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Hydrogen-bonding interaction in capillary electrophoresis using polyether matrices

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Abstract

Polyethylene glycol (PEG) serves as a novel matrix in capillary electrophoresis. The purpose of this work was to explore some evidence for hydrogen-bonding complex formation between analytes and PEG in the separation system using benzoic acids as model analytes. An increase in the column temperature resulted in a significant decrease in the interaction between PEG and substituted benzoic acids with hydrogen-donating groups. Addition of urea suppressed the interaction. NMR spectra of phenol and salicylic acid in the presence of PEG in C^2HCl_3 showed an obvious electrostatic interaction, probably a hydrogen-bonding interaction, between the hydroxyl protons of the analytes and PEG. These results strongly support the contention that hydrogen-bonding interaction between the polyether segments of PEG and the hydrogen-donating groups of analytes occurs in the separation systems. Some other minor interactions controlling the separation are also described.

1. Introduction

Increasing attention has been paid to the use of capillary electrophoresis (CE) in the biological and pharmaceutical fields [1–3]. CE techniques for separation include capillary zone electrophoresis (CZE), electrokinetic chromatography and capillary gel electrophoresis. Each of these modes has achieved characteristic separations of a variety of samples. However, the development of a new separation mode is much in demand for further extension of the applicability of CE.

One of the possible interactions to be utilized for separation would be electrostatic interaction. With this in mind, we focused our attention on polyethers, which are known to serve as electrostatic electron donors via their ether oxygen atoms. Typical examples are inclusion phenomena of cations by crown ethers and non-cyclic polyethers [4–12]. It is noteworthy that hydrogen bonding is also observed between a polyether ionophore and amine complexes in hydrophobic media [13,14]. These studies stimulated us to utilize the hydrogen-bonding ability of polyethers to develop a new "hydrogen-bonding mode" or "electrostatic mode" in CE [15].

An earlier study revealed that the addition of polyethylene glycol (PEG) as a free matrix can greatly improve the CZE separation of benzoic

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acid derivatives used as model analytes as a result of the interaction between PEG and the analytes [15]. The strength of the interaction is appropriate for controlling the mobility of the analytes and appears to depend on the hydrogen-donating activity of the substituents. Therefore, we described the phenomenon in terms of hydrogen-bonding interaction between PEG and the analytes.

In this study, we attempted to obtain further evidence for hydrogen bond formation between PEG and analytes during CZE separations. Our strategy was to investigate the effects of the temperature of capillary and of urea as an additive on the strength of the interaction between PEG and analytes, because hydrogen bonding is weakened at elevated temperatures and urea, a bifunctional hydrogen donor and acceptor, breaks hydrogen-bonding complexes as a result of hydrogen-bonding exchange. Furthermore, nuclear magnetic resonance (NMR) spectroscopy would provide direct evidence of hydrogen-bonding complex formation. We shall also discuss some other minor interactions controlling the separation.

2. Experimental

Three kinds of PEG with mean molecular masses of 400, 4000, and 20 000 (PEG 400, PEG 4000 and PEG 20 000) were obtained from Kishida Chemical (Osaka, Japan) and used as received. 4-Acetamidobenzoic acid ($4CH_3CONH$-BA), 4-acetoxybenzoic acid ($4CH_3COO$-BA), 4-aminobenzoic acid ($4NH_2$-BA), 4-hydroxybenzoic acid (4OH-BA), 4-methylbenzoic acid ($4CH_3$-BA), 4-carboxybenzaldehyde (4CHO-BA), 2-hydroxybenzoic acid (2OH-BA), 2-carboxybenzaldehyde (2CHO-BA), benzoic acid (BA) and [^{2}H]chloroform (C^2HCl_3, containing 1% TMS) were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical-reagent grade.

