Journal of Chromatography, 633 (1993) 251–259
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 752

Bidirectional isotachophoresis

I. Verification of bidirectional isotachophoresis and simultaneous determination of anionic and cationic components

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(First received June 16th, 1992; revised manuscript received November 10th, 1992)

ABSTRACT

Bidirectional isotachophoretic migration was confirmed by the direct measurement of the pH profiles and the boundary velocities of the separated zones. The anolyte used was 10 m*M* HCl–*β*-alanine (pH 3.6) and the catholyte was 10 m*M* KOH–acetic acid (pH 4.8). It was shown that bidirectional isotachophoresis could be achieved with an electrolyte system consisting of a leading electrolyte for an anionic analysis and one for a cationic analysis. The combination was not arbitrary but the pH difference between the anolyte and the catholyte was restricted to keep the effective mobility of the terminating ion not too small. The simultaneous separation and determination of anions and cations in a test mixture were demonstrated on the basis of the time-based zone length measured by the use of a dual detection system.

INTRODUCTION

As electrophoretic phenomena are bidirectional in principle, isotachophoretic migration must be also bidirectional. In fact, as pointed out by Thormann *et al.* [1], isotachophoretic stacking zones can be formed simultaneously for anionic and cationic components in a sample when a suitable electrolyte system is chosen. They demonstrated bidirectional isotachophoretic migration under an electrolyte system of sodium acetate and HCl. However, no practical separation utilizing bidirectional isotachophoresis has been reported in spite of its utility in the simultaneous determination of anions and cations.

In this work, bidirectional isotachophoretic migration was verified by the direct measurement of the pH profile and the boundary velocities of the separated zones. In the pH profile measurement, a free-flow apparatus (Bender and Hobein, Elphor Vap 22) was used and the pH values of the fractions were measured. For boundary velocity measurements, a capillary-type ITP analyser equipped with a position scanning ultraviolet detector was used.

The practical simultaneous determination of anions and cations was demonstrated for a test mixture by the use of a dual potential gradient detector system applied from two Shimadzu IP-2A ITP analysers.

Although the selection of the operational electrolyte system for bidirectional isotachophoresis is not very difficult, the difference between the pH of an anolyte and that of a catholyte is restricted. In the next section we outline how bidirectional iso-

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0021-9673/93/$06.00

tachophoresis works and how the restriction comes about.

THEORETICAL

Configuration of an operational electrolyte system

Fig.1 illustrates the configuration of a bidirectional operational electrolyte system in a separation tube. In bidirectional isotachophoresis, the leading electrolyte for anions must be simultaneously the terminating electrolyte for cations, and the leading electrolyte for cations must be the terminating electrolyte for anions. That is, the pH-buffering cations coexisting with the leading anion play the role of the terminating cation, and the pH-buffering anions coexisting with the leading cation plays the role of the terminating anion.

Fig.1 also shows an example of the electrolyte system. The anolyte [a 10 m*M* HCl solution buffered by adding 20 m*M* β-alanine (pH 3.6)] is a typical leading electrolyte for anionic analysis, where the leading ion is Cl^-. This solution can be the terminating electrolyte for a cationic analysis in the pH range 4–5, because β-alanine has a suitably small effective mobility in this pH range. On the other hand, the catholyte [a 10 m*M* KOH solution buffered by adding 20 m*M* acetic acid (pH 4.8)] is a typical leading electrolyte for a cationic analysis (leading ion K^+). Acetic acid in this solution is an acceptable terminator in the pH range 3–4. When these electrolytes are used in combination, bidirectional isotachophoretic migration will occur.

Limitation on the electrolyte combination

There are several typical leading electrolytes for a cationic analysis and for an anionic analysis, where the pH ranges from 3 to 10 [2–4]. However, these electrolyte systems cannot be combined arbitrarily, because the pH difference between the anolyte and the catholyte is restricted, as discussed below.

The pH of each electrolyte should be selected to ensure the pH-buffering ability as follows:

$$pK_{QA} - 0.5 < pH_{LA} < pK_{QA} + 0.5 \quad (1)$$

$$pK_{QC} - 0.5 < pH_{LC} < pK_{QC} + 0.5 \quad (2)$$

where pK_{QA} and pK_{QC} are the pK_a values of the pH buffer of an anolyte and that of a catholyte, respectively, and pH_{LA} and pH_{LC} are the pH_L of an anolyte and a catholyte, respectively.

