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Enhancement of the zone electrophoretic separation of 1-phenyl-3-methyl-5-pyrazolone derivatives of aldoses as borate complexes by concerted ion-interaction electrokinetic chromatography with Polybrene

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Abstract

It was confirmed that PMP derivatives of aldopentoses and aldohexoses gave anionic borate complexes in borate buffer and moved to the cathode in an uncoated fused-silica capillary by the combined effects of electroosmosis and electrophoresis. All epimers of each group were completely separated from each other at pH 9.5 because of the difference of the ease of complexation among epimers. Addition of Polybrene to the borate buffer caused reversal of moving direction of PMP derivatives of aldopentoses and aldohexoses due to the ionic association of the resultant borate complexes (ion-interaction electrokinetic chromatography) with Polybrene. The separation of peaks was greatly enhanced when appropriate conditions were used. For example the resolution was prominently increased for all neighboring peaks under the conditions of 50 m*M* borate and 1% Polybrene concentrations at pH 9.5. The migration order partly changed depending on the conditions employed. This change of resolution can be explained by alteration of the molar fractions of borate complexes, due to exertion of the ionic interaction before attainment of the complexation equilibria. © 1998 Elsevier Science B.V.

Keywords: Buffer composition; Phenylmethlpyrazolone; Aldoses; Polybrene; Carbohydrates; Borate complexes

1. Introduction

The high capabilities of capillary electrophoresis (CE) in both separation and detection are widely recognized nowadays in separation science and many related fields, so it will not be necessary to mention details of this method here. It will be enough to point out that it is also a powerful tool even for carbohydrates, which normally have no electric charge [1].

In CE, samples are usually analyzed in free solution using a running buffer of a specified composition, hence allowing separation in a single mode. Since the running buffer can be changed easily one after another, especially when the apparatus is equipped with an automatic sampler, CE permits analysis of a sample by multiple modes and thereby enables highly reliable identification of its components.

Carbohydrate analysis by CE is favored by this advantage, and a number of separation modes have hitherto been developed, especially for 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives, including

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direct zone electrophoresis [2], zone electrophoresis as borate [3] or metal [4] complexes, sodium dodecyl sulfate (SDS) micellar electrokinetic chromatography [4,5], hydrogen bonding with optically active additives [6], etc. However, all studies of carbohydrate analysis by CE have been made based on separation by a single mode, and there have been no studies based on separation by combined modes.

This paper describes improvement of separation of PMP derivatives of monosaccharides by combining zone electrophoresis as borate complexes and ion-interaction electrokinetic chromatography with Polybrene.

2. Experimental

2.1. Apparatus

CE was performed using a Waters Quanta 4000 apparatus equipped with an automatic sampler and a UV detector. A roll of a fused-silica capillary having an inner diameter of 50 μm was obtained from Polymicro Technologies (Phoenix, AZ, USA) and ca. 60-cm portions were cut from the role and each of them was installed on the apparatus. The polyimide coating at a 5-cm position from the outlet was removed by burning and positioned at the detector window. Each piece of capillary was flushed with 0.1 *M* sodium hydroxide for a sufficient period of time before each series of experiments and rinsed with a running buffer at least for 10 min before each run. The PMP derivatives of carbohydrates have an absorption maximum at 245 nm, but they were detected at 254 nm by using a mercury lamp, because the detector installed in the apparatus was of a discontinuous wavelength type. It had also a zinc lamp (λ_{max}=214 nm), but the absorption at this wavelength was weaker than that at 254 nm. Sample solutions were introduced to the capillaries by gravity by maintaining the sample solution level higher than the cathodic solution level by 10 cm for 10 s.

2.2. Chemicals

Polybrene was purchased from Sigma–Aldrich Japan (Tokyo, Japan) and used as obtained. PMP was obtained from Kishida (Osaka, Japan) and recrystallized twice from methanol before use. All other chemicals were of the highest grade commercially available. The carbohydrate samples were obtained from the following sources: arabinose, xylose and glucose from Nakarai Tesque (Kyoto, Japan); ribose and mannose from Wako (Osaka, Japan); galactose from Kishida; lyxose, allose, altrose, gulose, idose and talose from Sigma (St. Louis, MO, USA). All of them were of the D-series. Water deionized and distilled in a glass ware was used for preparation of electrophoretic solutions.

