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Capillary electrophoretic examination of underivatized O-linked and N-linked oligosaccharide mixtures and immunoglobulin G antibody-released oligosaccharide libraries

David Emlyn Hughes

Pharmaceutical Research Institute, Bristol–Myers Squibb Company, P.O. Box 4755, Syracuse, NY 13221-4755, USA

Abstract

A procedure for the analysis of mixtures of underivatized, cleaved O-linked and N-linked oligosaccharides by capillary electrophoresis has been developed. The species of interest are separated by two borate-based buffer systems with an uncoated capillary. The procedure is applied to hydrazinolysis-released oligosaccharides from mouse, rat, sheep and human IgG protein samples which are examined within 6 minutes. Selectivity with respect to sample variation is demonstrated by analysis of thermally stressed samples.

1. Introduction

Oligosaccharides are polyacetals that are comprised of typically 2–10 monosaccharide units. This class of carbohydrates is found in a large range of biological systems with functions ranging from plant differentiation to cell adhesion, proteolytic resistance, hormone–receptor binding and lymphocyte migration. Oligosaccharides are often found in nature associated with proteins through O-glycosidic or N-glycosidic bonds or lipids through an O-glycosidic bond. The release of the oligosaccharides from a protein or lipid (to form a library) by hydrazinolysis, enzymatic protocols, or other means presents a chemically heterogenous sample for analytical examination. A variety of methods have been employed to examine oligosaccharide mixtures, including ion-exchange chromatography with pulsed amperometric detection, derivatization followed by gas chromatographic analysis, liquid chromatography with low-wavelength detection, and enzyme/lectin procedures [1].

Due to the multiplicity of species and often severely limited sample availability, a highly selective and highly mass-sensitive analytical technique is desirable. Capillary electrophoresis (CE) with capillary efficiencies of 10^4–10^6 plates/m and a mass-sensitivity advantage [2] of at least 10^3 over reference ion chromatographic–pulsed amperometric procedures, is an ideal candidate technique. In light of the above discussion, it might be surmised that successful procedures for the separation of oligosaccharides would be plentiful. It is in fact the very promise of CE analysis, namely high selectivity and high mass sensitivity, which has been difficult to achieve experimentally.

In the most straightforward case to interpret, oligosaccharide-associated species (proteins in these examples) are examined by CE, which is often followed by removal of oligosaccharides and reexamination. Removal of oligosaccharides or sialic acid residues is expected to alter the total net charge of the protein thereby changing the electrophoretic mobility. Hence it was found

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that when recombinant human tissue plasminogen activator (rhtPA) was analyzed by CE that a number of species were present, indicating carbohydrate-mediated microheterogeneity [3]. These species, or glycoforms, were not present after treatment with neuraminidase; complimentary results were produced by capillary isoelectric focusing. Method optimization studies have been performed on recombinant human erythropoietin (rhEPO) glycoforms without subsequent desialylation [4]. In a second study on rhtPA, CE examination procedures for the native protein, glycopeptides resulting from tryptic digestion, and the associated oligosaccharides were developed [5].

Having shown that oligosaccharide microheterogeneity can be inferred by studies with the native and carbohydrate-modified proteins, separations of the cleaved oligosaccharides themselves have been reported [6]. To address the low absorptivity of carbohydrates and resulting low mass sensitivity, some authors derivatize the species of interest prior to CE separation, typically in a pH 9–10.5 borate buffer [7–9]. Derivatization followed by laser-induced detection has also been used to increase sensitivity [10]. By use of a basic borate buffer, selectivity is also enhanced by formation of charged borate–carbohydrate complexes.

Alkaline earth complexes of carbohydrates, which show a different selectivity than borate complexes, have also been investigated [11]. Other attempts to increase selectivity have been pursued by dramatically increasing the separation efficiency with higher capillary temperatures [12] and by the addition of diaminobutane [5,13]. With selectivity optimization and 190 nm detection, Hermentin *et al.* [2] were able to show CE results comparable to those obtained by ion-exchange chromatography with pulsed amperometric detection. The feasibility of pulsed amperometric detection for CE has been reported [13].

The examination of oligosaccharides cleaved from proteins for the purpose of "fingerprinting" or establishing the suitability of the cell culture conditions for glycoprotein production presents unique analytical constraints. An ideal procedure would be fast and yet sufficiently specific to discern changes in oligosaccharides resulting from altered culture conditions. Fast analysis times preclude most derivatization schemes. A selectivity issue, namely that oligosaccharides produced outside of the validated pool may not derivatize and hence may not be detected, also argues against derivatization. Procedures that produce modest resolution advantages at the expense of total migration (run) times would similarly be unsuitable. This paper presents procedures for the examination of underivatized complex oligosaccharide mixtures in borate and modified borate buffers with analysis times of 4–6 min/sample.

2. Experimental

2.1. Materials

Boric acid and sodium borate crystals were obtained from Fisher Chemical (Fair Lawn, USA) Z1-Methyl reagent (trimethylammoniumpropanesulfonate, TMAPS) was purchased from Waters Assoc. (Milford, MA, USA). Pretreated capillary cartridges were purchased from Beckman Instruments (Palo Alto, CA, USA). Lacto-N-difucohexaose I, 2′-fucosyllactose, LS-tetrasaccharide a, 3′-sialyllactose, disialyllacto-N-tetraose, and disialyltetraose milk-derived O-linked oligosaccharides were obtained from Oxford GlycoSystems (Abingdon, UK). Also obtained from Oxford GlycoSystems were N-linked reference panels: (1) oligomannose-type containing oligomannose 5-oligomannose 9 (Man 5–Man 9); (2) complex panel 1 containing asialo-, galactosylated biantennary (NA2); asialo-, galactosylated biantennary, core-substitued with fucose (NA2F); asialo-, galactosylated biantennary with bisecting N-acetyl D-glucosamine (NA2B); and asialo-, galactosylated biantennary, core-substituted with fucose and bisecting N-acetyl D-glucosamine (NA2FB); (3) complex panel 2 containing asialo, agalacto-, biantennary (NGA2); asialo-, agalacto-, biantennary, core-substituted with fucose (NGA2F); asialo-, agalacto-, biantennary (NGA2B); and asialo-,