Fig. 2 shows the simulated R_E of model cations and anions of weak electrolytes at the isotachophoretic steady state. R_E is one of the qualitative indices of isotachophoresis [3] defined for a sample S as follows:

$$R_{E,S} = E_S/E_L = \bar{m}_L/\bar{m}_S \quad (3)$$

where E_S and E_L are the potential gradient of the sample (S) and the leading (L) zones and $\bar{m}$ is the effective mobility. The leading anion used in the simulation was 10 m*M* Cl^- and the leading cation was 10 m*M* K^+. The simulated effective mobility of the leading ion ($\bar{m}_L$) was $74.7 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} for Cl^- and $71.4 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} for K^+. The absolute mobilities of the model samples were var-

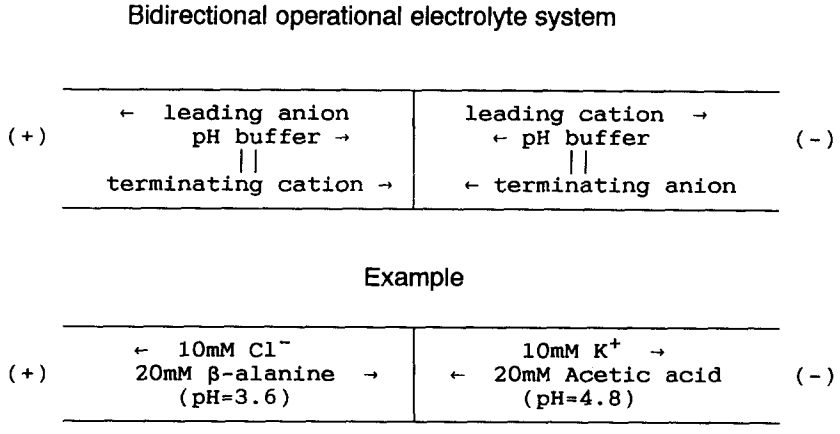


Fig. 1. Configuration of a bidirectional operational electrolyte system in a separation tube and an example.

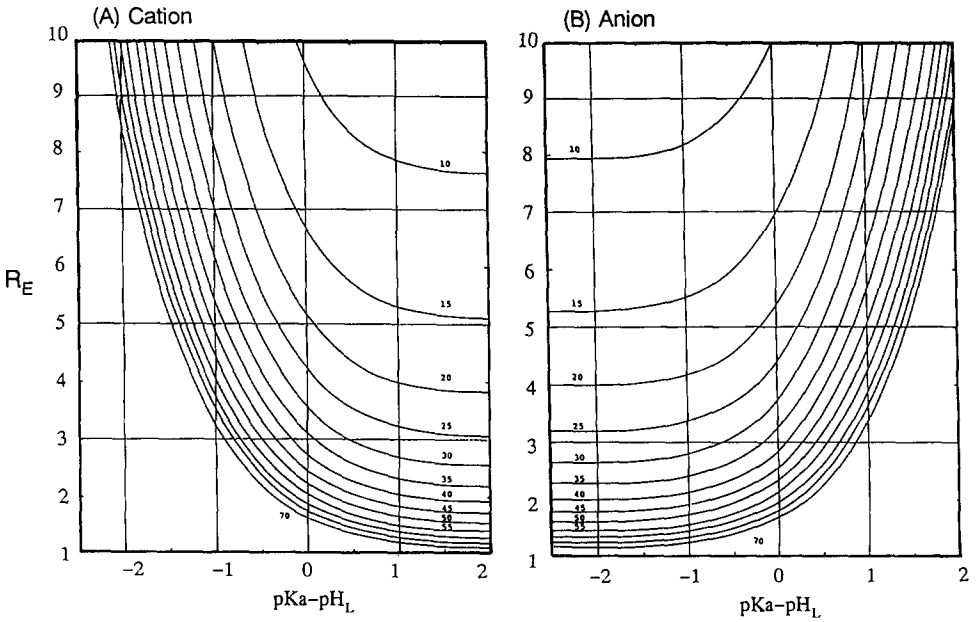


Fig. 2. Simulated R_E of model cations and anions of weak electrolytes. The leading anion was 10 mM Cl^- and the leading cation was 10 mM K^+. The absolute mobilities of the model samples were in the range $10 \cdot 10^{-5}$–$70 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}.

ied in the range of $10 \cdot 10^{-5} - 70 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}. The maximum buffering ability ($pH_{LA} = pK_{QA}$ and $pH_{LC} = pK_{QC}$) was assumed in the simulation and this is just the case for the electrolyte system illustrated in Fig.1.

As the effective mobility of a sample ($\bar{m}_S$) decreases with decrease in the degree of dissociation, R_E increases with decrease in $pK_a - pH_L$ for cations, and *vice versa* for anions, as shown in Fig. 2. If the pK_a value of the terminator used is different from the pH of the leading electrolyte, it is not a suitable terminator from the practical view point because the terminator should have an adequately small effective mobility.

