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Unusual separation of 1-phenyl-3-methyl-5-pyrazolone derivatives of aldoses by capillary zone electrophoresis

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Abstract

The 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives of aldoses showed unusual behavior in capillary zone electrophoresis in neutral buffers, giving excellent separation. Examination of the migration behavior of the PMP derivatives of aldopentoses and aldohexoses indicated that the derivatives of epimers having the 2,3-*trans* hydroxyl groups migrated faster than those having the 2,3-*cis* hydroxyl groups. This phenomenon can be explained by intramolecular ring formation by hydrogen bonding between the carbonyl group in the pyrazolone ring and the hydroxyl groups at C-2 and C-3 in the carbohydrate moiety. The 2,3-*trans* disposition will be favorable for ring formation and thereby cause reduction of the negative charge due to suppressed enolization. © 1997 Elsevier Science B.V.

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1. Introduction

Capillary electrophoresis (CE) is a powerful tool for the simultaneous analysis of biological substances, because of its high capabilities in separation and detection. It has been applied so widely that even neutral compounds, such as carbohydrates, have become the subject of analysis by this method.

In applying CE to carbohydrate analysis, pre-capillary derivatization plays an important role, since most of carbohydrates have neither chromophores nor fluorophores that can be detected by conventional methods. Pre-capillary derivatization has another significance in that it endows neutral carbohydrates with an electric charge and, thereby, enables their separation in an electric field.

A number of methods have been developed for the pre-capillary derivatization of carbohydrates, including those based on reductive amination with 2-aminopyridine [1], 4-aminobenzoic acid and its derivatives [2–4], 6-aminoquinoline [5], amino-naphthalene-mono- [6], -di- [7] or -trisulfonate [8] and 1-aminopyrene-3,6,8-trisulfonate [9]. A method involving reduction to glycamines, followed by reaction with 3-(4-carbobenzoyl)-2-quinolinecarboxaldehyde in the presence of cyanide [10] also belongs to this type of derivatization. We have developed a method based on condensation with 1-phenyl-3-methyl-5-pyrazolone (PMP) [11]. This method is excellent, because the derivatization proceeds under very mild conditions (30 min at 70°C in an almost neutral medium) and the derivatives absorb strongly (ε, ca. 20 000) in the ultraviolet region (λ_{max}, 245 nm) and are readily oxidizable on a glassy carbon electrode. For these reasons, we have been undertaking a study to develop a number of

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separation modes for carbohydrate analysis as PMP derivatives. In the course of this study, we observed an unexpected separation of PMP derivatives of small carbohydrate molecules. This paper describes this interesting phenomenon for PMP–aldoses under neutral conditions.

2. Experimental

2.1. Apparatus

CE was performed on a Waters Quanta 4000 apparatus equipped with a discontinuous-wavelength UV monitor. A fused-silica capillary (I.D. 50 μm, 60 cm) was obtained from Polymicro Technologies (Phoenix, AZ, USA). It was rinsed thoroughly with 0.1 *M* sodium hydroxide and equilibrated with the running buffer before use. Sample solutions were introduced at the anodic end of the capillary, using hydrodynamic pressure by raising the level of the sample solution 10 cm higher than that of the cathodic solution for 10 s. Although the absorption maxima of the PMP derivatives were at 245 nm, detection was carried out at 254 nm, because no lamp was available to irradiate the 245 nm light. Detection was carried out at a position that was 52 cm from the inlet.

2.2. Chemicals

PMP, obtained from Kishida Chemicals (Dosho-machi, Chuo-ku, Osaka, Japan), was recrystallized twice from methanol before use. The other chemicals were of the highest grade commercially available and were used as obtained. Reagent-grade specimens of carbohydrates were obtained from the following sources: Arabinose, xylose and glucose were from Nakarai Tesque (Nijo-karasuma, Nakakyou-ku, Kyoto, Japan); ribose and mannose were from Wako Pure Chemicals (Dosho-machi, Chuo-ku, Osaka, Japan); galactose was from Kishida Chemicals; lyxose, allose, altrose, gulose, idose and talose were from Sigma (St. Louis, MO, USA).