agalacto-, biantennary, core-substituted with fucose and with bisecting N-acetyl D-glucosamine; and (4) hydrazinolysis-released oligosaccharides from mouse, rat, sheep, and human IgG glycoproteins. Water from a Milli-Q filtration system (Waters) was used in the preparation of the buffer solutions.

2.2. Capillary electrophoresis system

A Beckman P/ACE 2100 CE system using an IBM PS/2 with P/ACE software and Microsoft Windows interface was used throughout this study.

2.3. Capillary electrophoresis procedures

The CE separation was performed using either 52 m*M* sodium borate, 1 *M* TMAPS, pH 9.38 buffer (procedure 1) or a 35 m*M* sodium borate, 130 m*M* boric acid, pH 8.35 buffer (procedure 2). Samples (diluted to 200–1000 μg/ml with water) were transferred to P/ACE micro vials contained in sample holders, and applied to the capillary as described below. Upon completion of each sample analysis, the capillary column was washed with 0.1 *M* sodium hydroxide solution, followed by a wash with separation buffer.

A pretreated capillary cartridge (50 cm × 75 μm I.D.) from Beckman was used in the separation. Samples were injected by a positive nitrogen pressure of 90 p.s.i. (1 p.s.i. = 6894.76 Pa) for 5 s and detected at 200 nm. The samples were then separated by a voltage of 20 kV for procedure 1 above or 30 kV for procedure 2 at 35°C.

Thermally degraded samples were prepared by placing *ca.* 200 μl of 1 mg/ml oligosaccharide solution in a 120°C oven for 30 min.

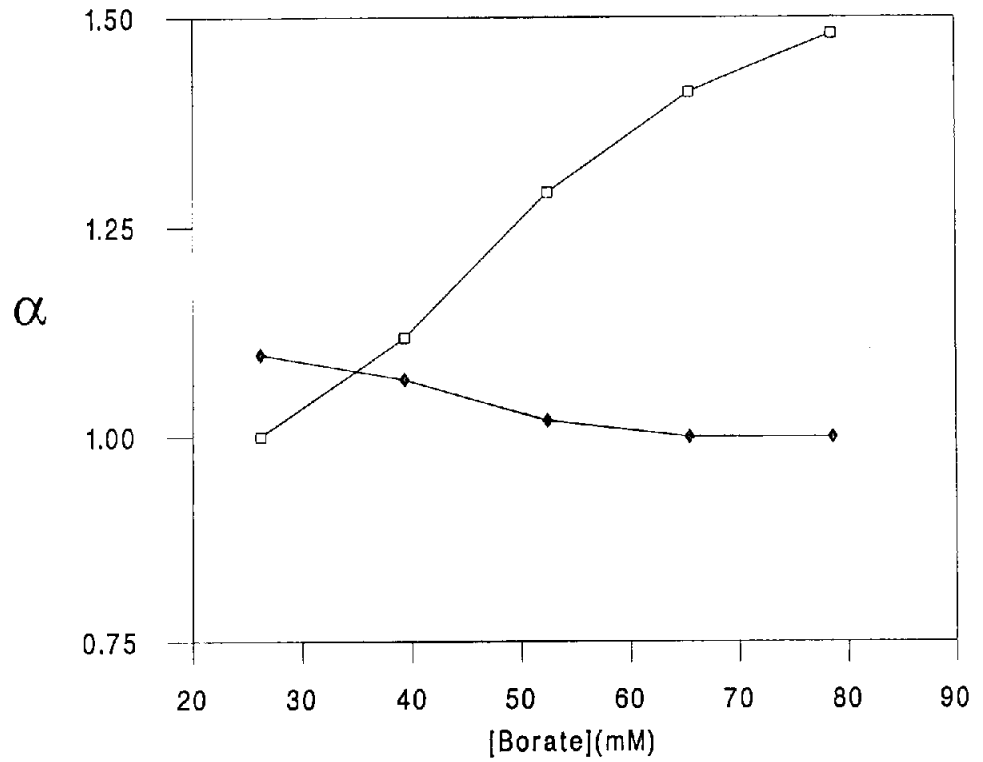

Fig. 1. Dependence of separation of six O-linked oligosaccharides (Table 1) on sodium borate concentration. □ = Neutral INDFH I; ◆ = disialylated DSLNT.

3. Results and discussion

The intention of method studies for CE analysis of oligosaccharide mixtures was to ascertain whether a relatively fast (less than 10 min) procedure with adequate selectivity could be developed. Such a procedure could then be used as a substitute or complementary method to the more well established ion chromatography–pulsed amperometric assays. The potential advantages of a CE procedure, when compared with ion chromatography–pulsed amperometric detection, include substantially less sample consumed by analysis, non-destructive analysis with the possibility of direct mass spectrometric interfacing, and more specific neutral oligosaccharide analysis. A method that could quickly examine complex oligosaccharide mixtures released from recombinant glycoproteins would yield valuable information for evaluating cell culture conditions over an entire production lifetime. It may be somewhat surprising that such an apparently straightforward analytical inquiry as to the existence of a single, general oligosaccharide analysis has not been definitively answered. The answer may be in part that much of what is known about CE oligosaccharide analysis is methodology to optimize derivatized, as opposed to unmodified, carbohydrates [14].

