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Review

Separation of neutral carbohydrates by capillary electrophoresis

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Abstract

The basic strategies for analysis of neutral carbohydrates by capillary electrophoresis are summarized. Neutral carbohydrates are dissociated in strong alkali to give anions, hence they can be separated directly by zone electrophoresis based on the difference between their dissociation constants. However, neutral carbohydrates are not electrically charged under normal conditions. Therefore, they should be converted to ions prior to or during analysis. Precapillary introduction of a basic or an acidic group to a neutral carbohydrate gives the derivative positive (in acidic media) or negative (in alkaline media) charge, respectively. The derivatives thus obtained can be separated by zone electrophoresis. Analysis of carbohydrates in a carrier containing an oxyacid salt (such as sodium borate) or an alkaline metal salt (such as calcium acetate) causes in situ conversion to anionic or cationic complexes, respectively, which are separated by zone electrophoresis. The effective uses of electrokinetic chromatography in sodium dodecyl sulfate micelles for hydrophobic derivatives (such as 1-phenyl-3-methyl-5-pyrazolone derivatives) and size-exclusion electrophoresis in gel-packed capillaries for size-different oligosaccharides are also discussed. Each separation mode has its inherent method(s) for detection, which are also described here.

Contents

1. Introduction

Capillary electrophoresis is a recently developed method for separation. It comprises all types of electrophoresis in narrow tubes having inner diameters less than ca. 250 μm. So far, zone electrophoresis, isotachophoresis and isoelectric focusing have been the major types of capillary electrophoresis. Zone electrophoresis is an electrophoretic version of elution chromatography, widely used in liquid chromatography. Isotachophoresis and isoelectric focusing corre-

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spond to displacement chromatography and chromatographic focusing, respectively.

The first paper on zone electrophoresis was by Hjertén [1]. In 1967 he reported an attempt to separate analytes in a horizontally placed tube rotating along its axis. Constant rotation was effective to prevent dispersion due to convective flow. A similar effect was obtained by using a very narrow tube without rotation. Mikkers et al. [2] succeeded in separating a number of carboxylic acids by this approach using a 200 μm I.D. PTFE tube with electrical field-strength detection. The high resolution power of capillary zone electrophoresis was further demonstrated by the skillful accomplishment by Jorgenson and Lukacs [3] by using a 75 μm I.D. glass tube and a fluorescence detector. The number of theoretical plates attained in this work exceeded $4 \cdot 10^5$.

The development of capillary electrophoresis in the last decade, mainly based on zone electrophoresis, is overwhelming; the number of papers published on the theory and the application of this method was not less than 2000. It has been applied to almost all kinds of compounds ranging from ionic to neutral compounds, as well as small molecules to macromolecules. Although it has not been widely applied to carbohydrates, there is enough data to make a few reviews.

Capillary electrophoresis is basically a method for ions, hence ionic carbohydrates such as sulfated and silylated oligosaccharides can be separated directly in the zone electrophoresis mode, based on the difference of their electric charge/molecular size ratio. Analyses of such ionic carbohydrates are reviewed in this issue by Linhardt and Pervin [4]. Most carbohydrates are, however, neutral and require their conversion to ions. Micellar electrokinetic chromatography is an extension of zone electrophoresis applicable to neutral hydrophobic substances. Application of this method to carbohydrates, however, also needs their transformation to hydrophobic derivatives. This article summarizes all methods to apply high-performance capillary electrophoresis to neutral carbohydrates. Detection of carbohydrates separated by capillary electrophoresis involves technological difficulties not encountered in liquid chromatography. Paulus and Klockow [5] have contributed a review article, focused on this problem. Application of capillary electrophoresis to carbohydrates in glycoconjugates, especially glycoproteins, is an important recent topic, hence it is reviewed separately by Kakehi and Honda [6].

Besides the four review articles concerning capillary electrophoresis of carbohydrates in this thematic issue other information on this subject can be obtained from various sources. Novotny and Sudor [7] wrote a review, with emphasis on the chemical derivatization of carbohydrates for sensitive detection. It also points out the great potential of this method for glycoconjugate research. Recently another review by Oefner et al. [8] has appeared. It also states the importance of this method as a powerful alternative for high-performance liquid chromatography. Other commentaries on capillary electrophoresis of carbohydrates are available from Refs. [9,10]. Convenient protocols for the analysis of carbohydrates in glycoproteins are also available [11–13].

2. Use of strong alkali

The hydroxyl groups in carbohydrate molecules have very small dissociation constants (in a range of 10^{-11}–10^{-13} M), as compared to the phenolic hydroxyl groups, but they are dissociated at extremely high pHs. Thus they behave as very weak anions in strongly alkaline solutions having pHs >11. Under such conditions carbohydrates can be separated by zone electrophoresis in intact state, as demonstrated by Garner and Yeung [14]. Electroosmotic flow, generated due to the electric double layer between the capillary inner wall and the carrier in the electrical field, drove the separated carbohydrates to the detector at the cathodic end, because the employed fused-silica capillary tube is negatively charged. Carbohydrates having greater dissociation constants migrated slower, since they were stronger retained to the anodic end by electrostatic force. This work was, however, tentative; sucrose, glucose and fructose, the only set of carbohydrates examined, were separated at pH 11.65. In this analysis the separated carbohydrates were moni-

tored by indirect fluorescence detection using a carrier solution containing Coumarin 343 which gave background fluorescence. This detection method is based on charge displacement, and the components of a sample are observed as troughs below the baseline. It is effective for detection of any compound having no fluorescence at the same wavelength as that of the background fluorescence. The details of this method were reviewed by Yeung and Kuhr [15]. The carbohydrate concentrations in this case were commonly 1 m*M*.

Another example of the separation of intact carbohydrates based on the same principle was presented by the group of Bonn [16,17] (Fig. 1). In this example six neutral carbohydrates (galactose, glucose, mannose, rhamnose, 2-deoxyribose, and raffinose) and four acidic carbohydrates (gluconic acid, galacturonic acid, glucuronic acid and mannuronic acid) were selected for separation. The separation of the acidic carbohydrates was satisfactory but the neutral carbohydrates were not baseline resolved at pH 12.1. In this analysis the separated carbohydrates were monitored by indirect UV detection at 256 nm using a 6 m*M* sorbic acid solution adjusted to this pH value, as carrier. Troughs were observed for individual carbohydrates in a way similar to that observed in indirect fluorescence detection and linearity was shown over the range 5–65 pmol injected.

Colón et al. [18] obtained better separation, as shown in Fig. 2. In this case separation was performed in aqueous 100 m*M* sodium hydroxide and the separated carbohydrates were monitored by an electrochemical method. Fifteen standard sugars were separated and detected with theoretical plate numbers ranging from 100 000 to

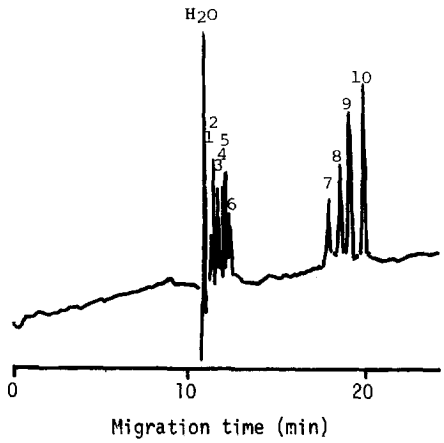

Fig. 1. Separation of neutral and acidic carbohydrates in intact state in strong alkali with indirect UV detection [17]. Capillary, fused silica (50 μm I.D., 120 cm); carrier, 6 m*M* sorbic acid adjusted to pH 12.1 with sodium hydroxide; applied voltage, 28 kV; detection, UV absorption at 256 nm. Peaks: 1 = raffinose, 2 = 2-deoxyribose, 3 = galactose, 4 = glucose, 5 = rhamnose, 6 = mannose, 7 = gluconic acid, 8 = galacturonic acid, 9 = glucuronic acid, 10 = mannuronic acid.

