Computer Simulation of Isotachophoresis

Application to the Determination of Physico-Chemical Constants and Separation Optimization

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A computer simulation of isotachophoresis is described for the purpose of determining physico-chemical constants and separation optimization. On the basis of the mobilities, dissociation constants and stability constants of the separands, the effective mobilities and the isotachopherogram in the steady state condition can be exactly simulated at a given electrolyte condition. When some of the necessary constants are unknown, they can be evaluated by means of a least-squares method on the basis of the observed qualitative indices. Since the sample amount necessary for electrophoretic separation is very small and good separability from coexisting substances is expected, this may be one of the useful methods for determining the physico-chemical constants. When the necessary constants are known, the optimum separation condition can be obtained by an iterative simulation that varies the electrolyte condition, such as the electrolyte pH, the solvent (water and methanol) and the concentration of the complex-forming agents to give the largest effective mobility differences among the separands.

Keywords Isotachophoresis, separation optimization, computer simulation, mobility, acid dissociation constant

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1 Introduction

Electrophoresis is one of the useful techniques used for analyzing ionic substances; its utility as an analytical technique has been widely accepted.¹⁻⁶ Recent developments involving capillary electrophoresis equipment have shown that electrophoresis is still a developing technique. Software concerning the preparation of the operational electrolyte system is very important to draw out optimum separation from recently developed hardware.

Electrophoretic separation is due to a difference among the electrophoretic mobilities of the sample components. As described later, the mobilities in an

actual electrolyte solution (effective mobility) depend on the atmosphere of the ions as well as several physico-chemical constants of the ions, such as the absolute mobilities, the acid dissociation constants and the stability constants. Since no packings are usually used, the separability of capillary electrophoresis depends directly on the electrolyte conditions, such as the electrolyte pH, the solvent used and the concentration of the coexisting complexing agent.

For separation optimization in electrophoresis, a theoretical approach is very useful. Especially in isotachophoresis, the pH and ionic strength of the separated zones are different from each other, as described later; no practical separability assessment is possible without a theoretical approach when the sample has a complex constitution.

The aim of this review is to describe our theoretical approach regarding separation optimization in

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isotachopheresis utilizing a computer-simulation technique. The optimum electrolyte system can be determined by iterative computer experiments in which the effective mobilities of the separands at the steady state are calculated using their physico-chemical constants; the differences of the effective mobilities are compared. This method may be called computer-aided separation optimization, which enables a determination of the electrolyte condition, such as the pH, without performing time-consuming "trial-and-error" experiments.

In addition to analytical utility, computer simulations make electrophoresis a measurement method of the physico-chemical constants of the separands. If we can exactly measure the qualitative indices related to the effective mobilities under some electrolyte systems, the unknown physico-chemical constants can be evaluated by solving simultaneous equations to express the unknown constants, or, practically, by a best-fitting procedure for the observed and simulated qualitative indices using the least-squares method. Since the sample amount necessary for electrophoretic separation is as small as 100 nmol or less, and good separability from coexisting substances is expected, this can be one of the useful methods for physico-chemical measurements.

Since these approaches greatly depend on the principle of electrophoresis, the theoretical aspects are described in detail, first.

2 Theoretical

2.1 Electrophoretic mobility

The effective mobility ($\bar{m}$) of an ionic substance in a solution can be expressed as a function of many factors as follows:

$$\bar{m} = f(m_0, k_a, K_s, T, \eta, \epsilon, \mu, \text{pH}, C_{\text{comp}}), \quad (1)$$

where m_0 is the absolute mobility, k_a the acid-dissociation constant, K_s the stability constants of the complexes or ion-pairs formed, T the temperature, η the viscosity of the solvent used, ϵ the dielectric constant of the solvent, μ the ionic strength, pH the pH of the solution, and C_{comp} the concentration of the complex-forming or ion-pair forming agent.

Apparently, from Eq. (1), the effective mobility is a rather complex function of the properties of the sample ion and the solvent. A basic concept of electrophoretic separation is to produce appropriate mobility differences among the samples by varying some of the factors in the function.

When the sample is a multivalent weak electrolyte and does not form any complexes or ion-pairs, the effective mobility ($\bar{m}$) at the acid-base equilibria can be expressed as follows:⁷

$$\bar{m} = \frac{m_1 \cdot [\text{AH}_{n-1}] + m_2 \cdot [\text{AH}_{n-2}] + \dots + m_n \cdot [\text{A}^{n-}]}{[\text{AH}_n] + [\text{AH}_{n-1}] + [\text{AH}_{n-2}] + \dots + [\text{A}^{n-}]}$$

$$= \frac{m_1 k_1 / [\text{H}^+] + m_2 k_1 k_2 / [\text{H}^+]^2 + \dots + m_n k_1 k_2 \dots k_n / [\text{H}^+]^n}{1 + k_1 / [\text{H}^+] + k_1 k_2 / [\text{H}^+]^2 + \dots + k_1 k_2 \dots k_n / [\text{H}^+]^n} \quad (2)$$

Here, m_n is the mobility of A^{n-} and k_n the acid-dissociation constant to form A^{n-} . For the simulation of the effective mobility in an actual electrolyte solution, m and k should be corrected by ionic strength using Onsager's equation and Debye-Hückel equation⁸, respectively.