2.3. Derivatization of aldopentoses and aldohexoses

This was performed according to our previous paper [7]. Briefly, the procedure was as follows. To a sample of an aldose (100 nmol) or a mixture of aldoses (100 nmol each) were added a 0.5 *M* methanolic solution of PMP (50 μl) and 0.3 *M* sodium hydroxide (50 μl). They were well mixed and the resultant solution was heated for 30 min at 70°C. After it was cooled to room temperature, it was neutralized with 0.3 *M* hydrochloric acid (50 μl), and evaporated to dryness. The sodium hydroxide and hydrochloric acid solutions were exactly normalized to the specified concentration, and exactly the specified volume of these solutions was taken by using a whole pipet. Water and chloroform (200 μl each) were added to the residue and the whole was vigorously shaken. The organic layer was discarded and the aqueous layer was re-extracted two more times with chloroform in a similar manner. The aqueous layer finally obtained was subjected to CE.

3. Results and discussion

3.1. Separation of PMP derivatives of aldopentoses and aldohexoses by zone electrophoresis as borate complexes

3.1.1. PMP–aldopentoses

We already reported the separation of 2-aminopyridine (AP) derivatives of carbohydrates in alkaline borate buffer [8]. When AP derivatives of monosaccharides were introduced to alkaline borate buffer in an uncoated fused-silica capillary, rapid

electroosmotic flow (EOF) generated and flowed towards the cathode. At the same time they are pulled back to the anode by electrostatic force, because the hydroxyl groups in the carbohydrate moiety reacted with the borate ion to give negatively charged borate complexes. The overall movement of AP-monosaccharides was towards the cathode, because the velocity of EOF to the cathode was higher than that of electrophoretic migration to the anode. They moved slower than a neutral marker, and retardation was more marked as pH increased, because the molar fraction of the resultant anionic complexes increased. Resolution between neighboring peaks (R_s) varied due to the variation of the ease of complexation among monosaccharide species.

PMP derivatives gave more strongly negative complexes than AP derivatives because of the additional dissociation of the enol group in the pyrazolone ring, which was formed from the keto group by tautomerism, and they were separated much more clearly than AP derivatives. The PMP derivatives of all aldopentose epimers were completely separated from each other at pH 9.5 without significant tailing, as shown in Fig. 1.

3.1.2. PMP–aldohexoses

The PMP derivatives of aldohexose epimers also gave anionic borate complexes, but the derivatives of all epimers were not completely separated from each other under the identical conditions as mentioned above for PMP–aldopentoses (50 m*M* borate buffer, pH 9.5), as shown in Fig. 2a.

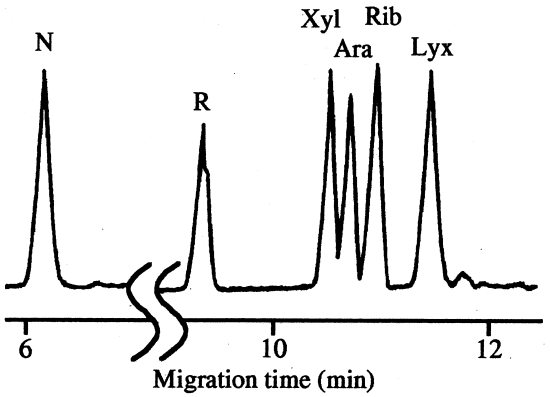

Fig. 1. Zone electrophoretic separation of PMP–aldopentoses as borate complexes. Capillary, uncoated fused silica (60 cm×50 μm I.D.); carrier, 50 m*M* borate buffer (pH 9.5); applied voltage, 10 kV; detection, absorption at 254 nm. The sample solution was introduced from the anodic end of the capillary. N, neutral marker (cinnamyl alcohol); R, reagent (PMP). Xyl, xylose; Ara, arabinose; Rib, ribose; Lyx, lyxose.

However, migration time increased and separation was complete, in accordance with our previous results [3], when 200 m*M* borate buffer of the same pH value was used (Fig. 2b). The difference of migration times was due to the difference of EOF, because the capillaries used were from different sources. In response to this increase of the borate concentration the migration order of PMP–allose and PMP–altrose was reversed. This result implies that the increase in the borate concentration caused increase in the molar fraction of the borate complexes but the increasing rate was varied among hexose epimers. The excellent separation of the derivatives of all aldohexoses achieved in 200 m*M* borate buffer was superior to any other reported separations performed by various analytical methods.