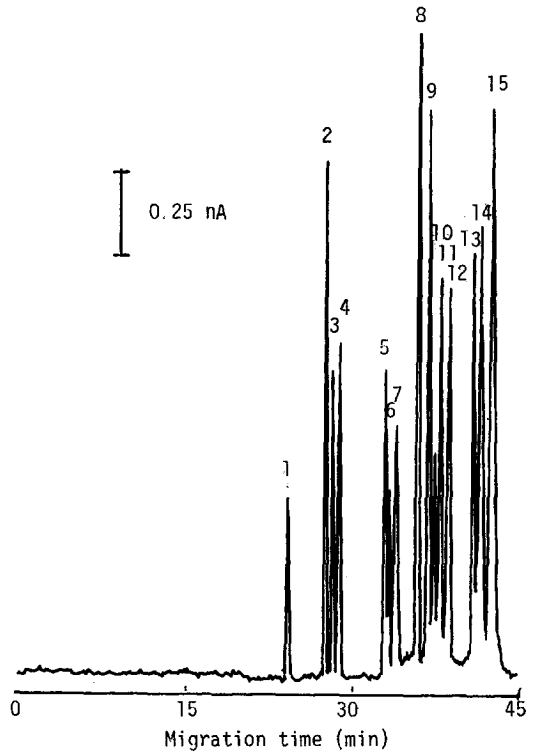

Fig. 2. Separation of simple mono- and oligosaccharides in strong alkali with electrochemical detection [18]. Capillary, fused silica (50 μm I.D., 73 cm); carrier, 100 m*M* sodium hydroxide; applied voltage, 11 kV; detection, electrochemical method using a copper microelectrode (working electrode) and a silver/silver chloride electrode (reference electrode); sample injection, hydrodynamic. Peaks: 1 = trehalose, 2 = stachyose, 3 = raffinose, 4 = sucrose, 5 = lactose, 6 = lactulose, 7 = cellobiose, 8 = galactose, 9 = glucose, 10 = rhamnose, 11 = mannose, 12 = fructose, 13 = xylose, 14 = talose, 15 = ribose.

200 000. Detection was performed by a special technique using the electrolytic current generated by applying a constant voltage between a microcylindrical (25 μm I.D.) copper wire as a working electrode and a silver/silver chloride reference electrode. In order to decrease the voltage used for electrophoresis to ground level at the detection end and maintain it at a constant value, an endcapillary device was used, previously described by Huang et al. [19]. Linearity of peak response was observed over a wide range of concentrations (1 μM–1 mM). Keeping the electrode at a constant position throughout the analysis was important to obtain high reproducibility of peak response.

The combination of strong alkali and pulsed amperometic detection (PAD) on a gold electrode, already established and commercialized for high-performance liquid chromatography by the Dionex group, has also been applied to capillary electrophoresis. According to the paper of O'Shea et al. [20] the PAD response to glucose in 10 mM sodium carbonate was linear over the range 10 μM–1 mM, when a 50-μm gold cylindrical wire was used as a working electrode.

3. Precapillary conversion to ions

Usually carbohydrates cannot be sensitively detected in their intact state in capillary electrophoresis, because they lack potential chromophores and fluorophores. This situation is similar to that in liquid chromatography. Thus precapillary derivatization plays an important role. It should be noted that such derivatization endows the derivatives with ionic properties the original carbohydrates did not have. For instance reductive amination of a reducing carbohydrate with a suitable primary amine in the presence of borohydride or a similar reductant (Fig. 3) gives the derivatives cationic nature, because the introduced imino group is positively charged in acidic media.

When a capillary tube of fused silica filled with an acidic buffer is used, such reductively aminated derivatives move to the cathode faster than

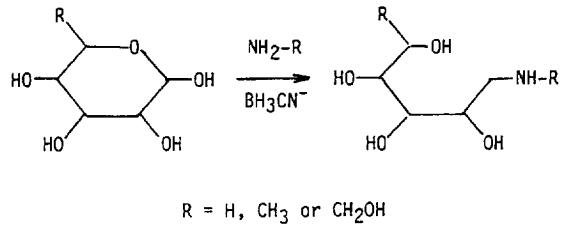

Fig. 3. Conversion of reducing carbohydrates to glycamine derivatives by reductive amination.

the neutral marker, since both electroosmotic flow and electrophoretic migration are directed toward the cathode. If the capillary inner wall is coated with non-charged hydrophilic substances such as linear polyacrylamide, electroosmotic flow is diminished and the derivatives move slower. Under such conditions reductively aminated carbohydrates having different degrees of polymerization (d.p.) are separated by the zone electrophoresis mode, as exemplified by reductively pyridylaminated dextran hydrolysate [21], as shown in Fig. 4.

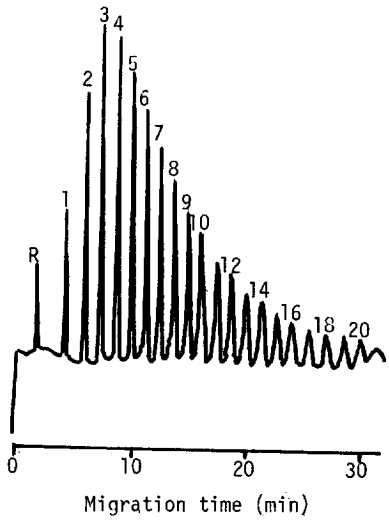

Fig. 4. Separation of AP derivatives of isomaltooligosaccharides having different d.p.s by zone electrophoresis in an acidic medium [21]. Capillary, fused silica coated with linear polyacrylamide (25 μm I.D., 20 cm); carrier, 100 mM phosphate buffer (pH 2.5); applied voltage, 8 kV; detection, UV absorption at 240 nm. R = reagent (AP). Peak numbers represent d.p.s.

Every 2-aminopyridine (AP) derivative has the same electric charge because one imino group is introduced to each oligosaccharide by this derivatization, while each derivative has a different molecular size. As a result the charge-to-size ratio is varied among the oligosaccharide derivatives, hence they are separated in the order of increasing d.p. by the direct zone electrophoresis mode in an acidic phosphate buffer. Nashabeh and El Rassi [22] examined the separation of the AP derivatives of maltooligosaccharides in acidic phosphate buffers and observed slight improvement of separation by addition of tetrabutyl ammonium bromide. The addition of this organic salt caused concurrent delay of migration by the decrease of the zeta potential due to the wall effect of the tetrabutylammonium ion. This improvement can be ascribed to the relatively increased contribution of electrophoresis as compared to electroosmosis. The AP derivatives can be detected sensitively by either UV absorption at 240 nm [11,12] or fluorescence intensity at 400 nm with irradiation at 320 nm [23]. Monitoring by UV absorption allowed detection of PA derivatives of carbohydrates in concentrations higher than 100 μM [23]. Fluorometric monitoring using a Xenon lamp as an excitation light source enabled detection at a level two orders of magnitude lower than that obtained in UV absorption, provided the excitation beam was focused on the detection window on the capillary tube by use of a collimator lens [23]. Using 6-aminoquinoline (AQ) instead of AP increased sensitivity in UV monitoring [24]. The AQ derivatives were reported to also fluoresce but relative fluorescence intensities of the AQ derivatives have been unknown.