The migration velocities of an ion (v)^{7,9,10} can be expressed as

$$v = \bar{m} \cdot E, \quad (3)$$

where E is the potential gradient of the separation field. Consequently, the difference in the electrophoretic velocities among sample components is the driving force of the separation.

Figure 1 shows the pH dependence of the effective mobility of some amino acids and peptides at infinite dilution ($\mu=0$). The curves were plotted using Eq. (2). The absolute mobilities (m_0) and thermodynamic acid dissociation constants ($\text{p}K_a$) used are tabulated later.

When a sample forms ion-pairs, the effective mobility is expressed by a different equation. Although the general equation for the effective mobility can be written and is useful in computations, we restrict ourselves to the following simple case in order to avoid complexity. Assume that Ca^{2+} coexists with tartrate anions (HTar^- and Tar^{2-}). The ion-pair forming equilibria in the solution can be written as follows:

and

The effective mobility of the tartrate ion ($\bar{m}_{\text{Tar}}$) can be written as follows:

$$\begin{aligned} \bar{m}_{\text{Tar}} &= \frac{m_1 [\text{HTar}^-] + m_2 [\text{Tar}^{2-}] + m_3 [\text{CaHTar}^+]}{[\text{H}_2\text{Tar}] + [\text{HTar}^-] + [\text{Tar}^{2-}] + [\text{CaHTar}^+] + [\text{CaTar}^0]} \\ &= \frac{m_1 k_1 / [\text{H}^+] + m_2 k_1 k_2 / [\text{H}^+]^2 + m_3 [\text{Ca}^{2+}] K_1 k_1 / [\text{H}^+]}{1 + k_1 / [\text{H}^+] + k_1 k_2 / [\text{H}^+]^2 + [\text{Ca}^{2+}] (K_1 k_1 / [\text{H}^+] + K_2 k_1 k_2 / [\text{H}^+]^2)} \end{aligned} \quad (5)$$

Here, m_1 is the mobility of HTar^- , m_2 is that of Tar^{2-} and m_3 is that of CaHTar^+ ($m_1 = 34.6 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$, $m_2 = 60.5 \times 10^{-5}$, $m_3 = 22.9 \times 10^{-5}$). Of course, the mobility of

Fig. 1 pH dependence of the effective mobility of carbonic acid (H_2CO_3), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), glycylalanine (Gly-Ala), glycyl- α -aminobutyric acid (Gly-Amin), glycine (Gly), glycylglycine (Gly-Gly), glycyl-L-leucine (Gly-Leu), glycylserine (Gly-Ser), glycylvaline (Gly-Val), histidine (His), leucylvaline (Leu-Val), taurine (Tau) and threonine (Thr) at infinite dilution.

CaTar^0 is zero and is not included in Eq. (5); k_1 and k_2 are the acid dissociation constants and K_1 and K_2 are the stability constants of CaHTar^+ and CaTar^0 , respectively ($\text{p}k_1=3.036$, $\text{p}k_2=4.366$, $\log K_1=1.59$, $\log K_2=2.88$). Obviously, from Eq. (5) $\bar{m}_{\text{Tar}}$ can be controlled by both the Ca^{2+} concentration and the solution pH. A few millimolar Ca^{2+} is effective to vary the effective mobility, as is shown in a later section.

2.2 Isotachophoresis

The name isotachophoresis comes from Greek, which means equal (iso, $\iota\sigma\omicron$) velocity (tacho, $\tau\alpha\chi\eta\omicron\varsigma$) sample dragging (phoresis, $\phi\omicron\rho\epsilon\epsilon\sigma\theta\alpha\iota$).¹¹ Although this name well characterizes its principle^{3,4,12}, if the sample velocities are the same throughout the migration process, there can be no separation.