3.2. Effect of the addition of Polybrene

3.2.1. PMP–aldopentoses

Separation of PMP–aldopentoses in borate buffer was greatly influenced by the addition of Polybrene, a polycationic water-soluble polymer. Although no peaks of PMP–aldopentoses were detected when they were introduced from the anodic end, well-resolved peaks appeared when introduced from the cathodic end (Fig. 3).

The migration times were longer than those in the Polybrene-free system with the borate concentration of 50 m*M* (Fig. 1) and all peaks were almost symmetrical.

At pH values of 7.0, 8.0 and 9.0 the resolution of the peaks of the arabinose–ribose (△) and ribose–lyxose (□) pairs increased with increasing Polybrene concentrations, but the resolution of the peaks of the xylose–arabinose pair (○) gave convex curves (Fig. 4a). At pH 10.0 all curves were almost convex. The highest R_s value (as high as ca. 13) was observed at a Polybrene concentration of 0.5% at pH 10.0.

On the other hand resolution increased at any Polybrene concentration as pH increased between 7 and 10 (Fig. 4b). The curves of the arabinose–ribose

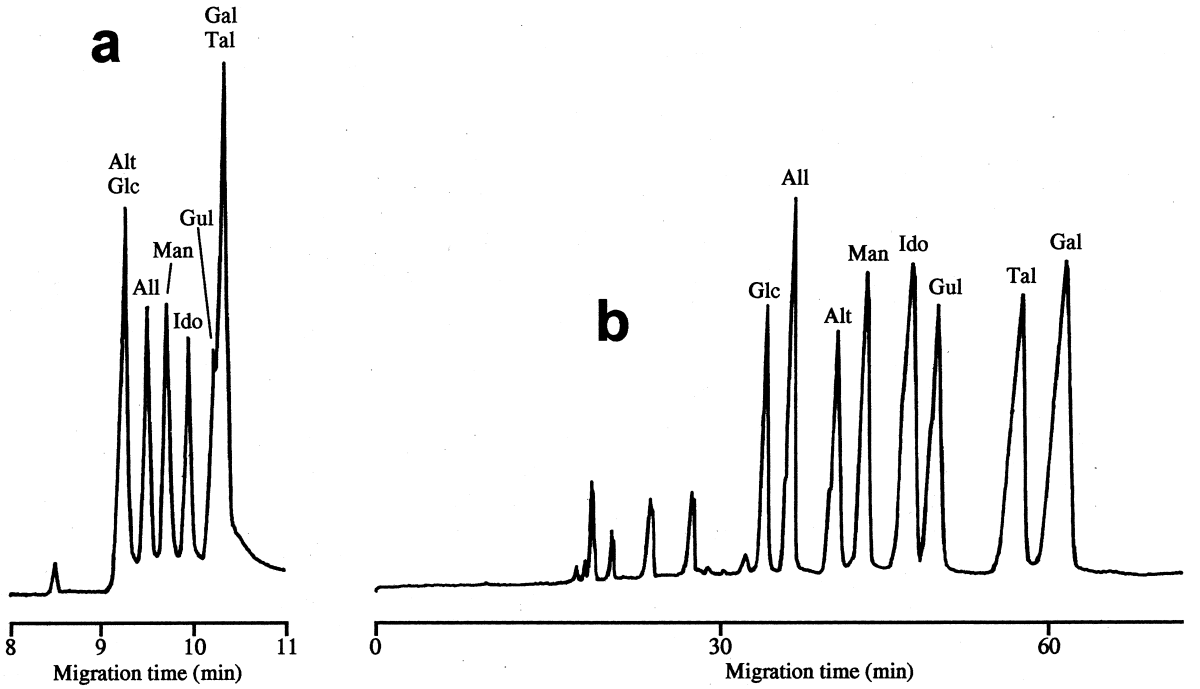

Fig. 2. Zone electrophoretic separation of PMP–aldohexoses as borate complexes. Capillary, uncoated fused silica (58 cm×50 μm I.D.); carrier, 50 (a) or 200 m*M* (b) borate buffer (pH 9.5); applied voltage, 15 kV; detection, absorption at 254 nm. Glc, glucose; Alt, altrose; All, allose; Man, mannose; Ido, idose; Gul, gulose; Tal, talose; Gal, galactose. Minor peaks were of impurities. The other conditions and abbreviations as in Fig. 1.