Electrophoretic separation was performed in a fused-silica tube (GL Science, Tokyo, Japan) of 0.05 mm I.D. and a column length of 750 mm, with an effective length for separation of 500 mm. Samples were introduced at the end to be connected to the positive high voltage by siphoning at a height of 15 cm, usually for a 5–10-s period. When PEG was used at higher concentrations, a longer time was required for sample injection because of the increased viscosity. Thermal control of the column was performed as follows. About 60% of the effective length of the column was passed through silicone rubber tubing of 1.0 mm I.D. covered with a heat insulating material and then water maintained at a given temperature was pumped with a peristaltic pump (Gilson Minipuls 2, ca. 2 ml min^{-1}) through the tubing during separations in the direction opposite to the electroosmotic flow. UV spectrophotometric detection was effected at the negative potential side. The detection wavelength was set at 210 nm. Other details were described in previous papers [15,16].

A series of ^{1}H NMR measurements of benzoic acids at various concentrations of PEG 20 000 were carried out in C^2HCl_3 with a JEOL GX-270 instrument operating at 270 MHz.

3. Results and discussion

Nine benzoic acids, $4CH_3CONH$-BA, $4NH_2$-BA, 4OH-BA, 2OH-BA, $4CH_3COO$-BA, 4CHO-BA, $4CH_3$-BA, 2CHO-BA and BA, were used as model samples in CZE experiments. PEG 400 and PEG 4000 were used at concentrations ranging from 1 to 10% in an electrolyte solution of 10 m*M* phosphate buffer (pH 7.8). Under the present separation conditions, all the analytes are considered to be in the form of univalent anions because of complete dissociation of their carboxyl groups. As reported in a previous paper [15], the addition of PEG drastically influences the migration time of these benzoic acids. The influence can be described in terms of the following two factors. The first is non-specific and is ascribed to the increase in viscosity, resulting in an increase in the migration time. In the case of 2CHO-BA, the change in the migration time can be simply described by this effect, because it appears to

exhibit no specific interaction with PEG (see later also) [15]. The second, in which we are interested, is specific to the analytes. The addition of PEG accelerates the mobility of some benzoic acids, especially four with hydrogen-donating substituents ($4CH_3CONH$-BA, $4NH_2$-BA, 4OH-BA and 2OH-BA), compared with 2CHO-BA. The acceleration of the migration time resulted from some attractive interaction between the hydrogen-donating benzoic acids and PEG, as PEG migrates in the direction of the negative potential at the electroosmotic flow-rate (V_{eo}). In order to eliminate the first non-specific factor, we shall describe the electrophoretic behaviour using relative values of the migration time and electrophoretic velocity against those of 2CHO-BA as a reference compound (see Eq. 1 also).

3.1. Effect of temperature

Fig. 1 shows electropherograms of the nine benzoic acids under thermostated conditions at (A) 3°C and (B) 80°C in the presence of 7.5% (v/v) PEG 4000. Comparison of the two electropherograms reveals that the relative migration time of the four hydrogen-donating benzoic acids ($4CH_3CONH$-BA, $4NH_2$-BA, 4OH-BA and 2OH-BA) increases with increase in temperature. This means that the increase in the column temperature weakens the interaction between PEG and the four benzoic acids having a hydrogen-donor active substituent. It is well known that an increase in temperature weakens and breaks hydrogen bonds. Hence this behaviour is in accord with our previous description in terms of the hydrogen-bonding complex formation between the hydrogen-donating benzoic acids and PEG.

When we can assume a stoichiometric and equilibrated complex formation between an analyte and PEG, the observed electrophoretic velocity of the analyte ion (V_{ep}) is expressed as a function of the PEG concentration [PEG], as described in a previous paper [15]:

$$V_{ep}/V_{ep,0} = V_{ep,f}/V_{ep,0} + K[(V_{ep,c} - V_{ep,f})/V_{ep,0}][PEG] \quad (1)$$

where $V_{ep,f}$ and $V_{ep,c}$ are the electrophoretic velocity of the free analyte ion and the analyte–PEG complex, respectively, K is the complex formation constant of the analyte with PEG and $V_{ep,0}$ is the electrophoretic velocity of a reference compound with $K \approx 0$ (2CHO-BA in our case). In our experiments, $V_{ep}/V_{ep,0}$ exhibited linear relationships against [PEG] up to 7.5% (v/v) (data not shown; see Fig. 2 in Ref. [15] as an example). Values of K can be easily estimated from the slopes of the linear plots on the basis of a reasonable assumption that $V_{ep,c} - V_{ep,f} \approx -V_{ep,f}$. Fig. 2 shows the dependence of the K