As was previously mentioned, the likelihood of encountering new (and hence analytically unvalidated) species during cell culture evaluation suggests that a reasonably non-specific form of detection is preferred. Hence low-wavelength detection of an underivatized sample, if the analysis could be performed with sufficient sensitivity, would yield data preferable to data produced by sample derivatization. Other detection techniques, such as indirect fluorescence [15], are similarly attractive since they examine unmodified species. Separatory requirements would include sufficient specificity to perform the

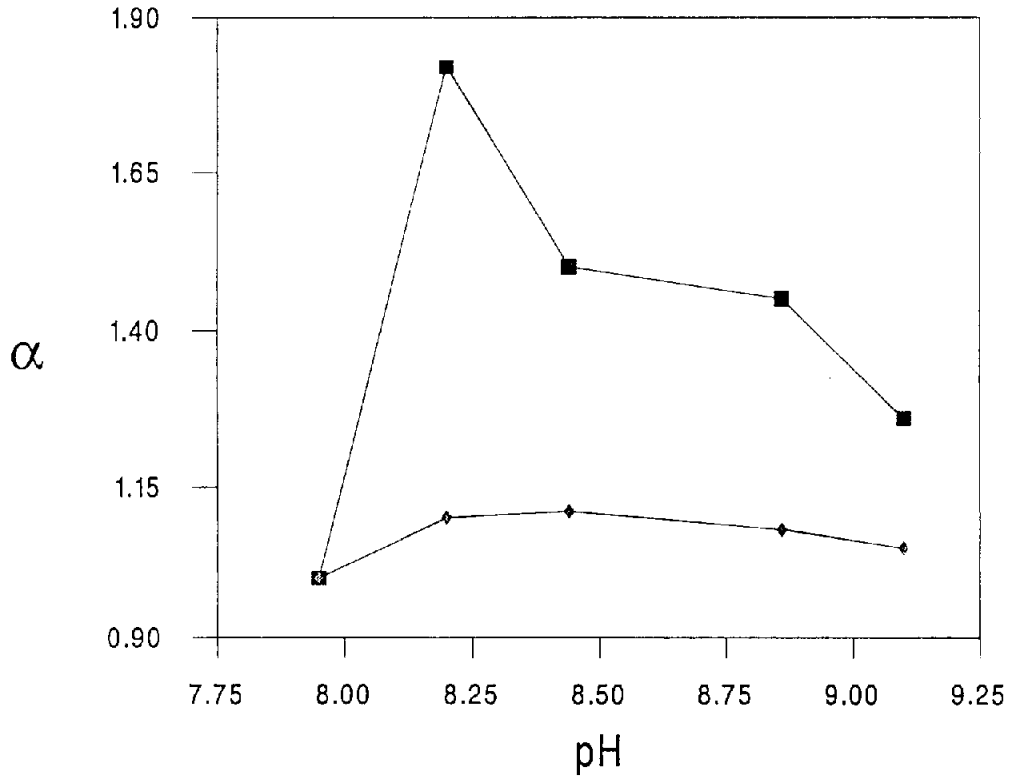

Fig. 2. Dependence of separation of six O-linked oligosaccharides (Table 1) on pH. ■ = Neutral INDFH I; ◆ = disialylated DSLNT.

analysis in the shortest possible time to accommodate a large throughput of samples for analysis.

The initial method optimization studies were performed on a general borate–additive system to attempt to converge to a single procedure which would demonstrate sufficient specificity with standard oligosaccharides to warrant cell culture sample analysis. Borate buffers have been used previously in sugar separation studies and it has been postulated that borate–sugar complexes are formed that increase the total net charge and hence the ionic mobility of the *in situ* derivatized species. There is also evidence that these complexes absorb 190–200 nm light more readily than unassociated sugars and hence yield a lower quantitative analytical range [12]. When borate concentrations were examined in the range of 26–79 m*M* sodium borate, collaborating evidence for oligosaccharide–borate complexes was found. The separation factor α was plotted for neutral oligosaccharide INDFH I (species 1) and disialylated oligosaccharide DSLNT (species 5) with respect to the nearest electrophoretic peak. Four other standard oligosaccharides were also included in the standard mixture (see Table 1). The resulting trends appear in Fig. 1 where it is seen that the separation between neutral species is enhanced by increased borate concentration whereas sialylated species are less well separated. The pH of the buffer was also varied from 7.95 to 9.10 with boric acid and the results appear in Fig. 2. It is seen that in the absence of the modifier TMAPS, neutral species are better separated at the lower end of the pH range investigated whereas the sialylated species are relatively unaffected. A third study (Fig. 3) with the additive TMAPS concentration varied from 0.25 to 1.0 *M* yielded no effect for the sialylated species, but resulted in an increase in separation

Table 1
Neutral and sialylated O-glycosidic linked oligosaccharides derived from human milk and urine

Species No.	Name	Abbreviation	Structure
1	Lacto-N-difucohexaose I	INDFH I	Galβ1–3GlcNAc–3Galβ1–4Glc 2 4 | | Fucα1 Fucα1
2	2′-Fucosyllactose	2′-FL	Fucα1–2Galβ1–4Glc
3	LS-tetrasaccharide a	LSTa	Galβ1–3GlcNAcβ1–3Galβ1–4Glc 3 | NeuNAcα2
4	3′-Sialyllactose	3′-SL	NeuNAcα2–3Galβ1–4Glc
5	Disialyllacto-N-tetraose	DSLNT	Galβ1–3GlcNAcβ1–3Galβ1–4Glc 3 6 | | NeuNAcα2 NeuNAcα2
6	Disialyltetraose	DST	NeuNAcα2–3Galβ1–3GalNAc 6 | NeuNAcα2

Gal = D-galactose, GlcNAc = N-acetyl D-glucosamine, Glc = glucose, Fnc = fucose, NeuNAc = N-acetyl neuraminic acid, GalNAc = N-acetyl D-galactosamine.