Figure 2 shows the isotachophoretic separation process. The sample solution is injected at the boundary of two electrolyte solutions: a leading electrolyte and a terminating electrolyte (Fig. 2a). The leading electrolyte contains both leading ions (L) with the same sign of the sample ions and appropriate counter ions with a pH-buffering ability. The terminating electrolyte contains terminating ions (T) together with appropriate counter ions. Their effective mobility is selected so as to fulfill the following relationship for the present model samples (A and B):

$$\bar{m}_L > \bar{m}_A > \bar{m}_B > \bar{m}_T. \quad (6)$$

The use of the two different electrolytes (L and T) is

Fig. 2 Separation process in isotachophoresis: a) before migration; b) separation process forming a mixed zone A+B; c) complete separation (a steady state). L, leading zone; A, B, sample zone; T, terminating zone.

decisively important in isotachopheresis.

During the initial stage of migration, a homogeneous mixed zone (A+B) of the separands is formed (Fig. 2b), in which components A and B migrate with different velocities under the same potential field: for example, $v_A > v_B$ in the mixed zone. Sample A forms its pure zone in the leading side of the mixed zone; sample B forms its pure zone in the terminating side of the mixed zone. After a while, the mixed zone diminishes and sample components A and B are separated to form independent zones (Fig. 2c). It should be noted that the migrating zones are stacked and this migration configuration is quite different from that of zone electrophoresis. Once they are separated, they never mix again and the zone lengths remain constant until the migration current is off. This is the isotachophoretic steady state.

When the migration order is as shown in Fig. 2c, the following relations are valid:¹³

$$\left. \begin{array}{l} \bar{m}_{A,A} > \bar{m}_{A,B} \\ \text{and} \\ \bar{m}_{B,A} < \bar{m}_{B,B} \end{array} \right\} \quad (7)$$

where $\bar{m}_{A,A}$ and $\bar{m}_{B,B}$ denote the effective mobility of A and B ions in the steady state zone, respectively, and $\bar{m}_{A,B}$ and $\bar{m}_{B,A}$ denote the effective mobility of ion A in the B zone and that of ion B in the A zone, respectively. This relation maintains the boundary between zones A and B to be very sharp (self-sharpening effect).

Figure 3 shows the isotachophoretic configuration of the leading zone (L), the sample zone (A) and the terminating zone (T) in an anionic analysis. Since the pH buffers (Q) are continuously supplied from the leading electrolyte, they regulate the pH of the sample zones. The following relation is usually valid for an anion analysis:

$$\text{pH}_L > \text{pH}_A > \text{pH}_B > \text{pH}_T. \quad (8)$$

The reversed relation holds in cationic analysis. The difference ($|\text{pH}_L - \text{pH}_T|$) is usually less than 1 when the pH buffering counter ion is selected properly. Since the pH of the zones is therefore not constant, in contrast to the case in zone electrophoresis, the effective mobility dependence of the samples on the pH of the leading electrolyte cannot be estimated in a straightforward way. This makes the separation optimization of isotachopheresis difficult, compared with the case regarding zone

Fig. 3 Configuration of isotachophoretic zones and acid-base equilibria. L⁻, leading anion; A⁻, sample anion; T⁻, terminating anion; and HQ⁺, pH buffering cation.

Table 1 pH buffers used in simulations for anionic analyses

Name	pK _a	pH range	No.
Glycylglycine	3.140	2.6– 3.6	(1– 6)
β-Alanine	3.552	3.0– 4.0	(7–12)
ε-Aminocaproic acid	4.373	3.8– 4.8	(13–18)
Creatinine	4.828	4.2– 5.4	(19–25)
Histidine	6.040	5.4– 6.4	(26–31)
Imidazole	7.150	6.4– 7.4	(32–37)
Tris	8.076	7.4– 8.4	(38–43)
Amediol	8.780	8.2– 9.2	(44–49)
Ethanolamine	9.498	9.0–10.0	(50–55)

Tris, tris(hydroxymethyl)aminomethane; Amediol, 2-amino-2-methyl-1,3-propanediol; No., number of electrolyte systems.

electrophoresis.

The pH buffers conventionally used are summarized in Table 1. To maintain a good buffering capacity of the leading electrolyte, the pH_L should satisfy the following relationship:

$$\text{pK}_Q - 0.5 < \text{pH}_L < \text{pK}_Q + 0.5, \quad (9)$$

where pK_Q is the pK_a of the buffers used. The maximum buffering capacity is obtained at pH_L = pK_Q.

At the isotachophoretic steady state, where the zone lengths of all samples are constant, and no mixed zone remains, the following relations are valid under a constant current:

$$v_L = v_A = v_B = v_T \quad (10)$$

and

$$E_L \cdot \bar{m}_L = E_A \cdot \bar{m}_A = E_B \cdot \bar{m}_B = E_T \cdot \bar{m}_T. \quad (11)$$

From Eqs. (6) and (11), the following relation holds among the potential gradient of the zones (Fig. 2c):

$$E_L < E_A < E_B < E_T. \quad (12)$$

The separated zones can therefore be detected by using a potential-gradient detector and a conductivity detector, in addition to the usual spectroscopic detectors.