In the bidirectional electrolyte system, the anionic terminator is the pH buffer for a cationic analysis and the cationic terminator is the pH buffer for an anionic analysis. Even if the selected pH buffers satisfied eqns. 1 and 2, they might not be suitable as terminators. The pK_a values of the pH buffer and the pH_L of the bidirectional electrolyte system should satisfy the following conditions:

$$0.5 \leqslant pK_{QC} - pH_{LA} \leqslant 1.5 \quad (4)$$
$$0.5 \leqslant pH_{LC} - pK_{QA} \leqslant 1.5 \quad (5)$$

The upper limiting value in eqns. 4 and 5 is to keep the effective mobility not too small as the terminator. If it is too small, the operational system is not practically useful owing to the limitation of the high-voltage power supply. It should be noted that the limiting values in the above equations depend on the absolute mobility of the terminator ion (the larger absolute mobility, the larger is the limiting value, and *vice versa*). Suppose a typical terminator of an anionic analysis, acetic acid ($pK_a = 4.756$), is used in combination with a leading electrolyte of 10 mM HCl–20 mM β-alanine ($pK_a = 3.55$, $pH_{LA} = 3.6$). In this instance, $pK_{QC} - pH_{LA} \approx 1.2$. According to our simulation, the effective mobility and R_E value of acetate ion were $12.5 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} and 5.99 at the steady state. Similarly, when a typical terminator β-alanine ($pK_a = 3.55$) is used in combination with a leading electrolyte of 10 mM KOH–20 mM acetic acid ($pH_{LC} = 4.8$), $pH_{LC} - pK_{QA} \approx 1.3$. The simulated effective mobility and R_E value of β-alanine were $12.5 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} and 5.73. The large value of $pK_{QC} - pH_{LA}$ or $pH_{LC} - pK_{QA}$ means a small effective mobility as the terminating ion.

On the other hand, the lower limiting value of 0.5

in eqns. 4 and 5 is to avoid an unnecessarily large mobility as the terminator. If the effective mobility is too large, the mobility range of analysable samples becomes narrow.

When eqns. 1 and 2 are satisfied as $pH_{LA} = pK_{QA}$ and $pH_{LC} = pK_{QC}$, eqns. 4 and 5 can be rewritten as follows:

$$0.5 \leqslant pH_{LC} - pH_{LA} \leqslant 1.5 \quad (6)$$

The above relationship expresses the adequate range of the pH difference between the catholyte and the anolyte: the pH of a catholyte should be appropriately higher than that of an anolyte.

EXPERIMENTAL

Samples

The cationic samples used were toluidine blue (TB), tris(hydroxymethylamino)methane (Tris), astrazone pink (AP), ethanolamine (EA), creatinine (CR) and ε-aminocaproic acid (AMC). The anionic samples were 4,5-dihydroxy-3-(*p*-sulfophenylazo)-2,7-naphthalenedisulphonic acid (SPADNS), monochloroacetic acid (MCA), picric acid (PA), *o*-chlorobenzoic acid (CB) and benzoic acid (BZA). Except for Tris and MCA, these samples absorb visible and/or UV light. The sodium salt of SPADNS was purchased from Dojin (Kumamoto, Japan) in the purest form. The other chemicals were guaranteed-grade reagents from Tokyo Kasei (Tokyo, Japan).

Three-component mixtures of SPADNS, MCA and PA and of TB, Tris and AP were used for the observation of the boundary velocities in the bidirectional electrolyte system. A six-component mixture of EA, CR, AMC, MCA, CB and BZA was used for the observation of bidirectional isotachopherograms.

Operational electrolyte system

Table I shows the operational electrolyte systems used. Electrolyte systems 1 and 2 (abbreviated as ES1 and ES2) were used for free-flow isotachophoresis. ES1 is a bidirectional electrolyte system. The pH of the ES1 anolyte was adjusted to 3.6 by adding β-alanine to 10 m*M* HCl and the pH of the ES1 catholyte was adjusted to 4.8 by adding acetic acid to 10 m*M* KOH solution. ES2 is a unidirectional electrolyte system for a cationic analysis and the pH of the anolyte was also adjusted to 3.6 by adding acetic acid to 20 m*M* β-alanine. Electrolyte systems 3, 4 and 5 were used for the boundary velocity measurements. ES3 is a bidirectional electrolyte system and ES4 and ES5 are unidirectional electrolyte systems for a cationic analysis and an anionic analysis, respectively. The use of 5 m*M* leading electrolyte was to avoid saturation of the UV detection system due to a high concentration of the sample in each zone. Hydroxypropylcellulose (HPC) (0.1%) was added to all of the operational electrolytes to suppress electroendosmosis.

pH measurements were carried using a Horiba (Tokyo, Japan) Model F7ss expanded pH meter.

TABLE I

OPERATIONAL ELECTROLYTE SYSTEMS

Electrolyte systems 1 and 3 are bidirectional and the others are the usual unidirectional electrolyte systems. Hydroxypropylcellulose (0.1%) was added.

