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Capillary electrophoresis of glycoconjugates in alkaline media

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ABSTRACT

Glycoconjugates were separated by capillary electrophoresis at alkaline pH and the migration order was investigated using different complex-forming agents in the electrolyte. Addition of cationic micelles, formed by cetrimide hydroxide, made the separation of anomers and element analogues possible. Boric acid gave the highest selectivity but the peak efficiency was slightly lower owing to slow kinetics of the complexation mechanism. Although the background electrolyte had very high pH values, no indications of instability of the fused-silica capillary or the analytes were observed under the separation conditions used.

INTRODUCTION

Carbohydrates pose separation and detection problems because of the large number of similar structures available and the inherent lack of chromophores and fluorescence properties. Extensive efforts have been made to separate carbohydrates, including the use of TLC, GC, LC, gel permeation, supercritical fluid and ion-exchange chromatography and electrophoresis [1]. In CE, solutes separate according to differences in electric charge and molecular size. For carbohydrates, this has been performed by derivatization [2,3] when charge and UV- or fluorescent-active groups were introduced into the solutes, thus enhancing the detection possibilities. Ionization of the solutes has also been achieved at alkaline pH [4] and by complex formation with borate [5,6] or alkaline earth metal ions [7].

Carbohydrates are weak acids [8] and partly charged at $pH \geq 12$. They can be separated depending on differences in their pK_a values, molecular size or complexation with species present in the electrolyte.

The aim of this study was to compare the selectivities obtained by the use of different separation modes at alkaline pH, including micellar electrokinetic capillary chromatography (MECC) with a cationic detergent, complexation with borate and with a cationic polymer. 4-Nitro- (4-NP), 4-amino- (4-AP) and phenyl-substituted carbohydrates (glycosides) were used as model substances for on-line UV detection. Separations of glycoconjugates including element analogues and nucleosides are presented.

EXPERIMENTAL

Equipment

The capillary electrophoresis system used was a Model 3 140 and a Model 3850 (ISCO, Lincoln, NE, USA), both equipped with a 67 cm $\times 50\,\mu m$ I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The polyimide coating was burned off 26 cm from the cathode end of the capillary to form the observation window for on-line UV absorption detection. Electropherograms from the ISCO Model 3850 were recorded with a Kipp & Zonen BD 40 recorder. Different polarity modes

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were used depending on the direction of the electroosmotic flow. Sample solutions diluted with water were introduced into the tube by gravity flow by raising one end of the tube 5 cm higher than the level of the opposite electrode solution for about 10 s. Analyses were carried out at ± 7 kV (-7 kV was used in the cetrimide and polybrene systems) and 27°C. Water obtained with a Milli-Q purification system (Millipore) was used to prepare all solutions.

Capillary conditioning

A new capillary was flushed with 1 *M* NaOH for 2 h, 0.1 *M* HCl for 30 min and 0.1 *M* NaOH for 30 min before the running buffer was introduced into the tube. Finally, the system was equilibrated by a pre-run for 10–15 min before analysis. The capillary was stored in running buffer overnight and rinsed with 0.1 *M* NaOH every morning and after changing the running buffer.

Chemicals

All test solutes except hexadecyltrimethylammonium (CTA) bromide (Fluka, Buchs, Switzerland) were obtained from Sigma (St. Louis, MO, USA). CTA bromide was converted into the corresponding hydroxide by shaking with silver oxide and extraction with dichloromethane. Any remaining bromide was tested for by adding silver nitrate in nitric acid to the solution. The concentration of CTA hydroxide was determined by titration with hydrochloric acid and phenolphthalein as indicator. Sodium hydroxide solutions were prepared from 1 *M* Titrisol solution (Merck, Darmstadt, Germany) and all hydroxide solutions were kept in plastic bottles.