Nashabeh and El Rassi [24] studied the relationship between electrophoretic mobility (mobility hereafter) and d.p. in N-acetylated chitooligosaccharides and found that the logarithm of the mobility linearly decreased with d.p., and both AP and AQ derivatives gave the same slope. They also examined the migration behavior of branched oligosaccharides derived from xyloglucan by digestion with cellulase. In this case oligosaccharides varied not only in size but also in the number of branches. Fig. 5 shows

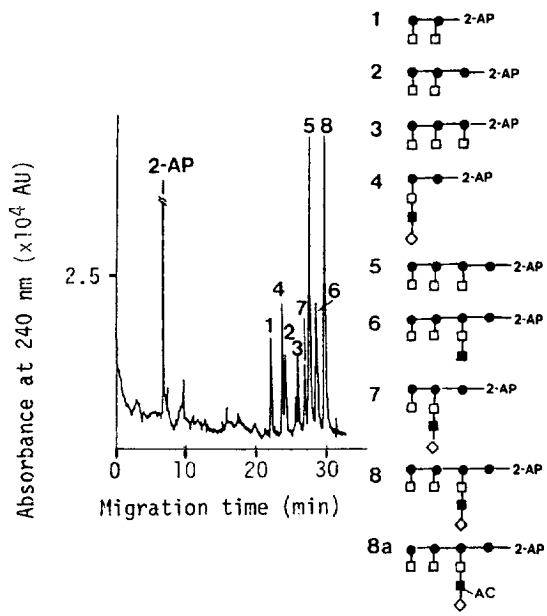

Fig. 5. Mapping of AP derivatives of xyloglucan oligomers reconstituted by mixing purified samples [24]. Capillary, fused silica coated with interlocked polyether (50 μm I.D., 80 cm); carrier, 100 mM sodium phosphate containing tetrabutylammonium bromide (50 mM), pH 4.75; applied voltage, 20 kV; detection, UV absorption at 240 nm. • = Glucose; □ = xylose; ■ = galactose; ◇ = fucose.

separation of such oligosaccharides as AP derivatives.

Since the capillary inner wall was coated with an interlocked polyether, electroosmotic flow was negligible and oligosaccharides having higher d.p.s were migrated slower. Moreover it was observed that in general mobility increased with extent of branching. The mobility index (M.I.) [defined as $100n + 100\ (\log\ \mu_s - \log\ \mu_{n+1})(\log\ \mu_n - \log\ \mu_{n+1})^{-1}$, where μ_s is the mobility of the AP derivative of a xyloglucan oligomer, and μ_n and μ_{n+1} are the mobilities of the two homologues with d.p. n and $n+1$ which elute before and after the xyloglucan oligomer, respectively] indicated that more branching gave a lower decrease in mobility in a way similar to the Kovats retention index in gas–liquid chromatography.

In contrast, reductive amination with primary amines containing strongly acidic functional

groups endows the derivatives with a negative overall charge. 7-Amino-naphthalene-1,3-disulfonic acid (ANDS) [25] and 8-amino-naphthalene-1,3,6-trisulfonic acid (ANTS) [26] present typical examples. The negative charge of the derivatives, especially the ANTS derivatives, is so strong that they are dissociated even in acidic media. Fig. 6 shows the separation of ANTS derivatives of maltooligosaccharides having various d.p.s, at pH 2.5, reported by Chiesa and Horváth [26].

Since electroosmotic flow is almost null under these conditions, the ANTS derivatives migrated to the anode in the order of increasing d.p.s almost solely by electrophoresis, where they were detected by UV absorption. The direction of migration was reversed to that of the PA derivatives, hence samples had to be introduced from the cathodic end. The use of such acidic conditions offered the advantage that the excess reagent appeared first, well isolated from the ANTS derivatives of the oligosaccharides. Chiesa and Horváth examined the relationship between electrophoretic mobility μ_{ep} and molecular mass M, and found that μ_{ep} is proportional to $M^{-2/3}$ (data not shown), similar to the correlation observed for peptides [27] and their derivatives

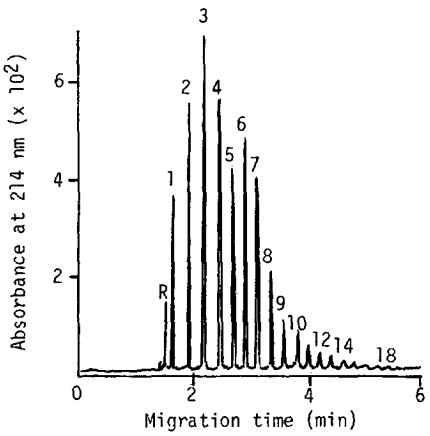

Fig. 6. Separation of ANTS derivatives of maltooligosaccharides having different d.p.s by zone electrophoresis in an acidic medium [26]. Capillary, fused silica (50 μm I.D., 27 cm); carrier, 50 m*M* phosphate buffer (pH 2.5); temperature, 25°C; applied voltage, 17 kV; detection, UV absorption at 214 nm. R = reagent (ANTS). Peak numbers represent d.p.s.

[28]. In order to understand this phenomenon completely further investigation will be necessary. Addition of triethylamine caused alteration of the direction and velocity of the electroosmotic flow by its adsorption onto the capillary inner wall. Addition of triethylamine to ca. 10% resulted in a change of direction of the electroosmotic flow; a higher concentration resulted in rapid analysis by increasing the electroosmotic flow in the same direction as the electrophoretic migration. When alkaline buffers were used as carrier instead of an acidic buffer as used in Fig. 6, ANTS derivatives of maltooligosaccharides moved to the cathode in a reversed order were separated from each other due to the difference in the electrophoretic migration velocities, but separation was not very good. In addition the peaks of the ANTS derivatives of higher maltooligosaccharides were interfered by a large peak of the excess reagent. The ANTS derivatives have an absorption maximum at 370 nm (ε = 5700, in water) and fluoresce at 515 nm with the excitation maximum at 370 nm. Laser-induced fluorescence detection was attempted by using the 325-nm beam of the helium–cadmium laser, but the sensitivity was only three orders of magnitude higher than that of UV absorption under these conditions due to the large deviation of the wavelength used for excitation. The long reaction time for derivatization is a drawback of not only the ANTS method but also of other methods based on reductive amination. The yield of the ANTS derivative of glucose was reported to be quantitative based on radioisotope measurement, but no details are known for oligosaccharides having high d.p.

Another example of precapillary conversion to anions is the condensation with 1-phenyl-3-methyl-5-pyrazolone (PMP). In this case two PMP groups are introduced rapidly and quantitatively to each reducing carbohydrate under mild conditions [29] (Fig. 7), and the derivatives are negatively charged in alkaline media due to the dissociation of the enol group at C-5 in the pyrazolone ring.

