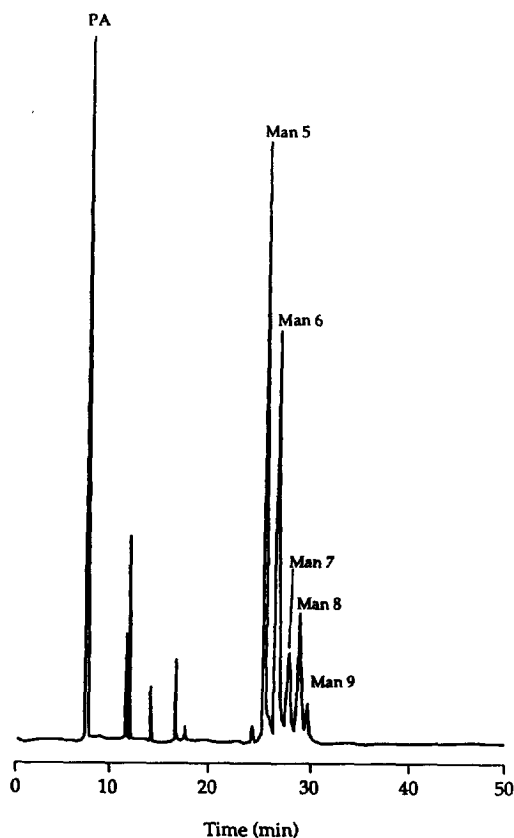


Fig. 5. CE separation of high-mannose oligosaccharides isolated from ribonuclease *b* (sodium phosphate buffer). A sample of high-mannose oligosaccharides isolated from ribonuclease *b* was separated in a third dimension orthogonal to the sodium acetate and PEG–borate systems. Electrophoretic conditions are described under Experimental.

deviation for each point were determined as described above. The orthogonality of this system compared to the previous two is illustrated by observed changes in elution order for some structures (e.g. Man 7 and A/B-Gal).

4. Conclusions

The described method of multi-dimensional mapping by CE of the relative migration values of oligosaccharides to identify these glycopro-

tein-derived structures has been demonstrated to be simple and reproducible. It is based on the use of a combination of orthogonal separations that each compensate for the other's limitations, and complement each other in their selectivities. The need for multiple orthogonal separations arises from the limitations in the resolution and selectivity of any single separation system. Davis [24] demonstrated theoretically that the certainty of analyte identification based on analytical separations increases geometrically with the number of orthogonal separation systems employed.

We have demonstrated a three-dimensional separation system using a combination of electroendosmotic flow-assisted CE, borate complexation CE, and a third dimension that emphasizes differences in mass. When plotted against one another the first two systems result in a scattered pattern indicating a high degree of orthogonality between the two separation systems (such a plot for highly similar separation systems results in a nearly linear array of mapping positions that runs diagonally across the plane of the graph).

Even though there is a high degree of orthogonality with this combination of two separation systems, not all classes of oligosaccharides could be well resolved. In the case of several of the neutral oligosaccharides studied, it was necessary to add a third dimension, where the separation was based primarily on differences in mass. Thus, all the neutral and charged oligosaccharides used in this study were resolved when using a combination of the three separation systems described.

It is proposed that multi-dimensional mapping of the relative migrations of oligosaccharides can become a valuable approach to the analysis of glycoprotein-derived oligosaccharides, requiring minimal quantities of analyte. The data presented suggest that identification could be assigned to a given oligosaccharide, with a high degree of confidence, by plotting its various migrations in multiple, orthogonal CE separation systems. CE is well suited for this multi-dimensional analysis approach due to the ease of switching from one separation system to another by simply re-filling a capillary with a new buffer.

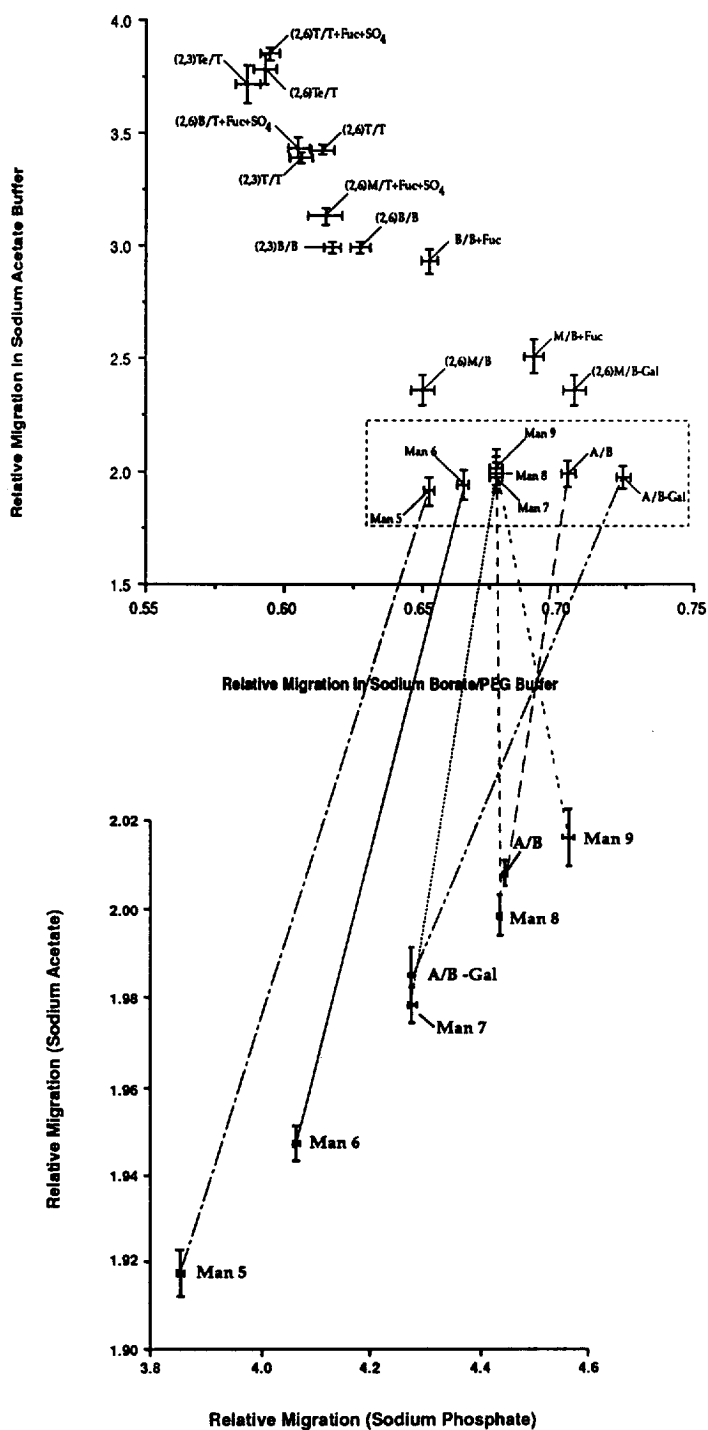


Fig. 6. Third-dimension (sodium phosphate buffer) mapping of PA-labeled oligosaccharides by CE. The relative mobilities of the high-mannose PA-oligosaccharides in the third dimension (shown in Fig. 5) are shown plotted against the sodium acetate dimension, resulting in a third-dimension map. Means and standard deviations for each sample in each separation system were calculated as described in the text and are represented by error bars.

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