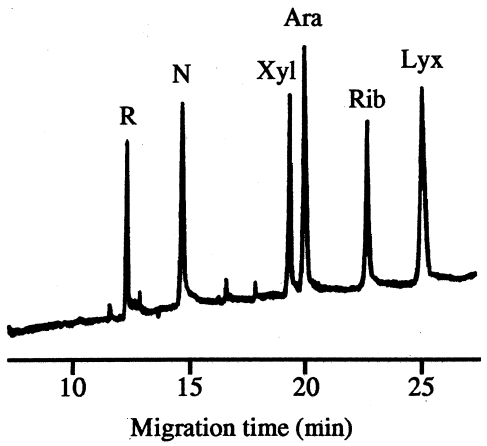

Fig. 3. Separation of PMP–aldopentoses in a borate buffer containing Polybrene. Carrier, 50 m*M* borate buffer (pH 9.5) containing Polybrene to a concentration of 1.0%. The sample solution was introduced from the cathodic end of the capillary. The other analytical conditions and abbreviations as in Fig. 1.

and ribose–lyxose pairs crossed each other at pH 9.0, at Polybrene concentrations of 0.5 and 1.0%.

The above observations for PMP–aldopentoses can be explained as follows. Added Polybrene will be adsorbed on the surface of the inner wall of the capillary to change the sign of the zeta potential between the inner wall and the electrophoretic solution. As a result EOF flowed toward the anode. PMP–aldopentoses will be complexed with the borate ion and drawn to the anode as in the Polybrene-free system. However, the situation is different from the Polybrene-free system in that the anionic borate complexes of PMP–aldopentoses will interact strongly with Polybrene which is moving rapidly to the cathode at a constant velocity. This interaction is considered to be so strong that the PMP–aldopentoses change the moving direction to the cathode. Thus, the PMP–aldopentose peaks appeared after the peak of cinnamyl alcohol as a neutral marker. The fact that PMP–aldopentoses were detected in the