Fig. 8 shows the separation of the PMP derivatives of isomaltooligosaccharides having various d.p.s in an alkaline phosphate buffer [23]. There

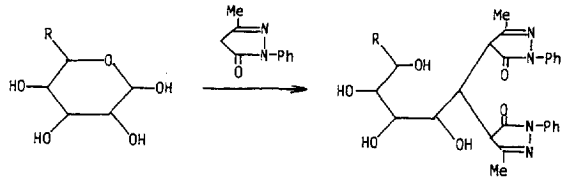

Fig. 7. Conversion of reducing carbohydrates to PMP derivatives [29].

is an equilibrium between the enol form and the keto form at C-5 in the pyrazolone ring. Thus, electrophoretic mobility increases as pH goes up, because the proportion of the enolate form increases, and better separation can be achieved at higher pHs. The PMP derivatives can be sensitively detected by UV absorption at 245 nm (PMP-glucose: $\varepsilon = 29\,400$ in ethanol; detection limit in capillary electrophoresis, ca. 10 μM).

All examples of the separation of precapillary-derivatized ionic species mentioned above for AP, AQ, ANTS and PMP derivatives are based on direct zone electrophoresis in a way similar to that for acidic carbohydrate analyses reviewed by Linhardt and Pervin [4]. These acidic carbohydrates have originally the ionic groups as their molecular constituents, while the neutral carbohydrates discussed here require precapillary derivatization to ions in order to be separated in this mode. Such derivatization commonly allows separation of size-different carbohydrates.

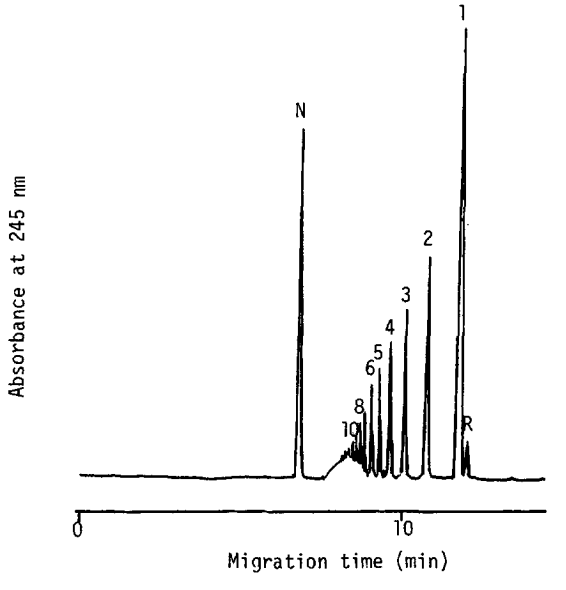

Fig. 8. Separation of PMP derivatives of isomaltooligosaccharides having various d.p.s by zone electrophoresis in an alkaline medium [23]. Capillary, fused silica (50 μm I.D., 70 cm); carrier, 50 mM phosphate buffer (pH 9.00); applied voltage, 15 kV; detection, UV absorption at 245 nm. N = neutral marker (cinnamyl alcohol), R = reagent (PMP). Peak numbers represent d.p.s.

4. In situ conversion to ions

One of the separation modes of liquid chromatography suitable for carbohydrate analysis is anion-exchange of carbohydrates as their anionic complexes. A typical example is the separation with the borate ion. The hydroxyl groups in carbohydrates rapidly react with the borate ion in the eluent to give anionic complexes, which can be separated on an anion-exchange column (e.g. Ref. [30]). The same principle can be applied to capillary electrophoresis of carbohydrates [31–38]. In a fused-silica capillary tube filled with a carrier solution containing a borate salt, carbohydrate components of a sample form anionic complexes. Upon application of voltage the anionic complexes migrate toward the cathode by the combined effect of electrophoresis and electroosmosis, as illustrated in Fig. 9 [31]. The separation mode is similar to that for acidic carbohydrates, but the migrating ionic species, i.e. anionic carbohydrate–borate complexes, are in equilibration with the intact carbohydrates.

Bruno et al. [32] reported the separation of three disaccharides (sucrose, cellobiose and lactose) and two N-acetylhexosamines (N-acetylgalactosamine and N-acetylglucosamine) in borate buffer (pH 9) at 27°C with refractive index monitoring using a helium–neon laser. Separation was good but an impurity peak overlapped the cellobiose peak. In principle the detection method is similar to that used in liquid chromatography, but required technological adaptation to capillary electrophoresis. The details of the

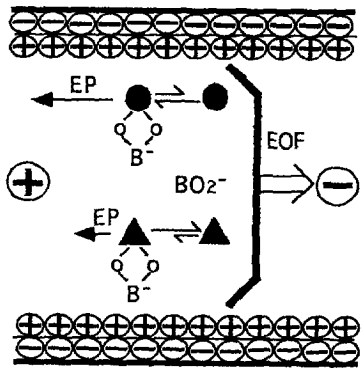

Fig. 9. Illustration of the separation of in situ formed carbohydrate–borate complexes by zone electrophoresis [31]. EOF = electroosmotic flow, EP = electrophoretic migration.

technique are described in the review by Paulus and Klockow [5].

Fig. 10 shows the separation of a few popular monosaccharides in 50 m*M* borate buffer (pH 9.3) [33], as monitored by UV absorption at low-wavelength (195 nm). These intact monosaccharides gave broad peaks at 20°C, but elevated temperature caused peak sharpening and higher resolution. At 20°C mannose and galactose were well separated though glucose and xylose gave a broad fused peak, whereas at 60°C they were completely separated from each other. Migration time decreased with increasing temperature. The rapid migration can be explained by decreased viscosity and possibly by increased permittivity.