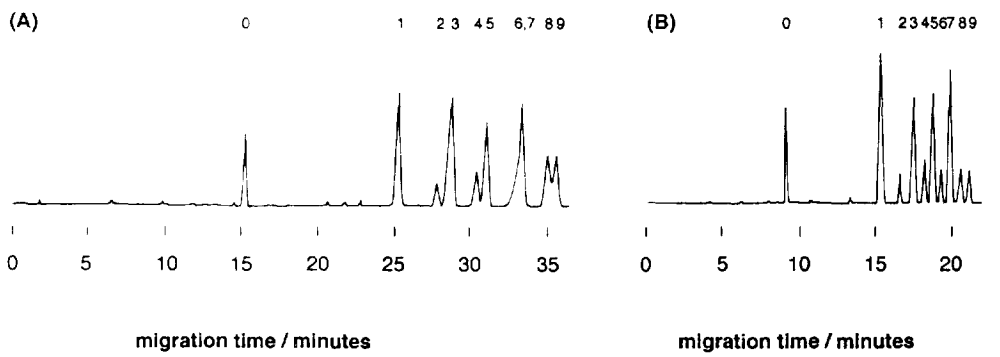

Fig. 1. Electropherograms of nine benzoic acids with 7.5% (v/v) PEG 4000 under thermostatic control at (A) 3°C and (B) 80°C. Electrolyte solution, 10 m*M* phosphate buffer (pH 7.8); capillary, 750 mm × 0.05 mm I.D. (500 nm effective length); applied voltage and current), (A) 14 kV and 4 μA and (B) 14 kV and 6 μA; detection wavelength, 210 nm. Peaks: 0 = mesityl oxide (electroosmotic flow marker); 1 = $4CH_3CONH$-BA; 2 = $4CH_3COO$-BA; 3 = 4OH-BA; 4 = $4CH_3$-BA; 5 = $4NH_2$-BA; 6 = 4CHO-BA; 7 = 2OH-BA; 8 = 2CHO-BA, 9 = BA.

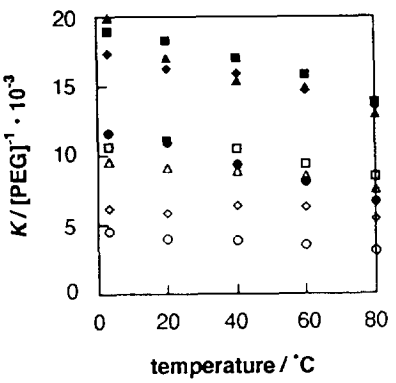

Fig. 2. Dependence of the complex formation constant (K) on the capillary temperature: ■ = $4CH_3CONH$-BA; ▲ = 4OH-BA; ◆ = 2OH-BA; ● = $4NH_2$-BA; □ = $4CH_3$-BA; △ = CH_3COO-BA; ◇ = 4CHO-BA; ○ = BA.

values of the analytes on the capillary temperature. In the cases of the four benzoic acids having a hydrogen-donating substituent ($4CH_3CONH$-BA, $4NH_2$-BA, 4OH-BA and 2OH-BA), the K values decrease significantly with increase in the capillary temperature. In contrast, in the case of 4CHO-BA, $4CH_3$-BA, $4CH_3COO$-BA and BA, which have no hydrogen-donating active substituent, the K values are almost independent of the capillary temperature or the dependence is very small. These results support the contention that the interaction between PEG and the four substituted benzoic acids with hydroxyl, amide or amino groups is predominantly governed by the hydrogen-bonding complex formation, in which the polyether oxygen atoms of PEG serve as hydrogen acceptors.

The above argument might mean that an increase in the capillary temperature is almost equivalent to a decrease in the PEG concentration. However, the two parameters controlling the experimental conditions are not exactly identical with each other. Therefore, from the point of view of practical application, both the capillary temperature and the PEG concentration can be useful parameters to improve separations in this method. In our experiment, the best separation was achieved at 80°C (Fig. 1B) for the samples used. Even when the use of a higher concentration of PEG is unavoidable to improve separation, an increase in the capillary temperature would be occasionally useful for rapid separation, because it results in a decrease in the viscosity and hence of the migration time.