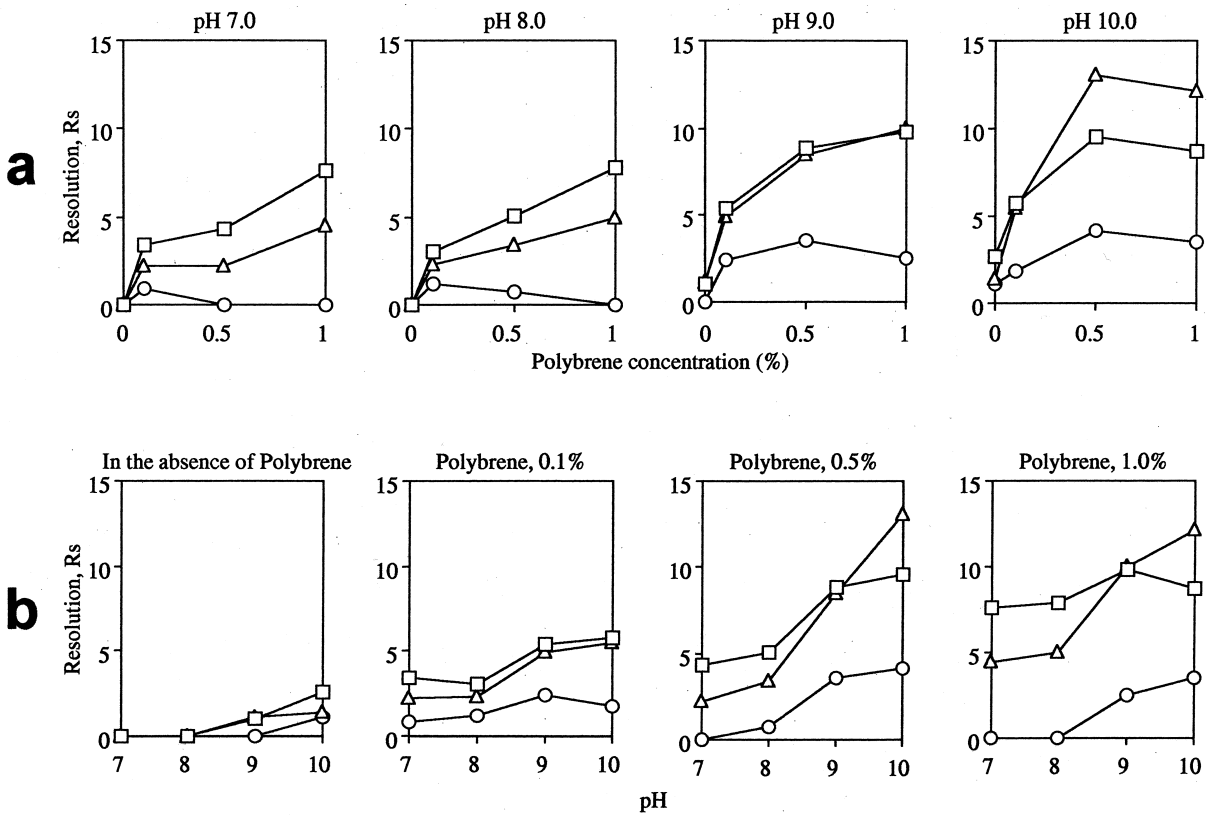

Fig. 4. Effects of Polybrene concentration (a) and pH (b) on the resolution (R_s) of the neighboring peaks of PMP–aldopentoses. Polybrene concentration of carrier, 0, 0.1, 0.5, 1.0%; pH of carrier, 7.0, 8.0, 9.0, 10.0. (○) Xylose–arabinose pair; (△) arabinose–ribose pair; (□) ribose–lyxose pair. The other analytical conditions as in Fig. 3.

same order as that in the Polybrene-free system under most conditions implies that more strongly negative complexes interacted more strongly with Polybrene.

We can assume that the observed electrophoretic mobility (μ_{obs}) of a solute interacting with an additive is the algebraic sum of its mobility due to zone electrophoresis (μ_{CZE}) and the hypothetical mobility due to such interaction ($\mu_{interaction}$). The value of μ_{CZE} can be estimated as the observed mobility of the solute in the additive-free system. Therefore, one can obtain the $\mu_{interaction}$ value by subtracting μ_{CZE} from μ_{obs} thus estimated. Fig. 5a indicates the effect of Polybrene concentration on $\mu_{interaction}$ of each PMP–aldose at various pH values, and Fig. 5b shows the pH dependence of $\mu_{interaction}$ at various Polybrene concentrations.

In both series of graphs the mobility corresponding to the movement from the cathode to the anode carries the plus sign, and vice versa. At pH values of 7.0 and 8.0 $\mu_{interaction}$ decreased (its absolute value increased) with increasing Polybrene concentrations for all PMP–aldopentoses, but it gave convex curves at pH 9.0 and 10.0 (Fig. 5a). It is also indicated that $\mu_{interaction}$ decreased rapidly in a wide range with increasing pH values at a Polybrene concentration of 0.1% (Fig. 5b). At a Polybrene concentration of 0.5% the decreasing rate became sluggish and the range of decrease was narrower. At a Polybrene concentration of 1.0% $\mu_{interaction}$ was almost flat above pH 8 (Fig. 5b). These relationships are presumably because the concentration of the interacting ionic species of Polybrene is insufficient at low Polybrene concentrations and low pH values.