2.3. Derivatization

Derivatization was performed according to our previous paper [11]. Briefly, a sample of an aldose (100 nmol), or a mixture of aldoses, was dissolved in 0.3 *M* sodium hydroxide (50 μl) and a 0.5 *M* methanolic solution (50 μl) of PMP was added. Since PMP was neutralized with sodium hydroxide, the resultant solution was almost neutral. The resultant solution was maintained for 30 min at 70°C, then neutralized with 0.3 *M* hydrochloric acid (50 μl), and the solution was evaporated to dryness under reduced pressure. The residue was dissolved in water (200 μl) and extracted three times with chloroform (200 μl). The aqueous layer was evaporated to dryness under reduced pressure, the residue was dissolved in water (500 μl) and the solution was analyzed by CE.

3. Results and discussion

3.1. pH Dependence of the separation

Fig. 1 shows the separation of PMP derivatives of aldopentoses of the D-series in 50 m*M* phosphate buffer at various pH values.

Under these conditions, PMP–aldopentoses were negatively charged, due to the dissociation of the enol group in the pyrazolone ring, which was formed by tautomerization of the keto form, and they were attracted to the anode by electrostatic force. Since a fused-silica capillary was used in this work, electroosmotic flow was towards the cathode. As a result, PMP–aldoses migrated to the cathode by the combined effect of electroosmosis and electrophoresis. Under these ordinary conditions for zone electrophoresis, the mobilities of PMP–aldoses were roughly proportional to the charge-to-mass ratio. Since all of the aldoses and their PMP derivatives are apparently considered to have the same charge-to-mass ratio under identical conditions, they would not be separated by zone electrophoresis. What happened was, however, different from this expectation, as seen from Fig. 1. They were separated at pH values between 6.0 and 9.0 with varying migration orders and peak resolutions. The best separation was observed at pH 7.5, where all of the derivatives were separated from each other to the baseline level.