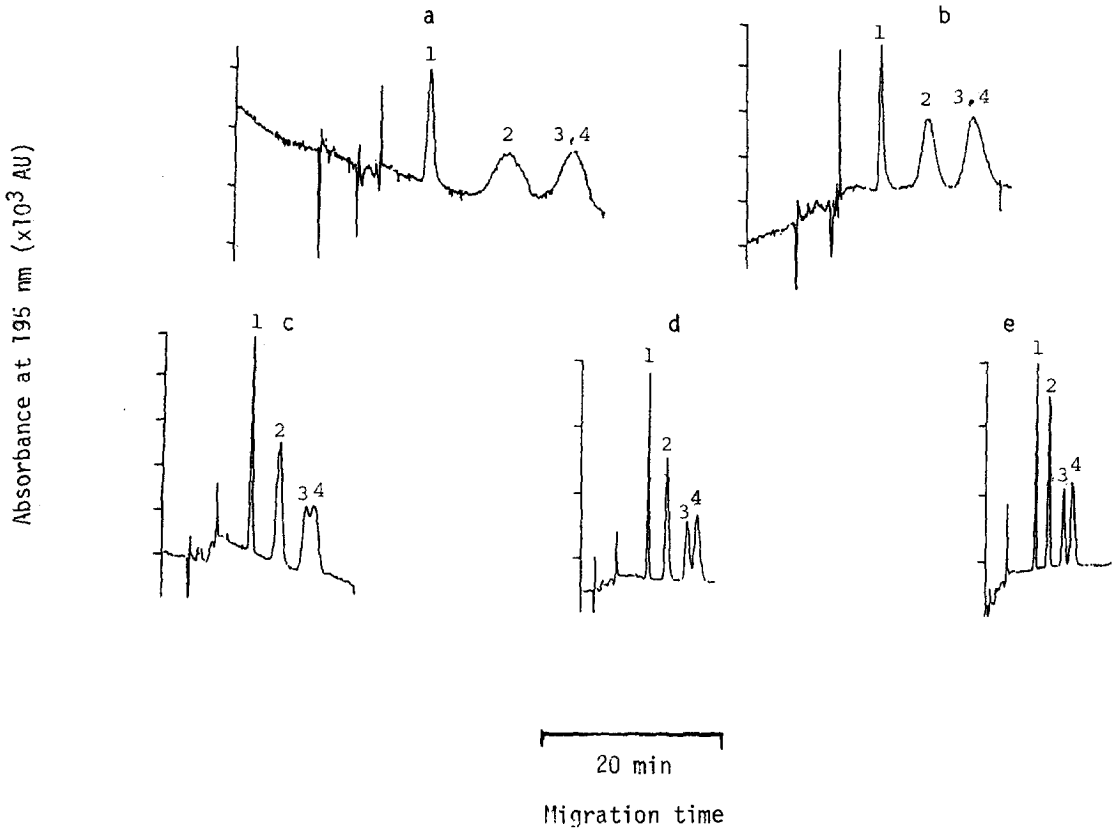

Fig. 10. Separation of popular monosaccharides as ionic borate complexes [33]. Capillary, fused silica (75 μm I.D., 94 cm); capillary temperature, 20°C (a), 30°C (b), 40°C (c), 50°C (d) and 60°C (e); carrier, 50 m*M* borate buffer (pH 9.3); applied voltage, 20 kV; detection, UV absorption at 195 nm. Peaks: 1 = mannose, 2 = galactose, 3 = glucose, 4 = xylose. Each division of the ordinates corresponds to $1 \cdot 10^{-3}$ A.U.

Hoffstetter et al. [33] attributed the improved separation at increased temperature to increased reactivity of each carbohydrate to the borate ion, but the extraordinary broadness of the peaks as compared to derivatized carbohydrates (see examples in Figs. 12 and 13) remains unexplained. However, this phenomenon cannot be understood without temperature dependence of mutarotation.

The electroosmotic flow velocity increases with pH when a fused-silica capillary tube is used. The electrophoretic migration velocity increases with higher rates than the electroosmotic flow velocity as pH and borate concentration increase, as observed from the example for AP derivatives of monosaccharides (Figs. 11a and 11b, respectively), since the molar fractions of the complexes increase [31].

The increase in the electrophoretic mobility differs slightly among the different carbohydrate species, hence optimization of the analytical conditions resulted in complete separation of almost all naturally occurring monosaccharides (arabinose, ribose, xylose, galactose, glucose, mannose, fucose, rhamnose, N-acetylgalactosamine, N-acetylglucosamine, galacturonic acid and glucuronic acid) under a simple condition of 200 m*M* borate buffer (pH 10.5), as shown in Fig. 12.

Although the analysis involved equilibrium reaction, the peaks were relatively sharp and the theoretical plate numbers were relatively high, e.g. 80 000 for the AP derivative of galactose. Glucose and mannose were the only pair of naturally occurring monosaccharides that was not separated under these conditions. Lyxose and cinnamic acid can conveniently be used as internal standards. These monosaccharides were derivatized by reductive pyridylamination prior to analysis, for sensitive detection. The analytical sample was a reaction mixture of reductive pyridylamination, and no clean-up was performed. It is an advantage that the large excess of reagent (AP) was well isolated from the AP derivatives of the monosaccharides, and did not interfere with their analysis. The good separation was based on the ease of complexation and the magnitude of the charge of the complex formed.

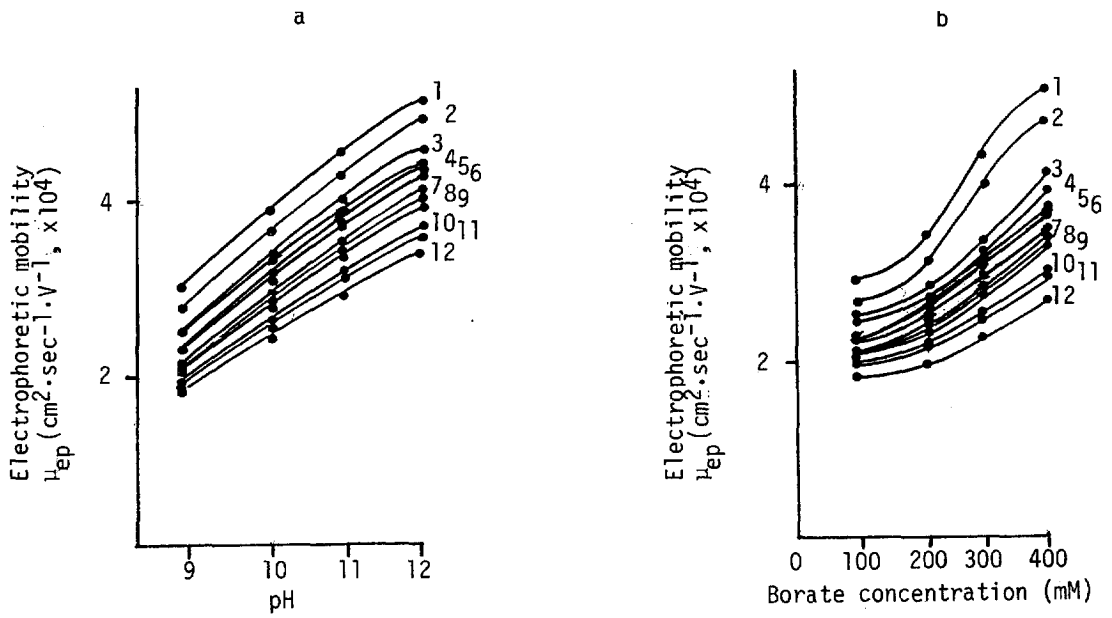

Fig. 11. Change of electrophoretic mobility with pH (a) and borate concentration (b) [31]. Curve assignment: 1 = galacturonic acid, 2 = glucuronic acid, 3 = galactose, 4 = fucose, 5 = arabinose, 6 = glucose, 7 = N-acetylglucosamine, 8 = ribose, 9 = xylose, 10 = rhamnose, 11 = lyxose, 12 = N-acetylgalactosamine.