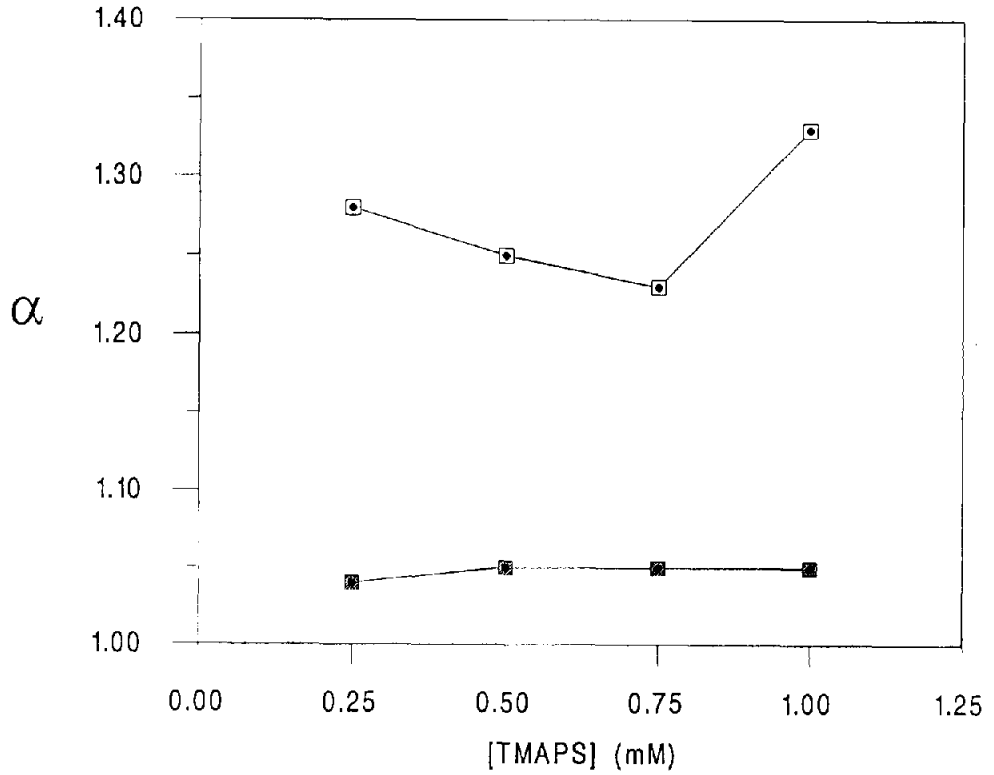

Fig. 3. Dependence of separation of six O-linked oligosaccharides (Table 1) on TMAPS concentration. □ = Neutral INDFH I; ■ = disialylated DSLNT.

factor for neutral species near 1 *M* TMAPS, the concentration recommended by the manufacturer.

The result of the initial method development studies was to suggest that the relative abundance of neutral and sialylated species would dictate different optimal separatory conditions. Two separatory conditions were then investigated further with sample analysis. A pH 9.35, 52 m*M* borate, 1 *M* TMAPS buffer (procedure 1) was chosen as a candidate method for oligosaccharide mixtures for which separation of multiple neutral species was important. A second buffer containing 35 m*M* borate, 130 m*M* boric acid, pH 8.35, no additive (procedure 2) was chosen to optimize the separation of sialic acid containing species. Electropherograms of the six O-linked standard oligosaccharides (see Table 1) separated by procedures 1 and 2 described above appear in Fig. 4A and B, respectively.

To demonstrate the ability of procedure 1 to separate mixtures of neutral oligosaccharides with superior resolution to procedure 2, several N-linked neutral oligosaccharide mixtures were examined. In Figs. 5–7, commercially available mixtures of N-linked neutral oligosaccharides released from sheep IgG antibodies were examined by procedures 1 and 2. Although individual identification of each species was not performed, the number and resolution of species present suggest in each case that procedure 1 was more promising for neutral oligosaccharide-rich mixtures than procedure 2. Examination of oligosaccharide samples resulting from the hydrazinolysis of sheep, human, mouse and rat IgG antibodies were then performed. The resulting electropherograms appear in Figs. 8 and 9.

Procedure 2 was then used to examine the sheep, human, mouse and rat IgG oligosaccharide samples. The resulting electropherograms appear in Figs. 10A, 11A, 12A, 13A. Comparison of Figs. 8, 9 and 10A–13A does not support the supposition made on the basis of N-linked neutral standard studies (Figs. 5–7)