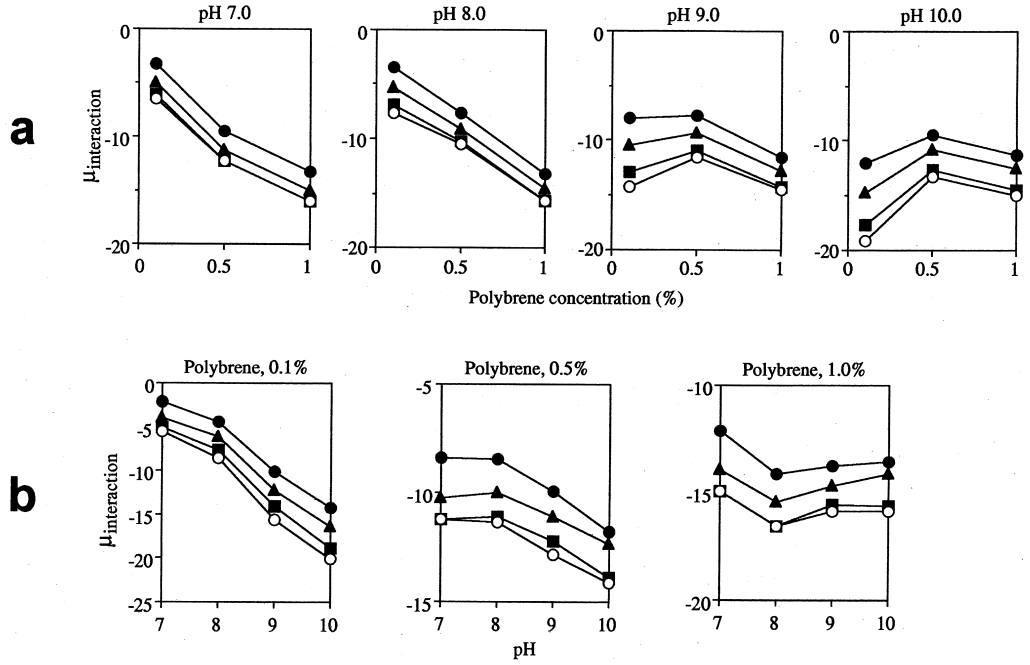

Fig. 5. Effects of Polybrene concentration (a) and pH (b) on the hypothetical mobility due to the ionic interaction between the anionic borate complex and Polybrene ($\mu_{interaction}$). The data source was the same as in Fig. 4. (●) Lyxose; (▲) ribose; (■) arabinose; (○) xylose.

Its concentration will become high enough as Polybrene concentration and pH increase.

3.2.2. PMP–aldohexoses

The separation of PMP–aldohexoses was also affected by the addition of Polybrene.

When Polybrene was added to the 50 m*M* borate buffer (pH 9.5), well-resolved peaks began to appear at a sufficient Polybrene concentration of 1.0% (Fig. 6), with migration times in the range of 15–24 min (Fig. 6). The sample of a PMP–aldohexose mixture was introduced from the cathodic end, as in Fig. 3. The range of migration times was longer than that in the absence of Polybrene (9–11 min).

It is noted that the migration order was somewhat different from that in the Polybrene-free system.

Fig. 7 compares the resolution of neighboring peaks between Polybrene-free and Polybrene-containing systems at pH 9.5, at borate and Polybrene concentrations of 50 m*M* and 1.0%, respectively.

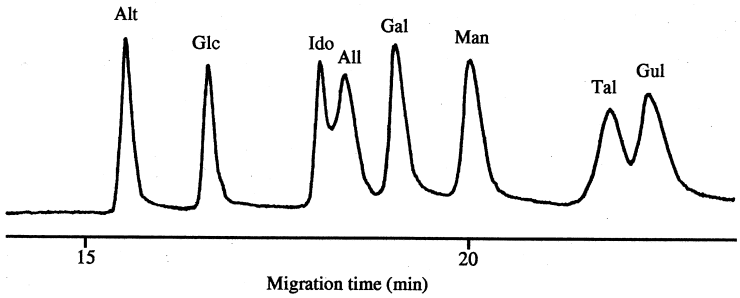

Fig. 6. Separation of PMP–aldohexoses in a borate buffer containing Polybrene. Carrier, 50 m*M* borate buffer (pH 9.5) containing Polybrene at a concentration of 1%. The sample was introduced from the cathodic end of the capillary. Other analytical conditions and abbreviations as in Fig. 2.

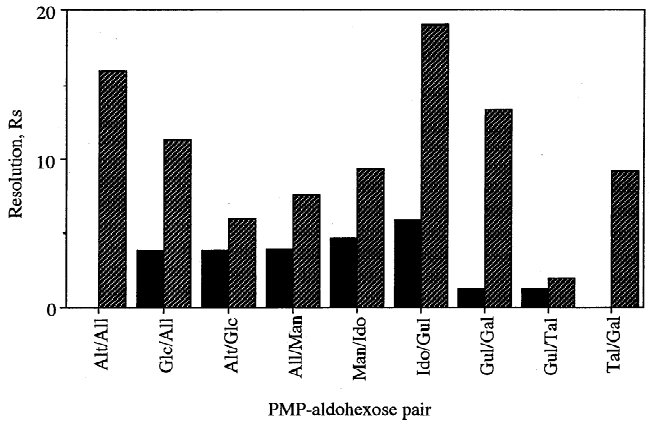

Fig. 7. Comparison of the change of resolution of the peaks of PMP–aldohexoses caused by addition of Polybrene. The black bars indicate the Polybrene-free system, and the bars with slanting lines Polybrene-containing system. The data were taken from Figs. 2 and 6.