Fig. 2a shows the pH dependence of electro-

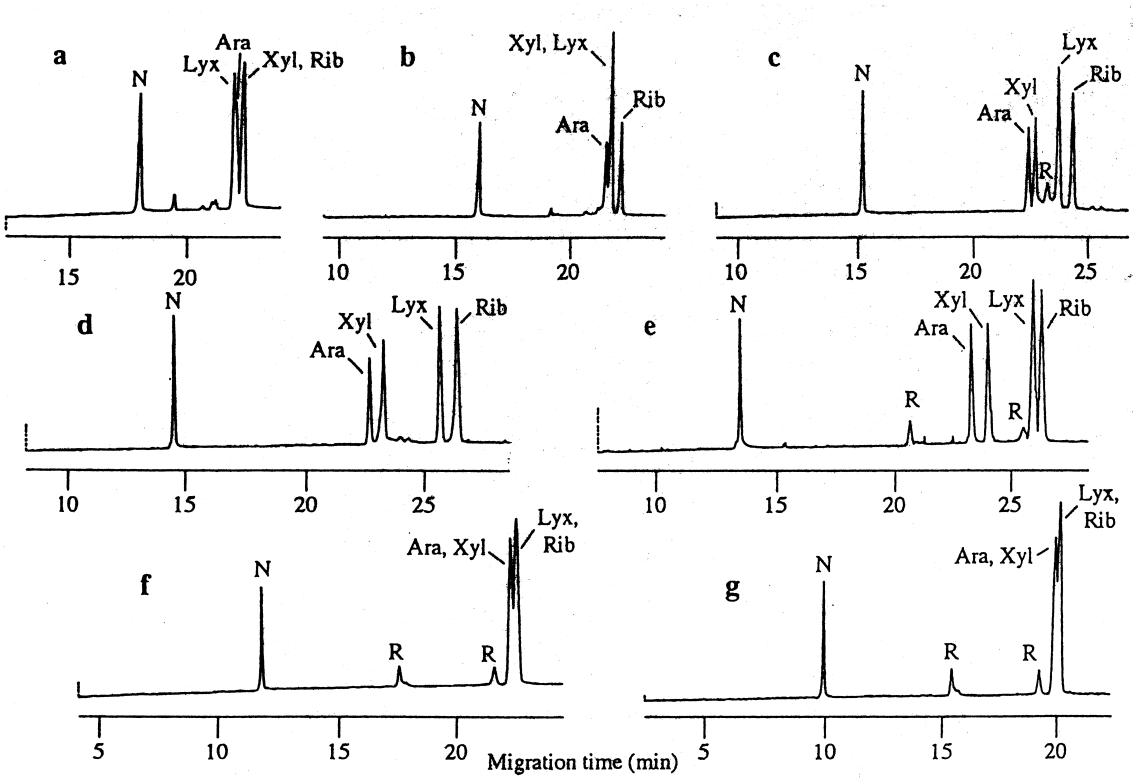

Fig. 1. pH Dependence of the separation of PMP–aldopentoses by zone electrophoresis in phosphate buffer. Capillary, fused-silica (50 μm I.D., 60 cm); running buffer, 50 m*M* phosphate buffer; applied voltage, 10 kV; sample concentration, equivalent to $2 \cdot 10^{-3}$ *M*; method of sample introduction, hydrodynamic (10 cm, 10 s). N=neutral marker (cinnamyl alcohol), R=reagent blank peak, Ara=arabinose, Lyx=lyxose, Rib=ribose, Xyl=xylose. (a) pH 6.0, (b) pH 6.5, (c) pH 7.0; (d) pH 7.5, (e) pH 8.0, (f) pH 8.5 and (g) pH 9.0.

phoretic mobility, calculated from the migration times of the individual derivatives and a neutral marker (cinnamyl alcohol), in the electropherograms of Fig. 1.

A tendency to separate could be seen as early as at pH 6.0, where dual peaks were observed. The front peak was assigned to the derivatives of lyxose and arabinose, and the rear peak to those of xylose and ribose. Mobility increased as the pH increased, but the increasing rate differed slightly among species. As a result, the resolution of the neighboring peaks changed, as shown in Fig. 2b. An outstanding change was observed for the resolution between xylose and lyxose; it reached a maximum value that was greater than ten at pH 7.5. The change in resolution for other pairs was not so drastic, but did exceed two at pH 7.5. At pH values lower than 6, mobilities were so small that the derivatives almost migrated with the neutral marker; at pH values higher than 9, the mobility was high with all derivatives, but no separation was observed.

Fig. 3 shows the separation of PMP–aldohexoses of the D-series under the same conditions (50 m*M* phosphate buffer, pH 7.5), where the best separation of PMP–aldopentoses was observed.

The eight aldohexoses were divided into two sets of four species, which was dependent on the configuration of the hydroxyl group at C-4. The derivatives of the first group (altrose, glucose, mannose and allose; structures in Fig. 4) were well separated from each other in this order (Fig. 3a), and the derivatives of the second group (galactose, idose, gulose and talose; structures in Fig. 4) migrated in this order and were also completely separated (Fig. 3b).