RESULTS AND DISCUSSION

Effect of pH

Carbohydrates are weak acids with pK_a values in the range 12-14, becoming increasingly charged with increasing hydroxide concentration, *i.e.*, pH. The linear relationship between the electroosmotic velocity, μ_{eo}, and the inverse square root of the ionic strength (data not shown: *cf.*, ref. 9) indicated that the performance of the capillary was compatible with the strong alkaline conditions used in this study. The influence of pH on the relative migration for 4-NP conjugates compared with water as a marker for the electroosmosis is shown in Fig. 1. The degree of solute ionization, at a specific pH, depends on the pK_a values of the sugars and pH is therefore an important parameter in the regulation of the selectivity. Generally, the selectivity increased with increasing hydroxide concentration and a separation of five glycoconjugates at pH 13.09 is shown in Fig. 2. The high current (≤ 70 μA) will give rise to temperature effects and was the limiting factor for further increases in pH. The migration times for the

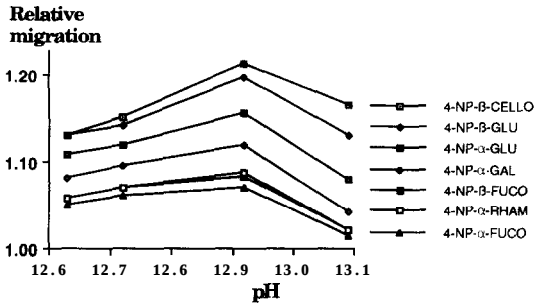

Fig. 1. Variation of the relative migration of seven 4-NP conjugates with pH. The electrolyte was 0.5 *M* Na_3PO_4 in NaOH. UV detection at 300 nm.

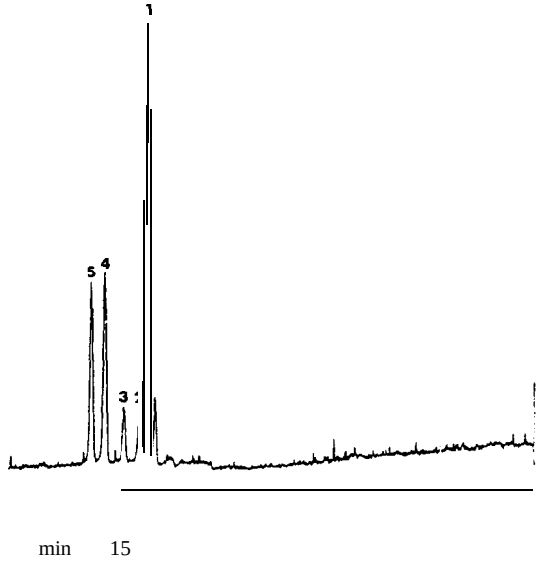

Fig. 2. Electropherogram of the separation of five 4-NP conjugates with 0.1 *M* NaOH in 0.05 *M* Na_3PO_4. Peaks: 1 = 4-NP-α-L- and 4-NP-β-L-fucosides and 4NP-α-L-rhamnoside; 2 = 4-NP-α-D-galactoside; 3 = 4-NP-α-D-glucoside; 4 = 4-NP-α-D-mannoside; 5 = 4-NP-β-D-cellobioside. UV detection at 267 nm: 0.01 a.u.f.s.

TABLE I

ADDITION OF ORGANIC MODIFIERS

Capillary: see Experimental. Electrolyte: 0.1 *M* NaOH (A) and organic modifier (MeOH = methanol and ACN = acetonitrile). Solute: 4-nitrophenyl-β-D-cellobioside.

Parameter	Electrolyte			
	A	A + 10% MeOH	A + 30% MeOH	A + 30% ACN
Electroosmotic flow ($cm^2/V\ s$)	5.69. 10^{-4}	3.88 10^{-4}	1.61 10^{-4}	2.71 10^{-4}
Number of theoretical plates	110000	75 000	36 000	55 000
Current (μA)	61	48	32	40

related phenyl-conjugates increased in the order -β-D-galactoside = -a-D-mannoside < -a-D-glucoside = -β-D-glucoside < -β-D-glucuronide.

Although very high pH values were used throughout this study, no detrimental effects on the separation system could be observed. The same fused-silica capillary was used for more than 1 month and showed the same performance. No indications of degradation of the analytes were found under the conditions used during the separations.