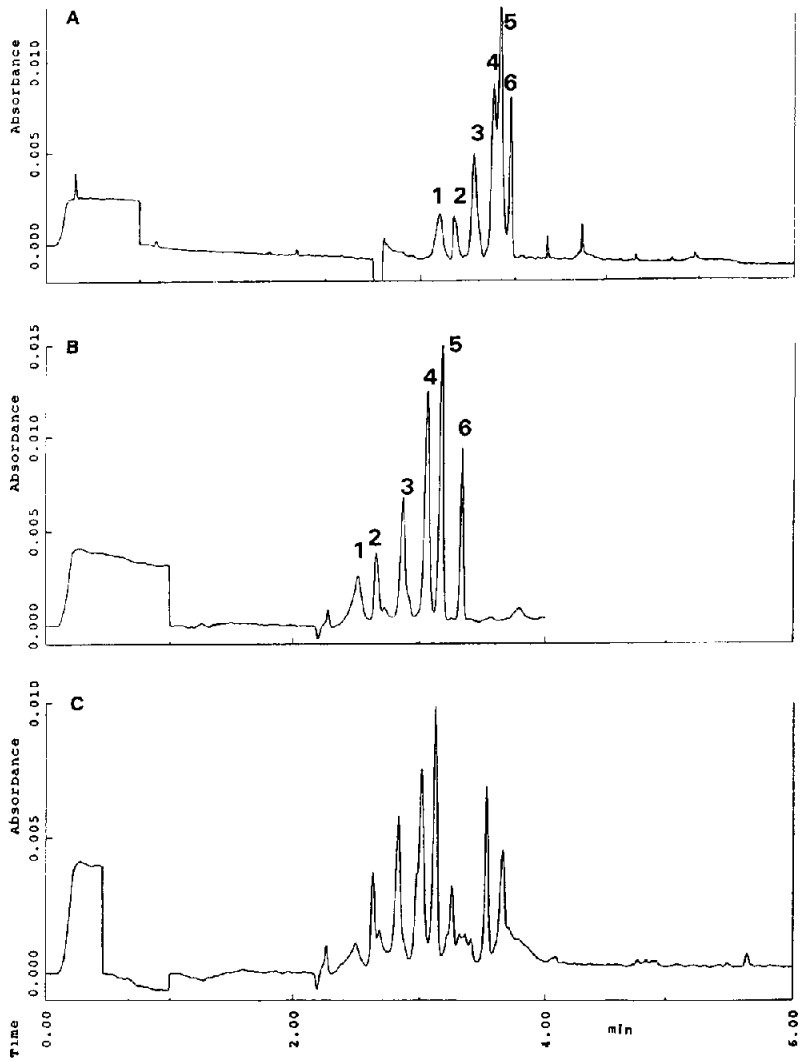

Fig. 4. Separation of six O-linked oligosaccharides (see Table 1 for identification). Conditions: (A) procedure 1: 52 m*M* sodium borate, 1 *M* TMAPS, pH 9.38; (B) procedure 2: 35 m*M* sodium borate, 130 m*M* boric acid, pH 8.35, 20 kV; and (C) 35 m*M* sodium borate, 130 m*M* boric acid, pH 8.35, 30 kV (procedure 2), sample degraded at 120°C for 30 min.

that samples will of necessity show better resolution of neutral species by procedure 1. It does appear that a rather wide set of separation conditions may be employed to examine the IgG-released oligosaccharides.

In order to evaluate the ability of procedure 2 to distinguish between similar mixtures of species, the sheep, human, mouse and rat IgG-released oligosaccharides were examined (Figs. 10A–13A), thermally degraded for 30 min at 120°C, and reexamined (Figs. 10B–13B). Under these conditions, the O-linked standard mixture was clearly degraded (Fig. 4C). The IgG-derived oligosaccharide mixtures showed varying sensitivities to the thermally induced changes. Whereas the sheep and human oligosaccharide electropherograms indicate significant sample alteration after heating, the rat and mouse sample electropherograms show less dramatic changes.

In summary, a wide range of separation conditions within the sodium borate buffer system yielded potentially useful results for the examination of underivatized N-linked and O-linked

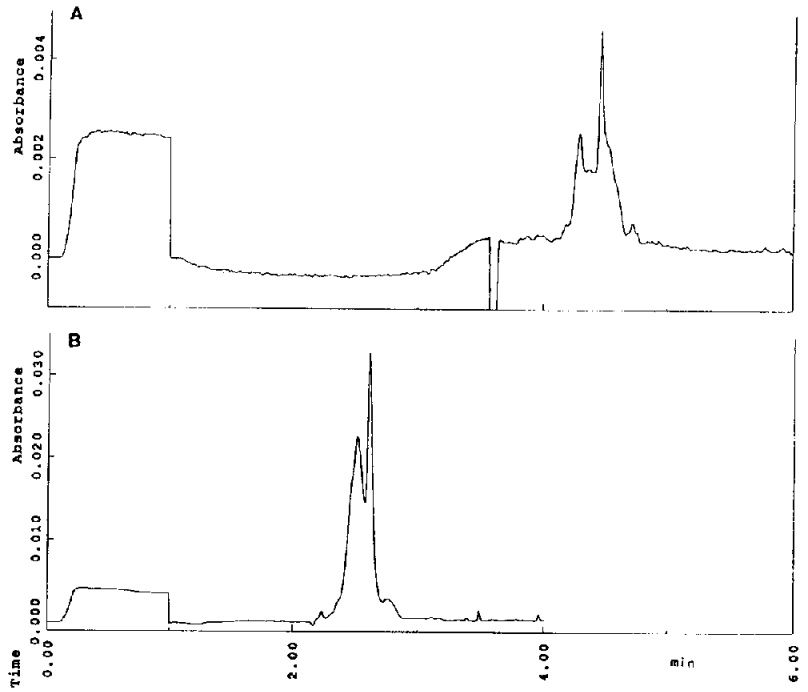

Fig. 5. Separation of neutral N-linked mannose series (5–9) oligosaccharides from ribonuclease B, by procedure 1 (A) and procedure 2 (B), see Fig. 4.