In this case the change of resolution for the neighboring pairs is discussed based on the Polybrene-free system (Fig. 2a), but not on the Polybrene-containing system (Fig. 7). It is indicated that in all these pairs the resolution of peaks increased by the addition of Polybrene, and the increase was the most outstanding for the allose–altrose pair which gave a ΔR_s value of ca. 17. Addition of Polybrene to 200 m*M* borate buffer also resulted in increase of the resolution for the peaks of the altrose–mannose, idose–gulose and gulose–talose pairs, but practically no change was observed for the talose–galactose pair (data not shown). The other pairs rather showed slight decrease of resolution. In this case the effect of the addition of Polybrene was relatively small as compared to the separation of PMP–aldopentoses mentioned above, which was performed in 50 m*M* borate buffer, as evidenced by the shorter migration times (6–10 min) than those (33–63 min) in the Polybrene-free system.

3.3. Speculation of the reason for the change of resolution in the combined modes

Terabe and Isemura [9] first reported fundamental observations on the effect the addition of Polybrene on the zone electrophoretic migration of some aromatic sulfonates and called this mode ion-exchange electrokinetic chromatography. However, they did not describe its effect on resolution of these acidic compounds [9]. Our preliminary experiments using similar compounds (aromatic carboxylates) indicated that there was no significant change of resolution by addition of Polybrene (data not shown).

In the borate–Polybrene mixed additive system the borate complexes of PMP–aldopentoses and PMP–aldohexoses were subjected to both zone electrophoresis and ion-interaction electrokinetic chromatography with Polybrene. These two effects counteracted each other, and it is suggested that in the 50 m*M* borate buffer-based systems the latter effect was greater than the former, as seen from the longer migration times of both PMP–aldopentoses and PMP–aldohexoses in the presence of Polybrene (measured after being introduced from the cathodic end) than those in the absence of Polybrene (measured after being introduced from the anodic end).

In borate buffer, the zone electrophoretic effect is associated with the in situ formation of an anionic borate complex, which is considered to be a relatively slow kinetic process and its velocity will be varied among aldose species. On the other hand, the ion-interaction electrokinetic chromatography with Polybrene is a mere ionic association between the inversely charged species (a negatively charged borate complex and a positively charged Polybrene ion), which is probably very rapid. Therefore, it is

possible that this rapid ionic association could cause a change of the molar fraction of the borate complex in the presence of Polybrene, by interrupting completion of equilibration. Since such interruption will affect borate complexation to varying extents depending on aldose species, it will cause alteration of molar fraction and, accordingly, relative migration time, resulting in change of resolution to varying magnitudes among aldose species.

Resolution is defined as the ratio of the difference in migration time between two peaks to the average peak width. Therefore, the change of peak width should also be discussed in parallel with the speculation on the change of migration time mentioned above. However, comparison of the corresponding peaks between the Polybrene-containing and Polybrene-free systems indicates that the former was generally wider than the latter; namely addition of Polybrene rather caused peak broadening, instead of peak sharpening. Consequently the migration time difference is considered to be the predominant factor to increase resolution.

4. Conclusion

The excellent separation of PMP derivatives of aldopentoses and aldohexoses in borate buffer was confirmed. The addition of Polybrene to the borate buffer caused reversal of moving direction and resulted in change of resolution. Selection of an appropriate Polybrene concentration and a relevant pH value enabled great enhancement of separation. The predominant role of the large increase of migration time difference in the increased resolution was ascertained, and this increase of resolution was presumably due to the alteration of the molar fractions of borate complexes.

The principle of separation enhancement realized in this work may be extended to improvement of separation of a number of other biological substances, such as amines, amino acids, peptides, carboxylic acids, etc., by adding an appropriate complexing agent and a selected macromolecular counterion.

Acknowledgements

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