Effect of organic modifiers

Uncharged organic modifiers added to the electrolyte have been shown to decrease the electroosmotic mobility [10–13]. The exact mechanism is not fully understood, but a decrease in the zeta potential, changes in the dielectric properties of the Stern layer and effects on surface charge generation and adsorption of ions in the compact layer are believed to account for this effect [14].

A decrease in the electroosmotic velocity, apparent number of theoretical plates and current was observed (Table I) on addition of methanol or acetonitrile to the buffer, the former giving the largest decreases in the electrophoretic parameters. Further, these changes were proportional to the decrease in the current.

Cationic micellar electrokinetic capillary chromatography

At high pH, the anionically charged carbohydrates can distribute to a micellar pseudo-phase present in the electrolyte. Possible mechanisms are ion-pair distribution to the micelle or ion exchange with the micellar charges. Both principles suggest that other anions present in the electrolyte will compete with the solute for distribution to the limited number of micelles. The cationically charged and hydrophobic detergent CTA hydroxide was investigated for this purpose (the critical micelle concentration is 0.9 m*M* [15]).

The electroosmotic flow was reversed by the addition of CTA to the electrolyte and, hence, the polarity of the electric field was reversed. The reversal has been claimed [16] to be due to adsorption of CTA to the negatively charged silica wall, thus creating a positively charged layer where the electroosmosis is generated by the migration of hydroxide ions towards the anode.

The influence of CTA concentration on the relative migration (compared with water as a marker for electroosmosis) for five phenyl glycosides (Fig. 3) showed that all solutes distributed to the micelles but to different extents. When CTA was added as its bromide salt at 40 m*M*, the relative migration decreased by 30–40%, expressing the competition from bromide for distribution to the cationically

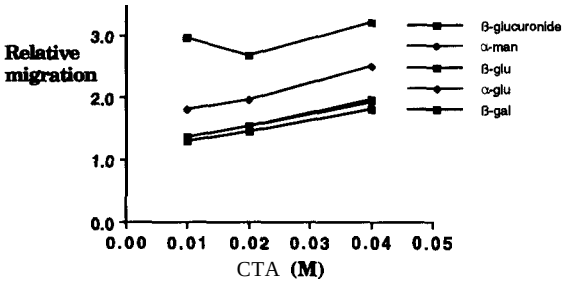

Fig. 3. Variation of the relative migration with CTA concentration at a constant hydroxide concentration (0.1 *M*).