The qualitative index is the ratio of the potential gradients or the conductivities of the separated zones. There have been various definitions proposed.²⁻⁴ We have proposed R_E , which is defined for component A as follows:

$$R_{E,A} = E_A / E_L = \bar{m}_L / \bar{m}_A. \quad (13)$$

Figure 4 shows the experimental definition of the R_E values using the step heights, where the asymmetric potential of the potential gradient detector is corrected using an internal standard.

In isotachopheresis, dilute components in a sample are

Fig. 4 Isotachopherograms and experimental definition of R_E values. L, leading zone; A, B, sample zone; std, internal standard sample; T, terminating zone; h , step height; δh , step height corresponding to an asymmetric potential of a potential gradient detector; $R_{E,A}$, corrected R_E value of sample A. $\delta h = (h_{std} - h_L R_{E,std}) / (R_{E,std} - 1)$; $R_{E,A} = (h_A + \delta h) / (h_L + \delta h)$.

concentrated according to Kohlrausch's regulating function¹² and, conversely, the concentrated components are diluted during migration. The concentration of the sample in the steady state zone is determined by the concentration of the leading ion and the effective mobility of the sample. The following relation is valid among the total equivalent concentrations of the samples (C^i):

$$C_L^i > C_A^i > C_B^i > C_T^i. \quad (14)$$

The quantitative index is the time-based zone length, as shown in Fig. 4.^{3,4,14} The absolute zone length of sample A (l_A /cm) can be defined as

$$l_A = 1000n / (C_A^i \cdot \pi r^2), \quad (15)$$

where n is the applied sample amount (mol) and r the radius (cm) of the separation tube at the detector.

The time-based zone length (t (s)) is equal to the actual zone length divided by the isotachophoretic velocity (v (cm/s)). When both the sample ion and the buffer ion are monovalent, t can be expressed as follows:¹⁴

$$t_A = Fn(1 + m_Q/m_A)/I, \quad (16)$$

where F is the Faraday constant, m_Q the mobility of the buffer ion, m_A the mobility of sample A, and I the migration current.

3 Computer Simulation of Isotachophoresis

3.1 Database of mobilities and acid dissociation constants

For a computer simulation of the isotachophoretic steady state, physico-chemical constants, such as the mobilities, acid-dissociation constants and stability

Table 2 Part of our database for the mobilities and acid-dissociation constants

No.	Formula	Sample name	m_0	pK_a	MW
34	H ₂ CO ₃	carbonic acid	-46.1	6.352	62.0
			-71.8	10.329	
143	HCl	hydrochloric acid	-79.1	-2.000	36.5
418	C ₄ H ₇ NO ₄	aspartic acid	30.1	1.990	133.1
			-30.1	3.900	
			-56.8	10.017	
421	C ₅ H ₉ NO ₄	glutamic acid	26.8	2.162	147.1
			-26.8	4.324	
			-55.7	9.966	
422	C ₅ H ₁₀ N ₂ O ₃	glutamine	30.1	2.170	146.1
			-30.1	9.308	
424	C ₆ H ₁₂ N ₂ O ₃	Gly-Amin	27.2	3.000	160.2
			-27.2	8.421	
426	C ₂ H ₅ NO ₂	glycine	37.4	2.350	75.1
			-37.4	9.780	
427	C ₄ H ₈ N ₂ O ₃	Gly-Gly	31.5	3.140	132.1
			-31.5	8.400	
438	C ₈ H ₁₆ N ₂ O ₃	Gly-Leu	25.1	3.180	188.2
			-25.1	8.432	
450	C ₆ H ₉ N ₃ O ₂	histidine	59.2	1.820	155.2
			29.6	6.040	
			-28.8	9.342	
461	C ₁₁ H ₂₂ N ₂ O ₃	Leu-Val	22.3	3.000	230.3
			-22.3	8.364	
469	C ₂ H ₇ NO ₃ S	taurine	37.9	1.500	125.1
			-37.9	9.182	
470	C ₄ H ₉ NO ₃	threonine	31.6	2.088	119.1
			-31.6	9.229	
621	C ₄ H ₁₁ NO ₃	Tris	29.5	8.076	121.1

m_0 , absolute mobility $\times 10^5$ (cm² V⁻¹ s⁻¹) at 25°C; pK_a , thermodynamic acid dissociation constant; MW, molecular weight.

constants are necessary. Although the number of such samples in which the mobilities and dissociation constants are available is limited (*ca.* 500), we compiled them in order to create a small database on a micro-computer. Most of them were taken from the literature^{8,15-17}, but a considerable number of the mobility and pK_a values were obtained using our isotachophoretic method (described in a later section).