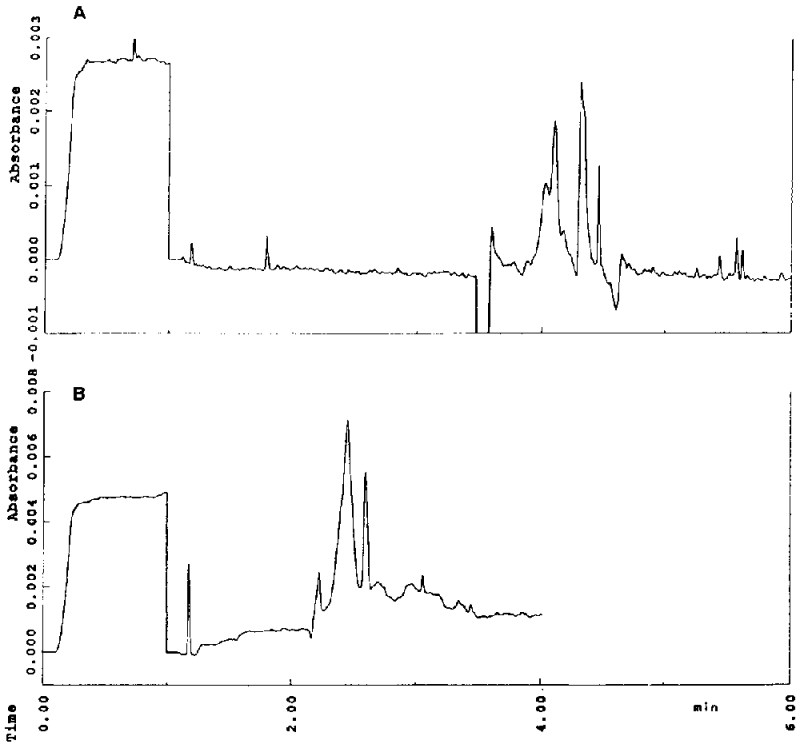

Fig. 6. Separation of neutral N-linked oligosaccharides (panel 1: NA2, NA2F, NA2B and NA2FB) from sheep IgG, by procedure 1 (A) and procedure 2 (B), see Fig. 4.

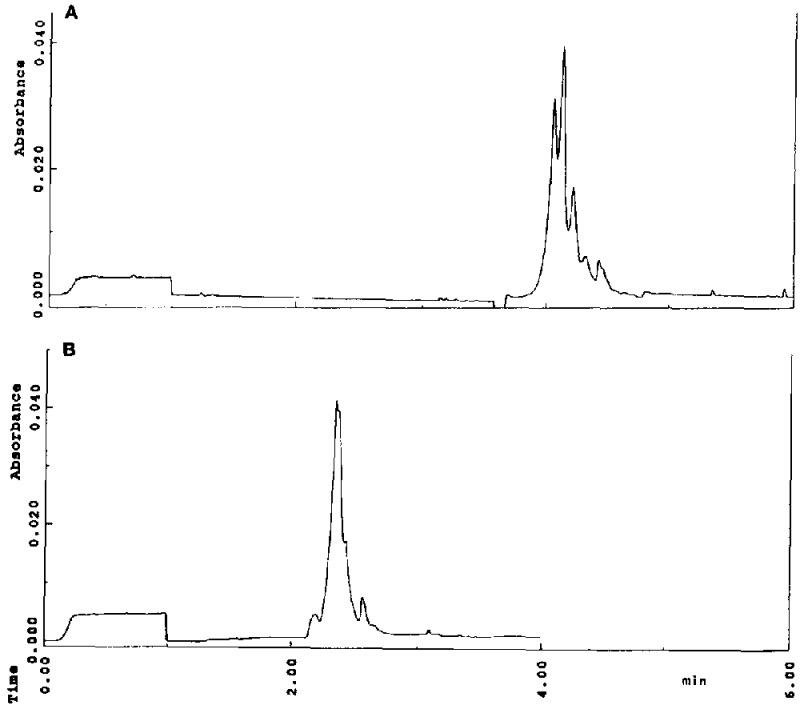

Fig. 7. Separation of neutral N-linked oligosaccharides (panel 2: NGA2, NGA2F, NGA2B and NGA2FB) from sheep IgG, by procedure 1 (A) and procedure 2 (B), see Fig. 4.

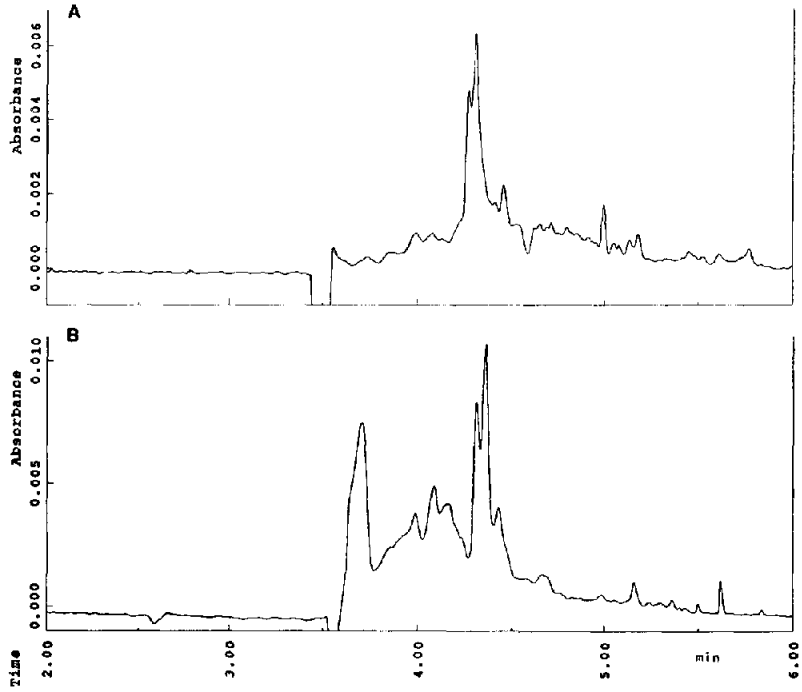

Fig. 8. (A) Separation of oligosaccharides from sheep IgG by separation procedure 1, see Fig. 4. (B) Separation of oligosaccharides from human IgG by procedure 1, see Fig. 4.