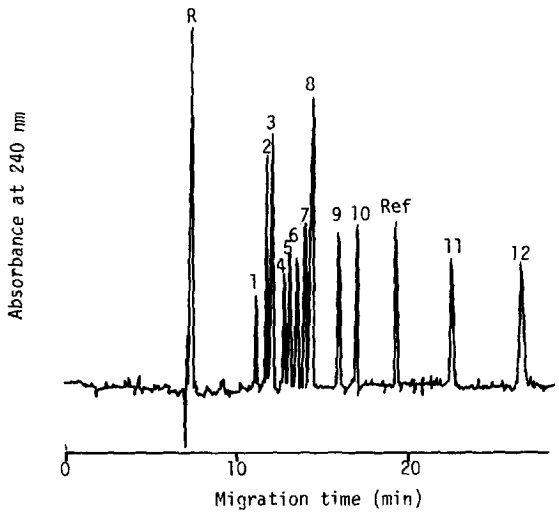

Fig. 12. Separation of naturally occurring monosaccharides as the in situ formed borate complexes of AP derivatives by zone electrophoresis [31]. Capillary, fused silica (50 μm I.D., 65 cm); carrier, 200 mM borate buffer (pH 10.5); applied potential, 15 kV; detection, UV absorption at 240 nm. Peaks: R = reagent (AP), Ref = reference (cinnamic acid), 1 = N-acetylgalactosamine, 2 = lyxose, 3 = rhamnose, 4 = xylose, 5 = ribose, 6 = N-acetylglucosamine, 7 = glucose, 8 = arabinose, 9 = fucose, 10 = galactose, 11 = glucuronic acid, 12 = galacturonic acid.

Since the uronic acids have an additional carboxyl group they were strongly retained at the anodic end and hence migrated slowest. The migration order of some monosaccharides, e.g. lyxose/xylose/ribose/arabinose, was somewhat different from that in anion-exchange chromatography as their borate complexes (lyxose/ribose/arabinose/xylose) [30].

Efficient separation in borate buffer was observed not only for AP derivatives but also for *p*-aminobenzoic acid (AB) [34], ethyl *p*-aminobenzoate (EAB) [35], and *p*-aminobenzonitrile (ABN) [36] derivatives. The migration order of aldoses commonly adopted in these four papers [31,34–36] was identical, and the theoretical plate numbers were at approximately the same level. The AB, EAB and ABNB derivatives can be monitored at 285, 305 and 285 nm, respectively, but they cannot be detected fluorometrically.

Addition of PMP groups to reducing carbohydrates causes easy complexation because the pyranose ring is ruptured to give an open-chain structure. Linearity of peak response was observed over the range 1 μM–1 mM, when detection was performed at the wavelength of maximum absorption (245 nm). Separation of the PMP derivatives in borate buffer was also excellent. The use of a slightly lower pH (pH 9.5) allowed complete resolution of all sets of aldopentose and aldohexose [37], as shown in Figs. 13a and 13b, respectively.

Under the conditions employed, the hydroxyl groups were complexed with the borate ion to give a negative charge. In addition the enol group in the PMP moiety was dissociated to yield another type of negative charge. Therefore, the migration times of the PMP derivatives of monosaccharides were somewhat longer than those of the PA derivatives, as seen in Fig. 12. It should be emphasized that this simple system allowed complete separation of all sets of aldose. The lower limit of detection was at the 10 μM level [23]. Separation of a few homologous series of glucose oligomers having various linkage types was also attempted. The slopes of the electrophoretic mobility vs d.p. plots slightly varied among the linkage types, presumably due to variation of the molecular shape of the carrier.

One more example to be discussed here is the separation of the isoindole derivatives by Liu et al. [38]. Since these derivatives, prepared by two-step reactions of reductive amination followed by condensation with 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) in the presence of the cyanide ion (Fig. 14), fluoresce intensely at 552 nm when excited by the 457-nm argon laser beam, the concentrations of the sample components can be lowered to the 10 nM level.

Thus the column efficiency was high (theoretical plate number 100 000–400 000) and better separation was obtained than with UV or ordinary fluorescence detection. For example, separation of glucose and mannose, which was not attained in the analysis of the PA derivatives, was achieved almost to baseline level by using a phosphate–borate buffer (pH 9.4) as carrier. Although further studies should be done for quantitative derivatization, the advantage of ultramicro analysis by laser-induced fluorescence

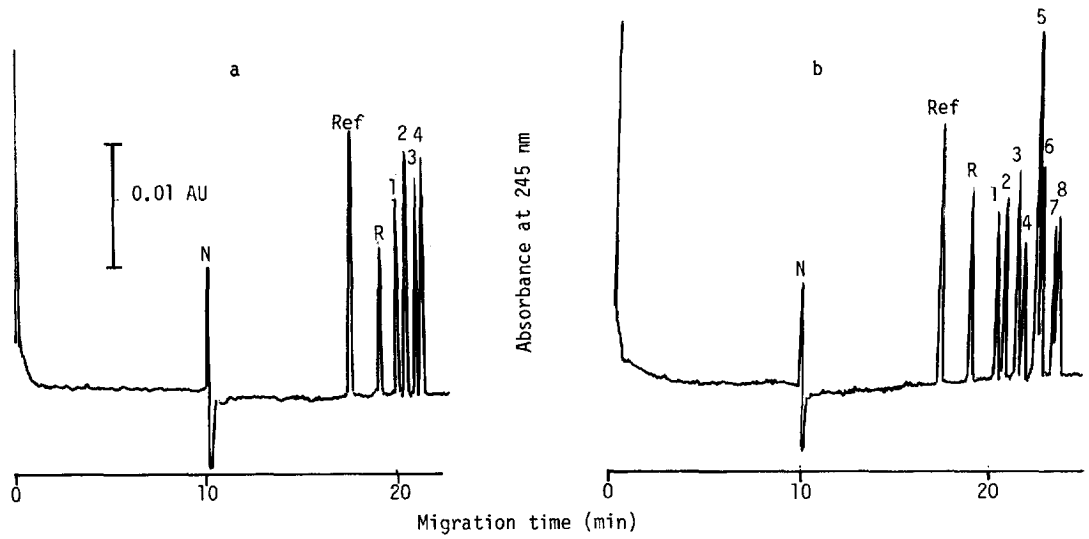

Fig. 13. Separation of aldopentoses (a) and aldohexoses (b) as the in situ formed borate complexes of PMP derivatives by zone electrophoresis [37]. Capillary, fused silica (50 μm I.D., 78 cm), carrier, 200 mM borate buffer (pH 9.5), applied voltage, 15 kV; detection, UV absorption at 245 nm. N = neutral marker (methanol), Ref = reference (amobarbital), R = reagent (PMP). Peaks in (a): 1 = xylose, 2 = arabinose, 3 = ribose, 4 = lyxose. Peaks in (b): 1 = glucose, 2 = allose, 3 = altrose, 4 = mannose, 5 = idose, 6 = gulose, 7 = talose, 8 = galactose. All carbohydrates are of the D-series.

detection will be of potential value in the analysis of biological samples.

The idea of in situ conversion to ions can be extended to other reaction types such as complexation with metal ions. For example, addition of a carbohydrate sample to an aqueous solution of an alkaline earth metal acetate causes complexation with the metal ion and the complexes formed from the component carbohydrates are separated from each other by the difference of their capability of complexation, as illustrated in Fig. 15. Fig. 16a shows the complete separation

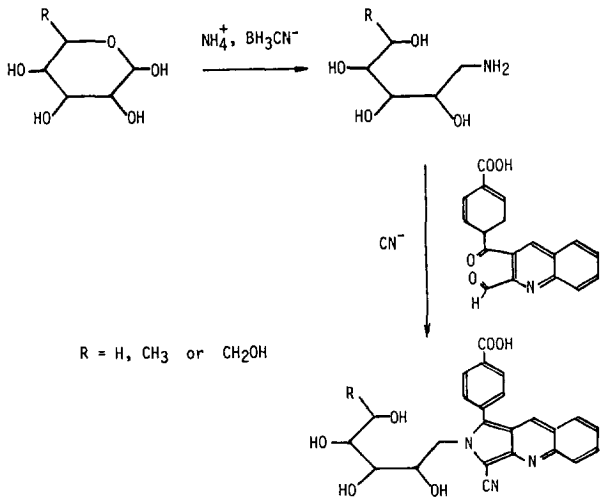

Fig. 14. Conversion of reducing carbohydrates to isoindole derivatives by transformation to glycamines, followed by condensation with 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde in the presence of the cyanide ion [38].