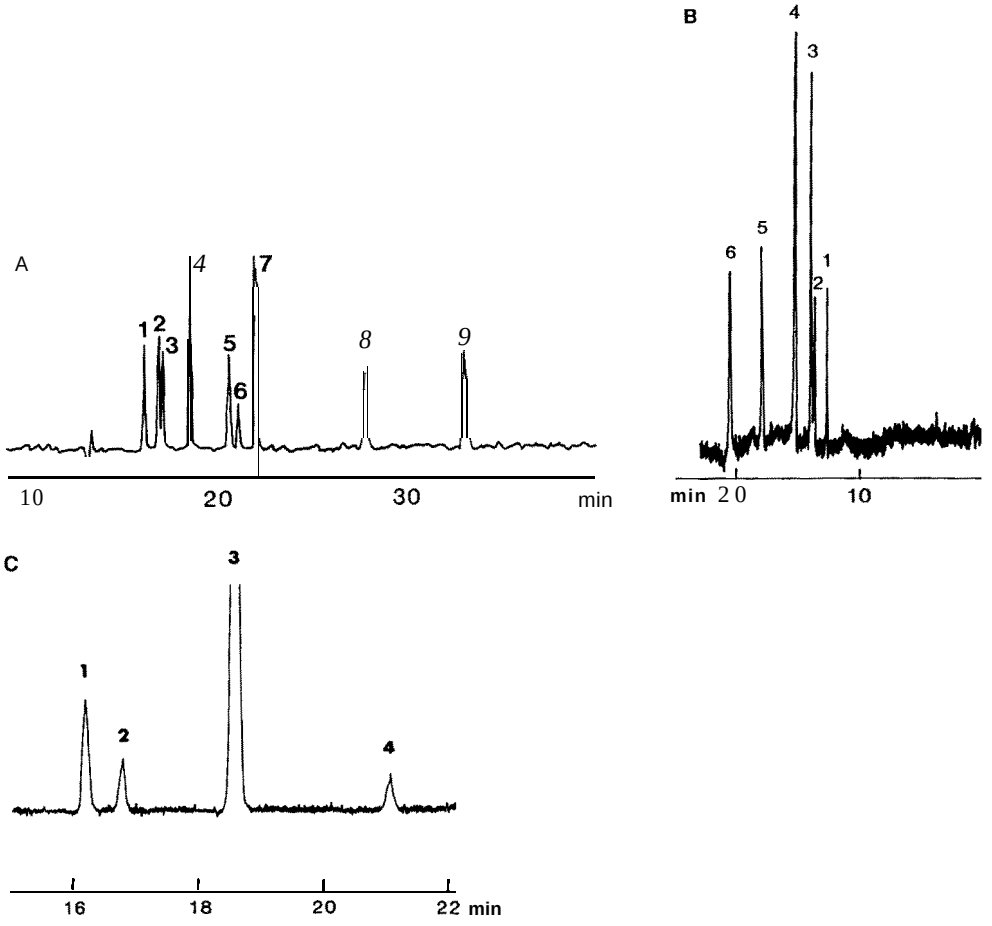

Fig. 4. Separation of (A) glyconconjugates. (B) nucleosides and (C) element analogues. (A) 1 = 4-AP-thio-β-D-galactoside; 2 = 4-AP-thio-β-D-glucoside; 3 = 4-AP-thio-β-L-fucoside; 4 = 4-AP-N-acetylthio-β-D-glucosaminide; 5 = phenyl-β-D-galactoside; 6 = 4-AP-thio-β-D-xyloside; 7 = phenyl-cc- and -β-D-glucosides; 8 = phenyl-α-D-mannoside; 9 = phenyl-β-D-glucuronide. 0.01 a.u.f.s. (B) 1 = uridine; 2 = cytidine; 3 = adenosine; 4 = thymidine; 5 = guanosine; 6 = inosine. 0.005 a.u.f.s. (C) 1 = 4-AP-β-D-xyloside; 2 = 4-AP-β-L-fucoside; 3 = 4-AP-β-L-thiofucoside; 4 = 4-AP-thio-β-D-xyloside. 0.01 a.u.f.s. Electrolyte: 40 m*M* CTA in 0.1 *M* hydroxide. UV detection at 267 nm

charged micelies. The relative migration increased with increasing CTA concentration, *i.e.*, micelle concentration, but the electroosmotic velocity was almost constant. The separations of nine glycoconjugates and six nucleosides (riboside derivatives), using 40 m*M* CTA hydroxide in 60 m*M* sodium hydroxide as electrolyte, is displayed in Fig. 4A and B. This system could also be used in the separation of element analogues (Fig. 4C) where the oxygen atoms in the ring (peaks 1 and 4) and in the glycosidic bond (peaks 2 and 3) have been replaced with a sulphur atom. When a capillary from another batch was used the differences in migration times were *ca.* 7%.

Electrolyte stability

Because of the high pH in the electrolyte, carbon dioxide is taken up, forming carbonate. This resulted in a gradual decrease in the electroosmotic velocity due to an increase in the ionic strength. Further, the selectivity decreased with increasing carbonate concentration and the separation of phenyl-a-D-glucose and phenyl-β-D-glucose (see Fig. 4A) was lost after about 2 h. This was probably due to com-

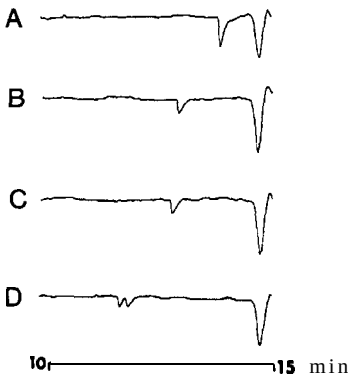

Fig. 5. Separation and indirect detection at 292 nm of (A) raffinose, (B) melibiose, (C) cellobiose and (D) galactosamine and glucosamine. The migration time of the system peak was 14.7 min. Electrolyte: 1 .O m*M* dimethylprotriptylin iodide in 40 m*M* CTA and 0.1 *M* hydroxide. 0.01 a.u.f.s.