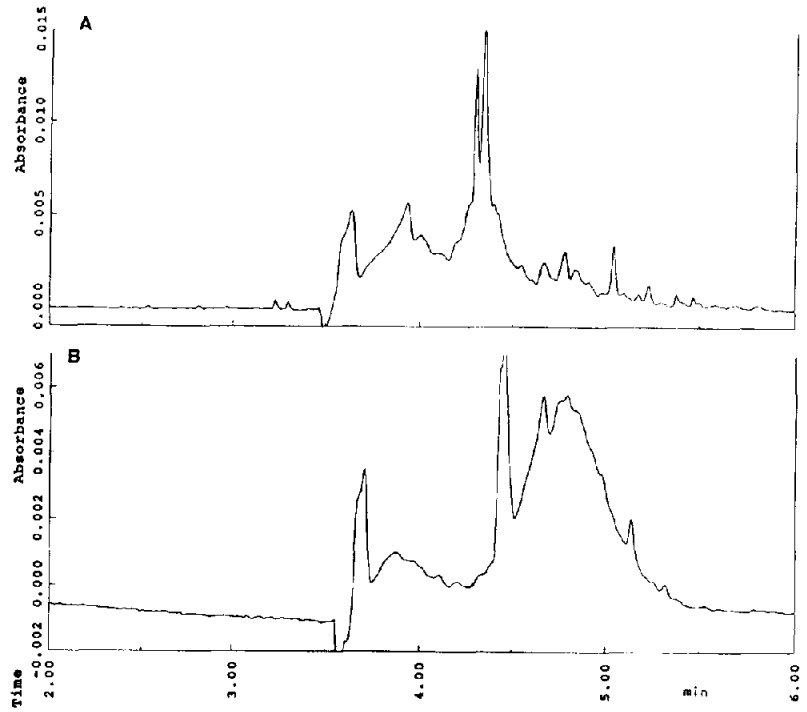

Fig. 9. (A) Separation of oligosaccharides from rat IgG by procedure 1, see Fig. 4. (B) Separation of oligosaccharides from mouse IgG by procedure 1, see Fig. 4.

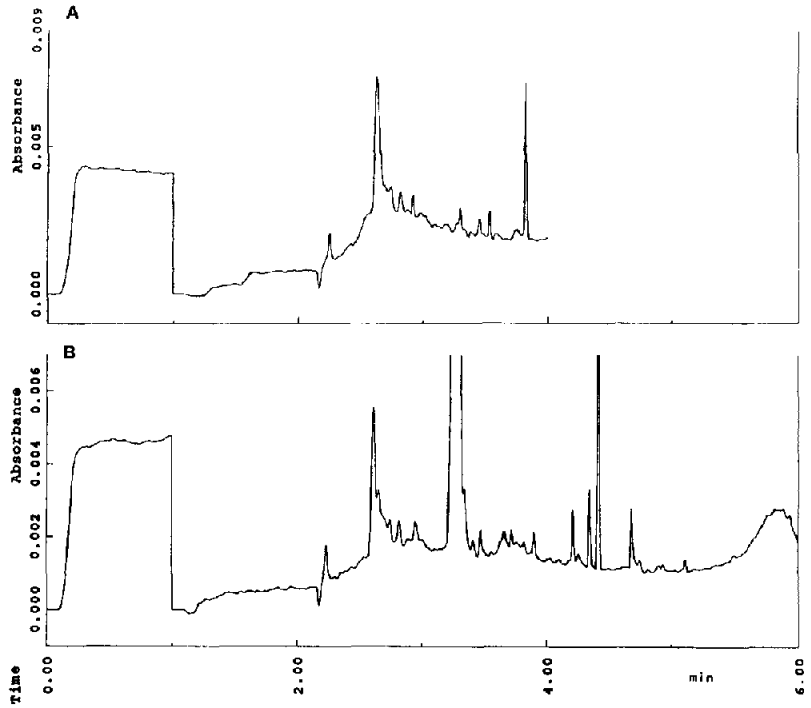

Fig. 10. (A) Electropherogram of oligosaccharides from sheep IgG by procedure 2, see Fig. 4. (B) Sample thermally degraded at 120°C for 30 min prior to analysis.