Table 2 shows part of our database of mobilities and acid-dissociation constants^{18,19} which were used to plot the curves given in Fig. 1. The samples are numbered as shown in Table 2; the data can be accessed by these numbers.

3.2 Isotachophoretic steady state

The isotachophoretic steady state is achieved after some time when the following four conditions are fulfilled: (1) the leading and terminating electrolyte are chosen properly to satisfy Eq. (11); (2) the constant migration current is applied; (3) the mass balance of the counter ion is maintained (the total molar amount of the pH buffer (HQ⁺+Q) flowed into the sample zone in a unit time should be equal to the amount flowed out the sample zone); and (4) the electroneutrality is maintained in each

zone ($[A^-] + [OH^-] = [HQ^+] + [H^+]$).

When the supporting media of operational electrolytes is a capillary tube of PTFE, fused silica, *etc.*, the separation field can be regarded as being a free-electrolyte solution. The acid-base equilibria in each zone and the above mentioned conditions (2)–(4) can be formulated. After an iterative calculation is performed in which the pH of the sample zones is varied, a steady state pH can be found, which satisfies all of the isotachophoretic conditions.^{3,4,20,21} As a result, the concentrations of the sample and pH-buffer, the effective mobilities, the conductivities of the zones, as well as the qualitative and quantitative indices can be calculated. This enables a precise simulation of the resultant isotachopherograms. We have developed a simulation code called SIPS for this purpose; it is useful in estimating the sample zone properties in the steady state.^{21,22}

By using the physico-chemical constants given in Table 2 and the operational electrolyte condition, isotachopherograms can be simulated. Figure 5 gives the simulated and observed isotachopherograms of several amino acids and dipeptides at pH=8 (Tris buffer).¹⁸ A tanh function was used to simulate the potential gradient steps, and a Gaussian function was used for the differential signal. Obviously, the simulated isotachopherogram is very useful for estimating the quality of separation under the electrolyte condition.

Concerning the time-based zone length, the simulated values agreed with the observed values with deviations of less than a few percent. For example, the observed time-based zone length for 150 nmol acetic acid at a migration current of 70 μ A was 358.5 s; the simulated was 347.3 s (dev.=−3.1%).¹⁴

Although the time necessary to calculate the steady state is usually less than 5 s for one sample on a 32 bit

micro-computer (CPU=80386, co-processor=80387, clock=20 MHz), the speed is not sufficient for a separation optimization covering a wide pH range (ex. 3–10). We therefore made a database of the simulated qualitative and quantitative indices for fifty-five electrolyte conditions (0.2 pH step) for anions and cations, respectively. The isotachopherograms can thus be simulated very rapidly, and the separation conditions can be optimized within a few minutes. Nine buffers and eleven buffers were used for anionic analyses and cationic analyses, respectively. The electrolyte conditions can be simply selected by number, as shown in Table 1.

Isotachophoretic qualitative and quantitative indices have been published for 287 ions in the pH range of 3–10 in a tabular form.¹⁹ The validity of our simulation has been confirmed for a large number of samples by many researchers. For example, Oefner *et al.* measured the qualitative and quantitative indices of 52 anions and compared them with the simulated values.²³ Correlation factors of 0.993 and 0.92 were obtained for the qualitative and quantitative indices, respectively.

Two different approaches have been reported concerning the simulation of electrophoretic processes.²⁴ One is based on solving a set of partial differential equations by applying a suitable approximation;^{24–29} the other is a direct analytical method that is used to solve several simultaneous equations that express the electrophoretic conditions which should be satisfied in the steady state zones.^{3,20} Our simulation method^{21,22} belongs to the latter group; the computer program SIPS is the only one capable of a practical simulation of isotachopherograms.

3.3 Determination of the physico-chemical constants

When some of the factors which affect the effective mobilities are unknown, but the others are defined exactly, we can evaluate the unknown ones on the basis of the observed qualitative indices, such as the R_E values (Eq. (13)).^{30–32} The qualitative indices should be obtained by varying experimental conditions, which are closely related to the unknown factors. For example, when the acid dissociation constant is unknown, the pH must be varied so as to change the effective mobility. A least-squares calculation is applied in order to minimize S , as follows:

$$S = \sum_i W_i (R_{E,obs} - R_{E,sim})^2. \quad (17)$$

Here W_i is the weight of the i th R_E observed value ($R_{E,obs}$). $R_{E,sim}$ is the simulated R_E value. The best-fitting procedure is repeated on a computer until the convergence of S .