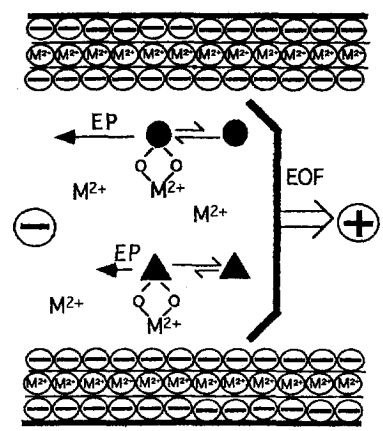

Fig. 15. Illustration of the separation of carbohydrates as in situ formed metal chelates by zone electrophoresis. EOF = electroosmotic flow, EP = electrophoresis, M^{2+} = alkaline earth metal ion.

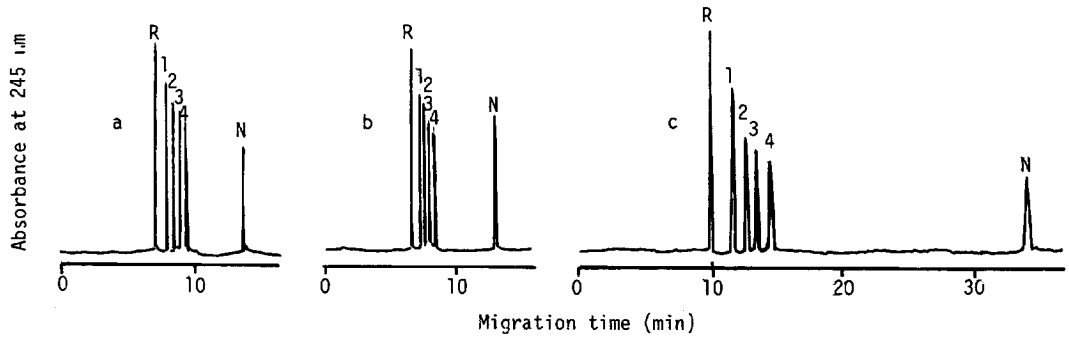

Fig. 16. Separation of aldopentoses as the in situ formed metal complexes of PMP derivatives by zone electrophoresis [39]. Capillary, fused silica (50 μm I.D., 53 cm); carrier, 100 m*M* aqueous solutions of calcium acetate (a), barium acetate (b) and strontium acetate (c); applied voltage, 10 kV; detection, UV absorption at 245 nm. Peaks: R = reagent (PMP), N = neutral marker (mesityl oxide), 1 = ribose, 2 = lyxose, 3 = arabinose, 4 = xylose. All carbohydrates are of the D-series.

of the PMP derivatives of aldopentoses in aqueous 100 m*M* calcium acetate [39].

Aqueous solutions of barium acetate and strontium acetate gave similar results, as can be seen in Figs. 16b and 16c, respectively. It should be noted that the electroosmotic flow was reversed in these analyses, because these metal ions are adsorbed on the capillary inner wall and thus alter the sign of the zeta potential. This reversion proceeds slowly and once an equilibrium is reached, the velocity of the electroosmotic flow becomes constant. The PMP derivatives reacted with these metal ions to give complexes. These complexes have a positive charge around the metals, but they also have a negative charge around the enol groups in the PMP moiety. The total charge is negative, but the degree of negativity depends on the aldopentose species. Consequently the excess reagent (PMP) surviving the cleanup appeared first, followed by the PMP derivatives of aldopentoses, separated in the order of increasing negativity, and finally the neutral marker (mesityl oxide) was detected.

5. Micellar electrokinetic chromatography

Micellar electrokinetic chromatography is an extension of zone electrophoresis and is a powerful method for the separation of non-ionic compounds. It is based on their solubilization in moving ionic micelles in the carrier and separation is achieved by the difference in hydrophobicity [40]. Thus, separation of intact carbohydrates, which are almost non-hydrophobic, is impossible. However, precapillary conversion to hydrophobic derivatives enables separation by this mode.

ABN derivatives were separated in a neutral carrier containing a high concentration of sodium dodecylsulfate (SDS). Fig. 17 shows an example of such a separation [36]. At the neutral con-

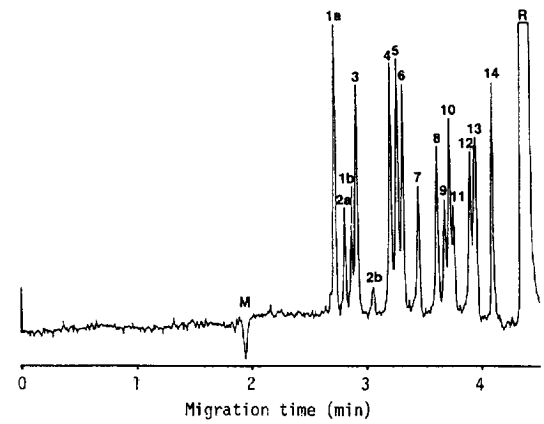

Fig. 17. Separation of ABN derivatives of mono- and oligosaccharides by SDS–micellar electrokinetic chromatography [36]. Capillary, fused silica (50 μm I.D., 55 cm); carrier, 25 m*M* Tris-phosphate buffer (pH 7.5) containing SDS to a concentration of 100 m*M*. Peaks: M = methanol, R = reagent (ABN), 1a and 1b = fructose, 2a and 2b = sorbose, 3 = lactose, 4 = melibiose, 5 = cellobiose, 6 = maltotriose, 7 = maltose, 8 = mannose, 9 = glucose, 10 = galactose, 11 = ribose, 12 = lyxose, 13 = arabinose, 14 = xylose.

ditions employed (pH 7.5) the ABN derivatives did not possess an electric charge, but hydrophobicity was increased by the introduction of the benzonitrile group. Because of the slight variation in hydrophobicity among the ABN derivatives, a number of mono- and oligosaccharides were well separated in a short time (ca. 5 min). However, a drawback of this method is that the derivatization reaction is not quantitative [36].

PMP derivatives of isomaltooligosaccharides were also well separated by this mode [23]. The hydrophobicity of the derivatives is caused by the PMP moiety, and the number of PMP groups is the same (two) for all derivatives independent of d.p. The overall hydrophobicity decreases with d.p. since the proportion of the carbohydrate moiety increases. On the other hand the SDS micelles migrated to the cathode at a constant velocity. As a result the PMP derivatives of isomaltooligosaccharides moved to the cathode in the order of decreasing d.p., since the derivatives having higher d.p.s are less well solubilized into the micelles. Thus the derivatives were separated by the differences in their d.p., apparently similar to direct zone electrophoresis at high pH.