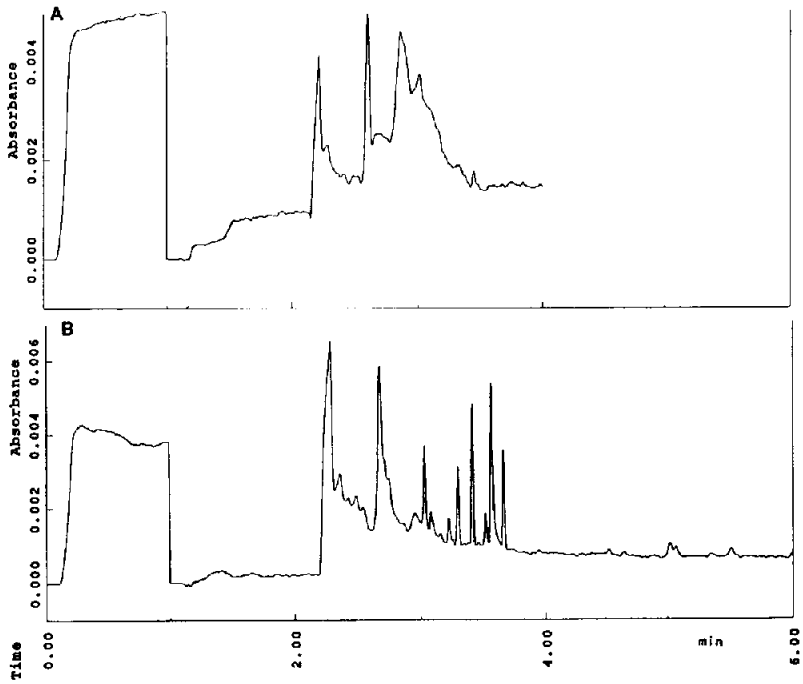

Fig. 11. (A) Separation of oligosaccharides from human IgG by procedure 2, see Fig. 4. (B) Sample thermally degraded at 120°C for 30 min prior to analysis.

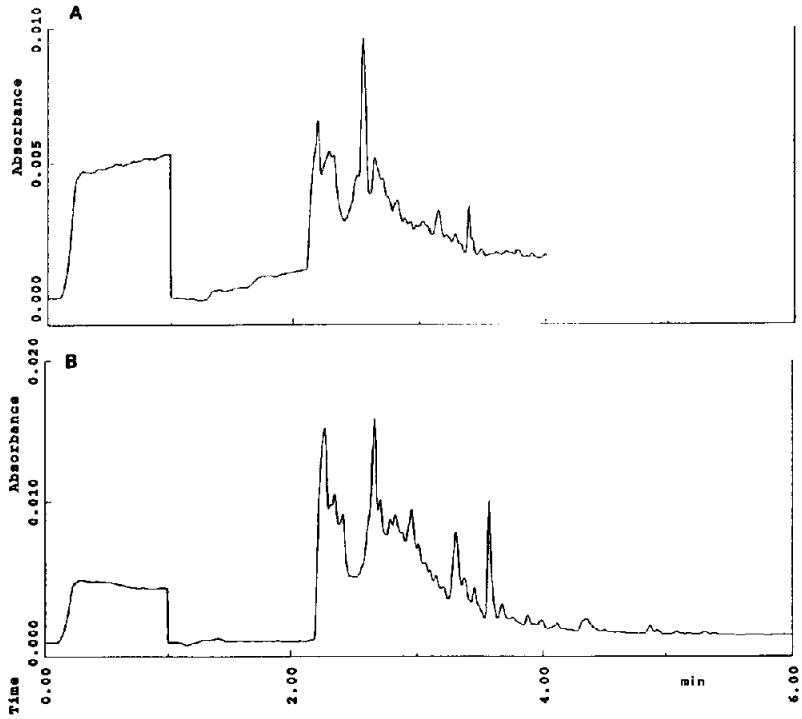

Fig. 12. (A) Separation of oligosaccharides from rat IgG by procedure 2, see Fig. 4. (B) Sample thermally degraded at 120°C for 30 min prior to analysis.

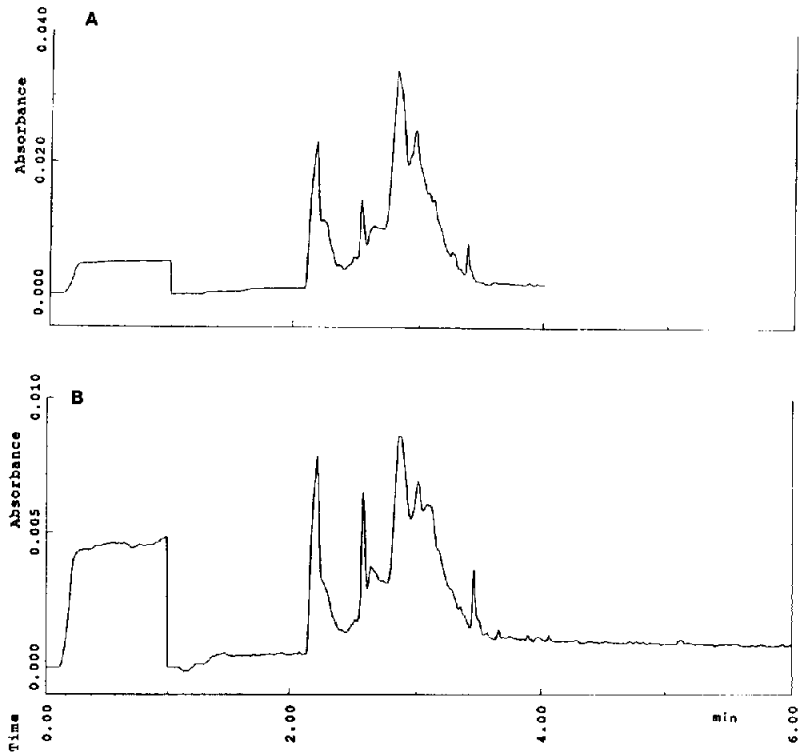

Fig. 13. (A) Separation of oligosaccharides from mouse IgG by procedure 2, see Fig. 4. (B) Sample thermally degraded at 120°C for 30 min prior to analysis.

oligosaccharide mixtures. When two model procedures were applied to IgG-released oligosaccharide samples, it was found that complex characteristic profiles were produced both for unmodified and thermally modified samples within 6 minutes. These studies suggest that CE may be a particularly efficient procedure for monitoring oligosaccharide uniformity in glycoprotein samples.

4. References

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