Figure 6 shows the observed R_E values of several dipeptides Leu-Val, Gly-Leu, Ala-Ala, Gly-Ser, Gly-Gly, Leu-Leu, Gly-Trp, Gly-Phe and Ala-Gly.¹⁸ Four pH buffers were used. The leading electrolytes were 10 mM HCl solutions and the pH_L were adjusted to 7.41, 8.30, 8.59, 8.77, 9.06, 9.33 and 9.52. The R_E values at high pH_L was almost constant. This suggests that the degree of dissociation was almost 1 at high pH, and that the R_E

Fig. 5 Simulated and observed isotachopherogram of several amino acids and peptides at pH_L=8 (Tris buffer). The abbreviations of the samples are as in Fig. 1. PVA, polyvinylalcohol.

Fig. 6 Observed R_E values of Leu-Val(1), Gly-Leu(2), Ala-Ala(3), Gly-Ser(4), Gly-Gly(5), Leu-Leu(6), Gly-Trp(7), Gly-Phe(8) and Ala-Gly(9). The curves were plotted using the best-fitted mobility and pK_a . The simulated R_E - pH_L curve for Gly(10) is also shown. The leading ion was the 10 mM chloride. Buffers: IM, imidazole; Tris, tris(hydroxymethyl)aminomethane; AM, amediol; EA, ethanolamine.

obtained values reflect the mobilities. On the other hand, the R_E values at relatively low pH_L , such as 7.4, reflect the acid dissociation constants. The least-square calculation to minimize S in Eq. (17) was carried out by varying the absolute mobilities and acid dissociation constants. The best-fitted constants have been already given in Table 2. It should be noted that the accuracy of the obtained constants is slightly worse than that of the usual conductivity method, depending on the accuracy of the observation and the used physico-chemical constants of the pH-buffers.³³ The most significant utility of the isotachophoretic method, compared with the classic conductivity method, is that the sample amount is small and mixed samples can be treated at the same time; in other words, the purification of objective samples is not necessary.

By using the simulational method we evaluated the mobility and pK_a values of many samples, such as amino acids³⁴, dipeptides¹⁸ and tripeptides.³⁵ By applying our method to the qualitative indices obtained at a different laboratory³, the mobility and dissociation constants of 62 anions were evaluated.³⁶ The best-fitted values coincided well with the previous values obtained by the classic methods, suggesting that the proposed method is useful for investigating the above-mentioned physico-chemical constants, and even complex stability constants.

Similar studies were carried out by Beckers³⁷, Pospichal *et al.*³⁸, as well as Kašička *et al.*³⁹, confirming the utility of the proposed method. Recently, the electrophoretic methods used for evaluating physico-chemical constants were reviewed.⁴⁰

4 Computer-Aided Separation Optimization in Isotachophoresis

When a sample has a very complex constitution, an experimental optimization of the separation condition is often very time-consuming. As expected from a simulation of the isotachopherogram as in Fig. 5, the operational electrolyte condition can be optimized to obtain the best separation quality by means of the simulation, or at least to limit the range of the experimental survey. Although the samples for which physico-chemical constants are available are presently limited, an important fact is that such a theoretical approach gives clear-cut insight for a strategy which can be used for separation optimization in isotachophoresis. Some examples were described concerning separation optimization utilizing the pH effect, the solvent effect and the complex-forming effect on the effective mobility.

Exactly speaking, although the separability depends upon the mobility difference in the mixed zone (see Fig. 2)^{41,42}, as a first approximation, the mobility difference at the steady state, or the difference of the R_E values, was used as a criterion for separation optimization, since a simulation of the transient mixed zone is rather complex and time-consuming.⁴²

4.1 Separation utilizing the pH effect on the effective mobility

When the sample contains weak electrolytes, the pH effect on the effective mobility should be utilized at first. This effect is practically important, and can be very easily simulated. An application of computer-aided separa-

Fig. 7 Simulated R_E of hypophosphorous acid (PO_2), phosphorous acid (PO_3), PO_4 , P_2O_7 , P_3O_9 , triphosphoric acid (P_3O_{10}), tetraphosphoric acid (P_4O_{12}), AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP, propionic acid and pelargonic acid at isotachopheric steady state. Buffers: BALA, β -alanine; EAC, ϵ -aminocaproic acid; CRE, creatinine; MP, 4-methylpyridine; IM, imidazole; MOR, morpholine; BA, butylamine and CHA; cyclohexylamine.

tion optimization was first reported regarding the analysis of trifluoroacetic acid metabolized from halothane, an anesthetic.⁴³ Another example of separation optimization utilizing the pH effect was described for a complex sample, a mixture of several phosphorous oxoacids and nucleotides. Since a considerable number of physicochemical constants were not available for the samples at that time, we first evaluated the mobilities and acid dissociation constants in order to optimize this separation.⁴⁴