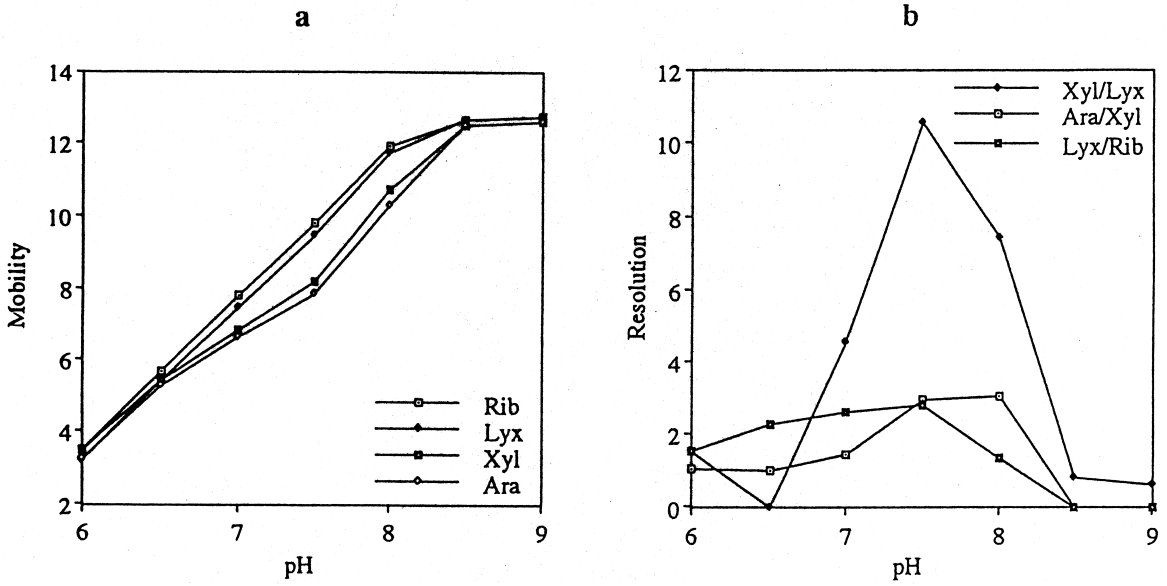

Fig. 2. pH Dependencies of mobility (a; in $cm^2\ V^{-1}\ min^{-1}$) and resolution (b) in the zone electrophoresis of PMP–aldopentoses. The data are based on Fig. 1. The analytical conditions and abbreviations are as in Fig. 1.

3.2. Behavior of other derivatives of aldoses

To examine whether such unexpected separation of PMP–aldoses was inherent to this kind of derivative, a few other derivatives, prepared by reductive amination, were similarly subjected to zone electrophoresis. The 8-amino-naphthalene-1,3,6-trisulfonic acid (ANTS) derivatives of aldopentoses were not separated from each other at any of the pH values examined (5.0–10.0), and gave only a single peak. The 2-aminopyridine (AP) derivatives of aldopen-

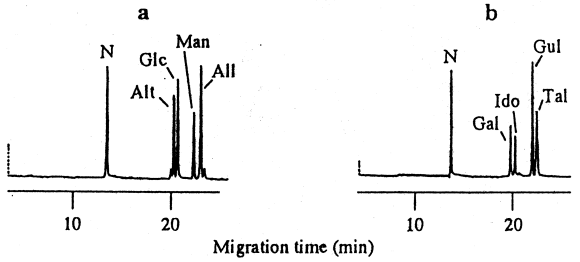

Fig. 3. Separation of PMP–aldohexoses by zone electrophoresis. N=neutral marker (cinnamyl alcohol), All=allose, Alt=altrose, Gal=galactose, Glc=glucose, Gul=gulose, Ido=idose, Man=mannose and Tal=talose. The analytical conditions were the same as in Fig. 1d.