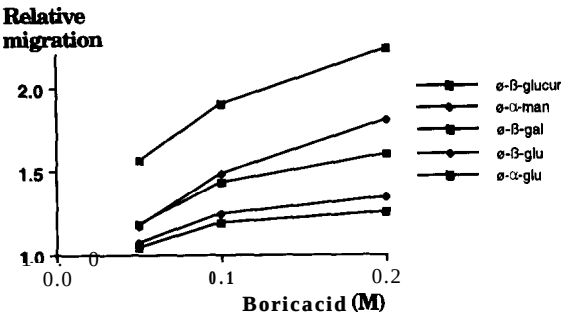

Fig. 6. Relative migration *vesus* borate concentration. Electrolyte: sodium hydroxide and borate (pH 11.2); UV detection at 267 nm.

petition from the divalent carbonate anion for distribution to the micelles as the separation was restored when a newly prepared buffer was introduced.

Indirect UV detection

Indirect detection has been used for many years [17] and the theoretical background has been described [18]. In this study, 1.0 m*M* dimethylprotriptyline (DMP) iodide was added to 40 m*M* CTA hydroxide in 60 m*M* NaOH. Electropherograms from injections of amino sugars and di-, tri- and tetrasaccharides are shown in Fig. 5. The responses were low and the detection limits were in the picomole range. The relative migrations increased with increasing size of the sugars but all solutes migrated faster than the electroosmotic flow, indicating no or very little interaction with the micelles. The migrations were unstable in this system and there was a pronounced degradation of DMP in the strongly alkaline solution.

Polymeric cation

Cationic polymers such as polybrene with the general structure

$$([-(CH_2)_6\overset{+}{N}(CH_3)_2(CH_2)_3\overset{+}{N}-(CH_3)_2-]_n\ [2\ Br^-]_n)$$

have been used in the ion-exchange electrokinetic chromatography of acids [19]. Both polymer and solute molecules are dragged along with the electroosmosis, but their electrophoretic mobilities are in opposite directions. Accordingly, anions can be separated based on differences in electrophoretic mobility and ion-pair formation constants with the cationic polymer. The electroosmotic flow was reversed, indicating that polybrene is adsorbed on the silica wall. Compared with the MECC system there was a change in the migration order for the phenyl glycosides, this being -β-D-glucuronide, -β-D-glucoside, -β-D-galactoside and -a-D-mannoside. The relative migration times for the solutes were close to 1, except for the glucuronide, indicating poor ion-pair formation with polybrene. This was probably due to the high concentration of bromide present in the system competing for ion-pair formation with the polymer and, preferably, the corrersponding hydroxide salt should be used in order to minimize this effect.

Boric acid

Borate has long been used as a complexing agent for carbohydrates and polyols. In this study, borate was included for comparison regarding selectivity. The relative migration times (Fig. 6) increased with increasing borate concentration as the degree of complex formation increased. The migration order (Fig. 7) was different to those with the other systems, thus offering further possibilities for carbohydrate separations. The lower peak efficiencies ($N \approx 65\ 000$) due to slow kinetics of the borate complexation were offset by the gain in the selectivity. Borate can complex to more than one polyol molecule and, in an attempt to increase the selectivity