3.2. Effect of urea

Urea works as a bifunctional hydrogen donor and acceptor and it is often employed to break intra- and/or intermolecular hydrogen bonds in biological molecules, such as proteins and DNA. Considering that the hydrogen-bonding interaction operates in the present separation mode for some analytes, addition of urea to the separation matrices would be expected to compete with PEG (hydrogen acceptor) to form hydrogen-bonding complexes with the analytes (hydrogen donors) and/or with analytes to form hydrogen-bonding complexes with PEG. Our assumption here is that the electrophoretic velocity of the urea–analyte hydrogen-bonding complex is not far from that of the free analyte, because the molecular mass of urea is sufficiently small compared with that of PEG. This assumption leads to an expectation that an apparent value of K evaluated by Eq. 1 will decrease at a sufficiently high concentration of urea.

The following experiments confirmed the expectation. Urea was added at 10 M to an electrolyte solution of 10 mM phosphate buffer (pH 7.8) containing several concentrations of PEG. In this work, PEG 400 was used, because urea is almost insoluble in an aqueous solution of PEG 4000 at 5% (v/v). The apparent K value was evaluated based on Eq. 1. Plots of $V_{ep}/V_{ep,0}$ vs. [PEG] gave straight lines in the range of [PEG] from 2.5 to 10% (v/v). Table 1 summarizes the K values of the samples in the absence and presence of 10 M urea. For all the analytes, the K values at 10 M urea decreased to 30–85% of those in the absence of urea. This decrease in K values is attributable to the suppression of the interactions, most significantly the hydrogen-bonding interaction, between PEG and the analytes. However, the urea effect was not restricted to the hydrogen-donating analytes. As will be mentioned later, we consider that several minor interactions exist in addition to the relatively

Table 1
Effects of addition of 10 *M* urea on *K* values

Analyte	$K \times 10^{-1}$ [PEG (%, v/v)]	
	No urea	10 *M* urea
4OH-BA	10.3	5.3
2OH-BA	9.8	5.6
$4CH_3CONH$-BA	9.5	3.9
$4NH_2$-BA	6.6	3.6
$4CH_3$-BA	7.0	3.6
$4CH_3COO$-BA	4.8	1.5
4CHO-BA	4.5	2.3
BA	2.7	2.3

strong hydrogen-bonding interaction between PEG and benzoic acids. Therefore, urea seems to suppress these minor interactions also by hydrogen-bonding complex formation with PEG molecules.

3.3. NMR spectral measurements

NMR spectroscopy is suitable for observing electrostatic interactions of molecules in solutions. When an electrostatic interaction of an analyte with PEG occurs, the distribution of the electron density of the analyte should change, resulting in a change in the chemical shifts. In this study, the interaction of the phenolic hydroxyl group with PEG 20 000 was followed in C^2HCl_3 using ^1H NMR spectroscopy. Phenol was used as a model compound, because 4OH-BA is insoluble in C^2HCl_3.

Fig. 3A shows the ^1H NMR spectra of 10 m*M* phenol in the absence and presence of PEG 20 000 at concentrations of 1–5% (v/v). The spectral change on addition of PEG was demonstrated by the signals of the hydroxyl proton. The ^1H signal of the hydroxyl group of phenol (4.8 ppm at 0% PEG) shifts significantly to lower field on addition of PEG. This provides direct evidence for hydrogen-bonding complex formation between the phenolic hydroxyl group and the polyether oxygen atoms of PEG. The electrostatic attractive interaction (hydrogen-bonding complex formation) between the phenolic hydroxyl group and PEG should increase the