Figure 7 shows the simulated R_E vs. pH_L curves for pyrophosphoric (P_2O_7), triphosphoric (P_3O_{10}), trimetaphosphoric (P_3O_9) acids, and fifteen nucleotides (AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP and UTP) plotted using the evaluated constants. Propionic acid as an internal standard and pelargonic acid as a terminator were also contained. The curves given in Fig. 7 overlap each other, thus suggesting that the separation of all these samples is difficult. In fact, Nukatsuka *et al.* utilized both complex-formation with Mg^{2+} and the solvent effect;⁴⁵ consequently, eleven nucleotides (AMP, ATP, CMP, CDP, CTP, GDP, GTP, IDP, ITP, UDP and UTP) and orthophosphate were separated. However, the simulated curves suggested that these samples might be separated at $pH_L \approx 4.7$. The optimum pH_L range may be quite limited, since CTP may form a mixed zone with UDP at a lower pH_L and with ATP at a higher pH_L .

Figure 8 shows the simulated and observed isotachopherograms at $pH_L = 4.7$ (creatinine buffer). As ex-

pected from the simulation, the fifteen nucleotides, P_2O_7 , P_3O_9 , P_3O_{10} and the internal standard (propionic acid) were practically separated. A satisfactory agreement between the simulated and observed isotachopherograms confirms the utility of a simulation concerning the separation optimization problem. In addition, an even better separation was obtained for phosphorous oxoacids when ion-pair formation with histidine was utilized.^{46,47}

In order to obtain high separability for weak electrolytes, the pH of the leading electrolyte should be carefully chosen. The present simulation technique is very useful for this purpose. At least, it gives a proper guideline for trying the solvent effect or the complex-forming effect for the expected separation.

4.2 Separation utilizing the solvent effect

Since the mobility is strongly affected by the viscosity of the solvent, and the dissociation constants depend on the dielectric constant of the solvent, the use of a nonaqueous solvent or a mixed solvent may improve the electrophoretic separation (the solvent effect).^{3,4} The other advantage of using nonaqueous systems is that it enables analyses of substances which are not sufficiently soluble in water. Many solvents have been successfully applied for isotachopheresis, such as methanol, ethanol, dioxane, acetone, propanols, and dimethylformamide.⁴

A simulation of the isotachopheric steady state is also possible for a nonaqueous solvent system. However, the number of substances whose mobility and pK_a

Fig. 8 Simulated and observed isotachopherograms of nineteen anions (AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP, UTP, pyrophosphoric acid (P_2O_7), triphosphoric acid (P_3O_{10}), tetraphosphoric acid (P_4O_{12}), and propionic acid (Prop)) at $pH_L=4.7$ (creatinine buffer). The terminator was pelargonic acid. The current was $75 \mu A$. The sample amounts were 1.2–3 nmol. The leading electrolyte contained 0.2% hydroxypropylmethylcellulose (HPMC). The buffer was creatinine.

data are available are presently extremely limited.⁸ Therefore, the mobility and pK_a of 60 anionic substances in methanol (99.5wt%) have been evaluated by the isotachophoretic method and then compared with those in water.⁴⁸

The migration behavior in a nonaqueous solvent is very different from that in an aqueous solvent. The use of a mixed solvent (aqueous-nonaqueous) gives an additional possibility of mobility control which could be used to improve the separation.

4.3 Separation utilizing the complex-forming effect

The use of a complex-forming agent is one of the traditional techniques used to improve the separability, especially in the case of metal ions.⁴⁹ Yoshida *et al.*⁵⁰ recently reviewed separations utilizing complex-forming electrolyte systems concerning their features and advantages. To achieve complex-forming equilibria, a constant amount of the complex-forming agent should be continuously supplied to the sample zone. The complexing agent can be supplied as follows: 1) as a counter ion in the leading electrolyte;⁵¹ 2) as a neutral ligand (crown ether and cyclodextrine);⁵² and 3) as terminating ions.^{53–55} Tazaki *et al.*⁵⁶ first elucidated the migration behavior of metal ions in the above-mentioned system 2 theoretically. Our theoretical approach was developed for system 1.²¹

Kaniansky *et al.* have reported on the isotachophoretic separation of several organic acids, including Krebs-cycle acids, by adding an appropriate amount of Ca^{2+} to the

leading electrolyte.⁵¹ We have analyzed the reported separation in order to investigate why the proposed electrolyte condition was optimum.⁵⁷

As shown in Fig. 9, the effective mobilities of several samples are very close to each other, thus suggesting that sufficient separation may not be expected when only the pH dependence of the effective mobility is utilized. The separation of formic acid and tartaric acid seems to be difficult throughout the pH_L range considered. In the 4.5–6 pH_L range, the separation of citric acid and α -ketoglutaric acid may also be difficult.