6. Size-exclusion in gel-packed capillaries

The use of gels for size exclusion has already been generalized in peptide and DNA fragment analyses, but in those cases slab or disc gels were used. The application of capillary electrophoresis in gel-packed capillaries is a recent topic in the analysis of the human genome (e.g. Ref. [41]), but there are only few examples of the application to carbohydrate analysis. Fig. 18 shows the separation of an autoclave-hydrolysed mixture of poly(galacturonic acid) as isoindole derivatives in a capillary tube packed with polyacrylamide gel in an alkaline Tris-borate buffer, as detected by argon laser-induced fluorescence [42]. The separation was excellent; oligomers having even-numbered d.p.s less than 66 could be discriminated from each other. The electroosmotic flow in such gel-packed capillaries can be neglected and the derivatives migrated to the anode solely by electrophoresis, since they were negatively charged due to the alkalinity of the medium. Separation was obviously because of size exclusion through micropores in the gel, but the different eases of complexation with the borate ion should also be taken into consideration. The high column efficiency was due not only to the anticonvective effect of the gel, but also to small amounts of introduced sample (at the amol level). Although data were not shown, the authors described that application to partial hydrolysates of a few glycosaminoglycans such as hyaluronic acid, chondroitin sulfate and heparin-like polymers also gave high resolution of oligomers. Chiesa and Horváth [26] followed the work of Liu et al. [42] and found that the electrophoretic mobility was approximately proportional to the molecular mass to the $-2/3$ power, as in free solution analysis.

7. Conclusions

Neutral carbohydrates, which are non-ionic and least hydrophobic, are the most difficult compounds to be separated by capillary electrophoresis, but various devices mainly based on chemical treatment, permit rapid, high-resolution separation. The high capability makes capillary electrophoresis as convenient as or more convenient than high-performance liquid chromatography. It should be pointed out that capillary electrophoresis is performed in free solution in most cases, which means that separation is done simply in a carrier solution, i.e. a single phase. This is comparable to various types of chromatography in which separation is based on the differences in sample distribution between solid and mobile phases. This may bring about many advantages for capillary electrophoresis. Above all, the easy operation and change of carrier solution, especially when automated apparatus are used, allows rapid multiple-mode analysis using the same capillary tube over and over again. The high reliability of multidimensional analysis for identification of a number of series of compounds, e.g. oligosaccharides in glycopro-

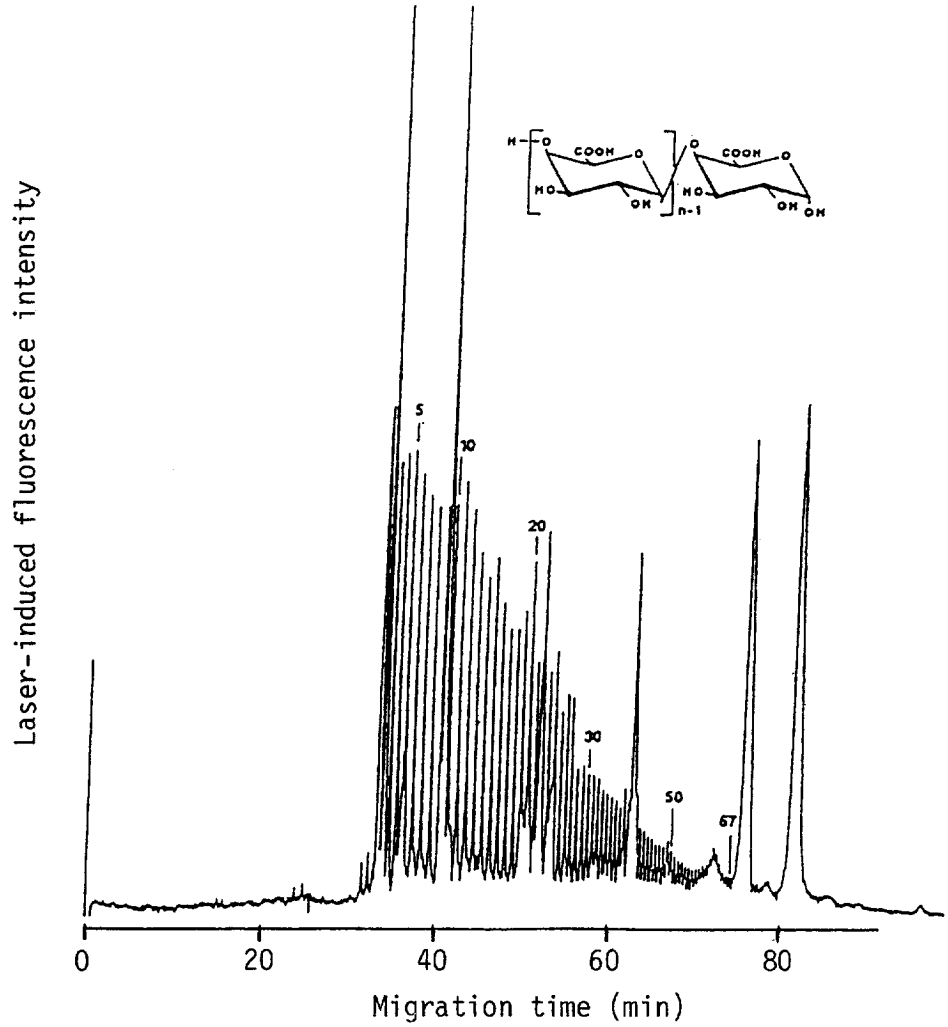

Fig. 18. Separation of an autoclave-hydrolysed poly(galacturonic acid) by size-exclusion electrophoresis in a polyacrylamide gel-packed capillary [42]. Capillary, 50 μm I.D., 32 cm (effective length, 23 cm); gel, 18% T, 3% C, impregnated with 1×100 m*M* Tris–250 m*M* borate–2 m*M* EDTA (pH 8.48); injection, electromigration (5 kV, 25 s); applied voltage, 234 V/cm. Peak numbers represent d.p.s.

teins, will be described in another review in this issue [6].

As generalized in books (e.g. Ref. [43]) and reviews (e.g. Ref. [44]) on capillary electrophoresis, migration time is not a suitable index to characterize compounds, the main cause being the variation of electroosmotic flow among analytical runs. Under conditions that ensure constancy of temperature, however, electrophoretic mobility is a good index for identification, because it is reproducible, provided that impurities adsorbed on the capillary inner wall are completely removed by rinsing with appropriate washing fluid(s) and the tube is equilibrated with the running carrier before each run. The electrophoretic mobility can be calculated by $L \cdot I \cdot V^{-1}$ $(t_0^{-1} - t^{-1})$, where L, I, V, t_0 and t are total tube length, effective tube length (between the inlet and the detector), applied voltage, migration time of a neutral marker and migration time of

the compound to be examined, respectively. The relative standard deviation (R.S.D.) of the electrophoretic mobility is normally less than 1% under thermostated conditions. This is not higher than that found in high-performance liquid chromatography. In situ conversion to ions during analysis does not cause significant increase of the R.S.D. Although quantification in capillary electrophoresis was not described in detail in this article, it is not special for carbohydrate analysis; it is as reproducible as in high-performance liquid chromatography using an internal standard.

This article has focused on the basic aspects of capillary electrophoresis, and the analytes described were confined to simple model carbohydrates. For practical applications the review by Kakehi and Honda should be consulted [6]. For sensitive and ultrasensitive detection the review by Paulus and Klockow gives useful information [5].

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