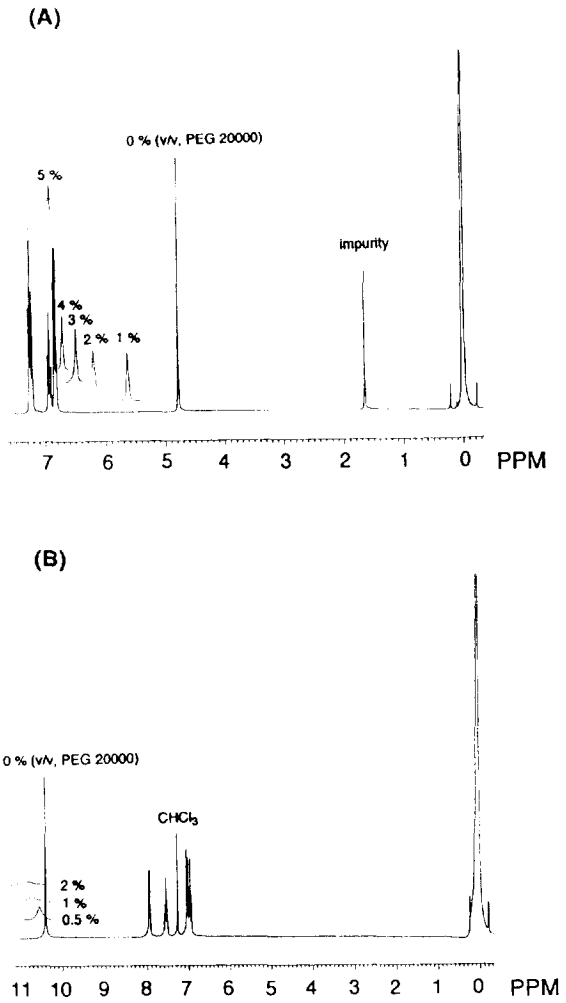

Fig. 3. ^1H NMR spectra of (A) phenol and (B) 2OH-BA in C^2HCl_3 with increasing concentration of PEG 20 000.

O–H bond length. Thus the interaction decreases the electron density around the proton, resulting in a lower-field shift owing to the deshielding. Similar lower field shifts of phenolic and alcoholic ^1H signals are observed with increasing concentration as a result of intermolecular hydrogen bonding with themselves [17].

The PEG molecule has two hydroxyl groups at the ends, and these end-groups are reasonably considered to serve as hydrogen donors. This consideration can be supported by NMR experiments. The NMR signal of the hydroxyl end-groups of PEG is broadened and shifted from 2.3

ppm to lower field with addition of BA (data not shown). This indicates the possibility of the occurrence of a hydrogen-bonding interaction between the carboxylate group of BA and the hydroxyl end-groups of PEG at least in C^2HCl_3. The numbers of hydroxyl groups in PEG molecules are generally far less than those of ether oxygen atoms. Hence the hydrogen-donating property of the end-groups of PEG would not be so significant compared with the hydrogen-accepting property of the polyether segments. In the following, we shall refer to the hydrogen-bonding interaction derived from the polyether segments and the hydroxyl end-groups as the major and minor hydrogen-bonding interactions, respectively.

3.4. Hydrophobic interaction

Judging from the K values in Table 1, $4CH_3$-BA seems to interact attractively with PEG at a strength comparable to that of $4NH_2$-BA. This could not be explained simply in terms of the hydrogen-bonding interaction, because the methyl group is inactive in hydrogen bonding. PEG can afford more or less hydrophobic surroundings. This property seems to work as an another sub-mode in addition to the hydrogen-bonding mode for separation in the CE system using PEG. Such a hydrophobic interaction with PEG could occur with the other analytes, but it was not as large as in the case of $4CH_3$-BA.

In addition to the direct effect of the hydrophobicity of PEG, a secondary effect might play a role in the hydrogen-bonding complex formation between the analytes and PEG, namely a solvation effect. Hydrogen-bonding interaction with PEG will occur more effectively in hydrophobic surroundings than in an aqueous phase. The benzoic acids used here are more or less hydrophobic in nature even in their monoanion state. Hence the analytes will be surrounded by the hydrophobic segments (or hydrophobic pockets) of PEG in part. The hydrophobic environments will enhance the hydrogen-bonding interaction in this separation system.