Ca^{2+} was then added to the leading electrolyte. As exemplified by tartaric acid in the former section, the mobility of organic acid decreases due to the ion-pair formation (Eqs. (4) and (5)). Since the decrement depends on the stability constants of the components, there might be an optimum concentration of Ca^{2+} and pH_L for the optimum separation.

From a simulation, the optimum separation was expected at $pH_L=6$, when the Ca^{2+} concentration was 2 mM. Figure 10a shows the Ca^{2+} concentration dependence on the R_E values of the samples at $pH_L=6$ and 4.5. Obviously, the R_E increment of citric acid was most significant, due to the largest stability constants ($\log K_{CaHCit}=2.9$ and $\log K_{CaCit}=4.8$). At a different pH_L (4.5), a similar separation was expected when $[Ca^{2+}]$ was 1 mM, as shown in Fig. 10b.

Figure 11 shows the observed and simulated isotachopherograms obtained at $pH_L=6$. A good agreement was obtained in both cases when no Ca^{2+} was added (A) and

Fig. 9 The pH dependence of the R_E values of Krebs-cycle acids at the isotachophoretic steady state. The samples are perchloric acid (ClO_4), formic acid (For), tartaric acid (Tar), citric acid (Cit), α -ketoglutaric acid (α -Ket), acetic acid (Ac), lactic acid (Lac), 2-hydroxybutyric acid (2HB) and caproic acid (Cap). The leading ion (chloride (Cl^-)) and the terminating ion (N -morpholinoethanesulfonic acid (MES)) are also shown. Buffers: β -Ala, β -alanine; ϵ -AMC, ϵ -aminocaproic acid; His, histidine; Tris, tris(hydroxymethyl)aminomethane. $\text{pH}_L = \text{pH}$ of the leading electrolytes.

Fig. 10 Ca^{2+} concentration dependence on the R_E values at $\text{pH}_L=6$ (a) and $\text{pH}_L=4.5$ (b). The samples are as in Fig. 9.

when 2 mM Ca^{2+} was added to the leading electrolyte. In addition to the above example, the complex-forming equilibria is very useful for separating metal ions. A typical example of isotachophoretic separation of metal

Fig. 11 Observed and simulated isotachopherograms obtained at $\text{pH}_L=6$ when no Ca^{2+} was added (A), and 2 mM Ca^{2+} was added to the leading electrolyte (B). The observed pherograms were from ref. 51.

ions utilizing the complex-forming effect has been reported for the lanthanides by Nukatsuka *et al.*⁵⁸, in which α -hydroxyisobutyric acid (HIB) was used as the complex-forming agent. The concentration of a complex-forming agent and the pH of the leading electrolyte should be optimized in order to obtain good separation.

Although the complex-forming equilibria in a lanthanide zone is very complex, due to the coexistence of many different ionic species in a zone, we analyzed the separation behavior of lanthanides theoretically.⁵⁹

Figure 12 shows both the observed and simulated isotachopherograms of 14 lanthanides. The important point is not the agreement between them, but that the evaluated constants for the simulation are useful for computer experiments at different electrolyte conditions. In fact, our recent study concerning a separability evaluation of lanthanides, the theoretical estimates agreed well with the observed values, even though the electrolyte conditions were different.⁶⁰

In addition to the stability constants, the coordination number of the formed complexes can be determined in a similar manner.^{61,62} This depends on the fact that if the coordination number is not correct, quantity S in Eq. (17) for the qualitative indices obtained by varying a

Fig. 12 Observed and simulated isotachopherograms of 14 lanthanides. HIB, the complex-forming agent α -hydroxybutyric acid. The leading ion, 9.85 mM K^+ ; pH-buffer=acetic acid ($pH_L=4.77$). The terminator= ϵ -aminocaproic acid (ϵ -AMC).

complex-forming reagent is large, even after the stability constants and mobilities are best-fitted to give a minimum S .

5 Conclusion

It was described how computer simulations of isotachopheresis are useful for separation optimization utilizing the pH effect, the solvent effect and the complex-forming effect on the effective mobility. It was also shown that the simulation method was useful regarding physico-chemical measurements of the mobilities, acid-dissociation constants and stability constants of ionic substances.

Undoubtedly, the fundamental data of the mobilities and pK_a are very important in our simulation methods. It should be noted that by using only a few constants for a sample as input data we can carry out enormous number of computer experiments during a very short time. Namely, a chemical diagnosis which includes separation optimization is possible by using a few constants and a simulation code. As pointed out by Gohshi⁶³, simulation and prediction will be important areas of analytical chemistry in the future. The

compilation of such basic constants to make a creative database is important work for the future analytical chemistry.

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