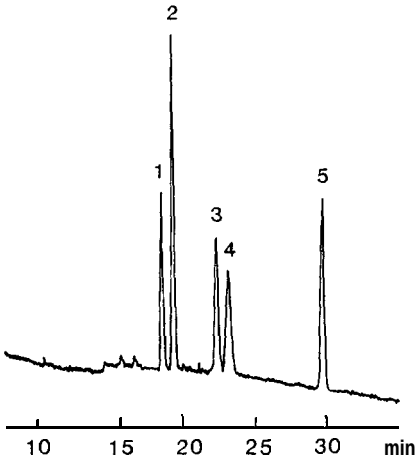

Fig. 7. Separation of phenyl conjugates as borate complexes. Peaks: 1 = phenyl-α-D-glucoside; 2 = phenyl-β-D-glucoside; 3 = phenyl-β-D-galactoside; 4 = phenyl-a-u-mannoside; 5 = phenyl-β-D-glucuronide. Electrolyte: 0.1 *M* boric acid in 0. 1 *M* NaOH at pH 11.2. UV detection at 267 nm; 0.01 a.u.f.s.

further, mannitol (a polyol giving strong complexes with borate) was added to the electrolyte. The aim was to investigate the selectivity of the complexation between the borate-mannitol complex and the glycoconjugates. Extremely broad peaks were obtained, however, for the galactose and mannose derivatives ($N \approx 400$), probably owing to even slower complexation kinetics.

CONCLUSIONS

Glycoconjugates were separated as anions using highly alkaline buffer solutions. The selectivities were governed by the pK_a value and the size of the glycoconjugate and by electrolyte additives. A change in the migration order was obtained by addition of a cationic detergent to the buffer. owing to differences between the solutes in distribution to the micelles. Further shifts in migration order could be obtained by addition of a polymeric cation or borate as complex-forming agents in the electrolyte. The study demonstrated that there are several ways to control selectivity and, hence, the separation of glycoconjugates by the addition of complex-forming agents to the electrolyte.

REFERENCES

1 S. C. Churms. *J*. Chromatogr., 500 (1990) 5555583.
2 J. Liu, 0. Shirota and M. Novotny, *Anal. Chem*., 63 (1991) 413–417.
3 J. Liu, 0. Shirota and M. Novotny, *J. Chromatogr*.. 559 (1991) 223-235.
4 T. W. Garner and E. S. Yeung. *J. Chromatogr*., 5 15 (1990) 639–644.
5 S. Honda, S. Iwase, A. Makino and S. Fujiward. *Anal. Biochem*., *I76 (1989)* 72–77.
6 S. Hofstetter-Kuhn, A. Paulus, E. Gassman and H. M. Widmer, *Anal. Chem*., 63 (1991) 1541-1547.
7 S. Honda, K. Yamamoto, S. Suzuki, M. Ueda and K. Kakehi, *J. Chromatogr., 588 (1991) 327-333.*
8 J. A. Rendleman, Jr.. *Adv. Chem. Ser*.. 1 17 (1973) 51–69.
9 A. J. Bard and L. R. Faulkner, *Electrochemical Methods. Fundamentals and Applications*, Wiley, New York, 1980.
10 S. Fujiwara and S. Honda, *Anal. Chem., 59 (1987)* 487–490.
11 J. Liu, K. Cobb and M. Novotny, *J. Chromatogr*., *468 (1988)* 55–65.
12 K. Salomon, D. S. Burgi and J. C. Helmer, *J*. Chromatogr.. 559 (1991) 69980.
13 C. Schwer and E. Kenndler, *Anal. Chem*., *63* (1991) 1801–1807.
14 J. W. Jorgenson and K. D. Lukacs. *Anal. Chem*., *53 (1981) 129X-1302.*
15 P. Mukerjec and K. H. Mysels, *Nat. Stand. Ref. Data Ser*., Nat. Bur. Stand., Washington, DC. 20 234 (1971).
16 K. Otsuka, S. Terabe and T. Ando, *J. Chromatogr*., *332 (1985)* 219–226.
17 S. Hjerttn, *Chromatogr. Rev., 9* (19767) 122-2 19.
18 H. Poppe. .I. *Chromatogr*., *506 (1990)* 45–60.
19 S. Terabe and T. Isemura, *J. Chromatogr*., *515 (1990) 667-676.*