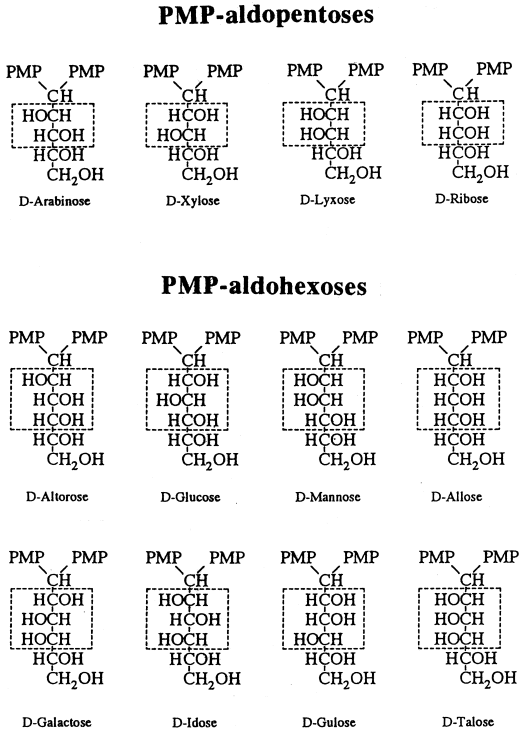

Fig. 4. Structures of PMP–aldopentoses and PMP–aldohexoses. Both groups of compounds are arranged in the order of migration.

toses were not resolved, even at low pH values, where protonation was effected. This is evidence supporting the selectivity of PMP derivatives in such an unusual separation.

3.3. Speculation on the mechanism of separation

As pointed out above, any two components of a sample solution with the same charge-to-mass ratio will not be separated from each other by zone electrophoresis. The unexpected separation of PMP–aldoses mentioned above has made us reconsider the magnitude of charge that these derivatives might have possessed under the analytical conditions employed. We can postulate that there is an intramolecular formation of rings for a PMP–aldose, as depicted in Fig. 5, which involves two hydrogen bonds being formed between the keto groups in the pyrazolone rings of the PMP moiety and the hydroxyl groups in the aldose moiety in the PMP–aldose.

The formation of such rings will make ionization of the keto group (via the enol group) disadvantageous, and the more strongly the ring structure is stabilized, the greater will be the reduction in the negative charge. Since the 2,3-*trans* disposition is obviously advantageous for ring stabilization, which will decrease the negative charge, the derivatives having such a disposition will migrate faster.

The structures of PMP–aldopentoses (see Fig. 4) are arranged in the order of migration. It is clearly shown that the derivatives of the 2,3-*trans* epimers (arabinose and xylose) migrated faster than the derivatives of the 2,3-*cis* epimers (lyxose and ribose). Comparison of the structures of the PMP–aldohexoses in Fig. 4 also indicates that this rule is true. In the first group, the 2,3-*trans* epimers (altrose and glucose) migrated faster than the 2,3-*cis* epimers (mannose and allose) (Fig. 3a), and in the second group, the 2,3-*trans* epimers (galactose and idose) moved faster than the 2,3-*cis* epimers (gulose and talose) (Fig. 3b).

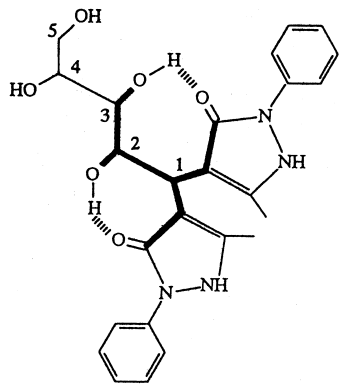

Fig. 5. Proposed intramolecular ring formation in a PMP–aldose through hydrogen bonding. PMP–aldoses having more stable rings will migrate faster, due to reduced negativity.

Although further evidence will be necessary to completely elucidate the mechanism of this unusual separation, the proposed ring formation hypothesis (by intramolecular hydrogen bonding) appears to be plausible, based on the principle of zone electrophoresis that the migration velocity is proportional to the charge-to-mass ratio. In a much more strict sense, the charge-to-hydrodynamic volume ratio should be considered instead of the charge-to-mass ratio. However, there will be practically no difference in the hydrodynamic volume ratio among monosaccharide species, in so far as the carbohydrate moieties take open-chain structures, as evidenced by our observation that ANTS and AP derivatives were not separated at all.

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