3.5. Comparison between ortho- and para-isomers

The K values of $4CH_3COO$-BA and 4CHO-BA were much larger than those of the *ortho*-isomers ($2CH_3COO$-BA and 2CHO-BA, respectively) (the K values of $2CH_3COO$-BA were $1.4 \cdot 10^{-3}$[PEG 400]$^{-1}$ and $3.8 \cdot 10^{-3}$[PEG 4000]$^{-1}$) [15]. This seems to suggest steric hindrance against the hydrophobic interaction of the analytes with the polyether segments of PEG and/or the minor hydrogen-bonding interaction in part (note that the major hydrogen-bonding interaction is not expected for these analytes). This steric effect seems to be a minor one, but it is occasionally useful for separation. A typical example is the separation between 2CHO-BA and 4CHO-BA. The two analytes could not be separated in the absence of PEG under our experimental conditions because of their hydrodynamic radius close to each other. However, the addition of PEG 4000 [more than 2.5% (v/v)] achieved the complete separation between them with the use of this steric effect. This would mean in turn that 2CHO-BA hardly interacts with PEG. Thus 2CHO-BA is the best reference substance for the determination of the complex formation constant of analytes with PEG (K) (see above).

A complicated situation is observed with 2OH-BA. Even though 2OH-BA is the *ortho*-isomer of 4OH-BA, the K value of 2OH-BA was comparable to that of 4OH-BA (Fig. 2, Table 1). This result can be interpreted as follows. The hydroxyl group in both 2OH-BA and 4OH-BA is strong hydrogen donor. Hence the major hydrogen-bonding interaction is reasonably considered to govern the overall interaction with PEG and is larger than the steric hindrance effect observed for other *ortho*-isomers. Another reason may be related to the hydrodynamic radius of 2OH-BA. In the absence of PEG, 2OH-BA migrates faster than 4OH-BA [15]. This is in contrast to the case with 2CHO-BA and 4CHO-BA, which migrate simultaneously in the absence of PEG (see above). This result means that the hydrodynamic radius of 2OH-BA is smaller than that of 4OH-

BA. Fig. 3B shows the ^1H NMR spectra of 10 m*M* 2OH-BA in the absence and presence of PEG 20 000 at concentrations of 0.5–2% (v/v). The chemical shift of the hydroxyl proton of 2OH-BA is observed at 10.4 ppm. This low-field resonance indicates intramolecular hydrogen-bond formation between the hydroxyl group and the dissociated carboxyl group. Therefore, the small hydrodynamic radius compared with 4OH-BA can be attributed to the intramolecular hydrogen-bond formation. With the addition of PEG, the ^1H signal of the hydroxyl group broadened and disappeared. This indicates the formation of an intermolecular hydrogen-bonding complex with PEG. Once the interaction with PEG has occurred, the hydroxyl group bonded to the carboxylate group of 2OH-BA will turn out to form a complex with PEG. This will result in a larger increase in its apparent hydrodynamic radius than that expected for 4OH-BA. Such an amplified effect may cancel the steric hindrance effect observed for other *ortho*-isomers.

4. Conclusion

The use of PEG as a matrix is very useful in CE separations. The present NMR experiments have demonstrated that PEG works as a hydrogen acceptor via its polyether segments and in part as a hydrogen donor via its hydroxyl end-groups. The effects of temperature and urea addition can be described fundamentally in terms of the hydrogen-bonding interaction between PEG and analytes. The hydrogen-bonding interaction in the aqueous phase will not be as strong as demonstrated by NMR in C^2HCl_3. However, the strength is fortunately appropriate for improving the separation in CZE. Hence the CE method using PEG as a matrix would open up routes to develop electrostatic capillary electrophoresis. From a physico-chemical point of view, this method might be applicable to the determination of the hydrogen-donating properties of analytes or hopefully of the hydrogen-accepting properties of analytes using a hydrogen-donating matrix.

This study suggested the occurrence of some other minor interactions in addition to the hydrogen-bonding interaction, such as hydrophobic and steric interactions. These interactions work in conjunction for hydrogen-donating analytes and sometimes effectively for hydrogen-donating inactive analytes to improve the separation.

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