No.	Anolyte[a]	Catholyte[a]
1	10 m*M* HCl–β-Ala (pH 3.6)	10 m*M* KOH–Ac (pH 4.8)
2	20 m*M* β-Ala–Ac (pH 3.6)	10 m*M* KOH–Ac (pH 4.8)
3	5 m*M* HCl–β-Ala (pH 3.6)	5 m*M* KOH–Cap (pH 4.8)
4	10 m*M* β-Ala–Cap (pH 4.5)	5 m*M* KOH–Cap (pH 4.8)
5	5 m*M* HCl–β-Ala (pH 3.6)	10 m*M* Cap–β-Ala (pH 3.6)

[a] β-Ala = β-Alanine; Ac = acetic acid; Cap = caproic acid.

Apparatus

Three different types of apparatus were used. The first was a free-flow isotachophoretic analyser (Bender and Hobein, Elphor Vap 22), which was used for pH profile measurements. The separation chamber was 50 cm high 10 cm wide and 0.5 mm thick. The sample and operational electrolytes were supplied continuously to the separation chamber and the sample was separated owing to the electric field applied perpendicular to the flow. The separated sample zones were fractionated at the end of the sample chamber using a 90-fold peristaltic pump. The total flow-rate of the operational electrolytes through the separation chamber was 160 ml/h. The migration current applied was 15 mA and the separation chamber was thermostated at 15°C. The anolyte and catholyte used were 2 l in volume and they were circulated by pumps through the electrode compartments during migration.

The second was a capillary-type ITP analyser equipped with a position-scanning UV spectrophotometric detector [5], which was used for the observation of separation process and boundary velocities. It was operated at λ_{max} = 330 nm. The separation tube used was a fused-silica capillary (32 cm × 0.7 mm O.D. × 0.53 mm I.D.). All experiments were carried out at 25°C. A single cycle to scan the 32-cm tube took 7.025 s and the number of data in a single scan was 5333. The resolution was 0.06 mm per datum, which was sufficient to trace the separation process accurately. Data were acquired by the use of a NEC (Tokyo, Japan) PC9801VX microcomputer (80286-80287, clock 10 MHz) and were processed for boundary velocity evaluation.

The last type was two Shimadzu isotachophoretic analysers (IP-2A), which were used in combination, on each side of the sample injection port, for the measurement of bidirectional isotachopherograms. The other components necessary for isotachophoresis such as the separation tube and high-voltage power supply were single units. Potential gradient detectors were used. The PTFE separating tube used was 40 cm × 0.5 mm I.D. The driving current applied was 50 μA.

For the simulations of steady-state isotachophoresis, our program SIPS was used on an NEC PC-9801RA2 (CPU 80386, coprocessor 80387, clock 20 MHz). SIPS permits the precise simulation of qualitative and quantitative indices [3] and isotachopherograms. A small database of mobility and pK_a values for *ca.* 500 samples can be used for sample data input.

RESULTS AND DISCUSSION

Verification of bidirectional isotachophoresis

As the steady state of an isotachophoretic zone is usually not affected by the counter ion contained in the terminating zone, there is no fundamental difference between the steady state of bidirectional isotachophoresis and that of unidirectional isotachophoresis. Therefore, bidirectional isotachophoretic migration can be simulated as the combination of the respective simulations for an anionic analysis and a cationic analysis.

Fig. 3 shows the configuration of the bidirectional electrolyte system studied and the sample zone properties simulated for the steady state (the pH of the zones, the total concentration of acetic acid and β-alanine and R_E). The anolyte was 10 m*M* HCl buffered by 20 m*M* β-alanine (pH 3.6) and the catholyte was 10 m*M* KOH solution buffered by 20 m*M* acetic acid (pH 4.8). It should be noted that the components of the anionic terminating zone developed (TA in Fig. 3B) were the same as those of the cationic terminating zone developed (TC in Fig. 3B), but according to our simulation the concentrations were different from each other, as shown in Fig.3C.

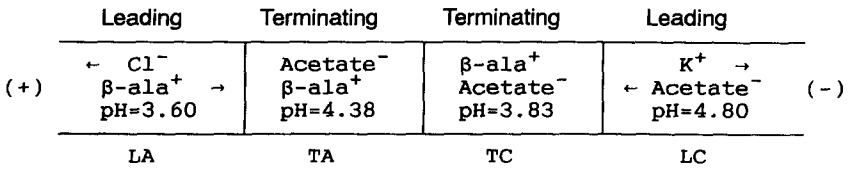
Fig. 3B shows the pH of zones simulated for the steady state. The values were significantly different from each other and this simulation was proved experimentally by measuring the pH of fractions obtained by the use of a free-flow isotachophoretic apparatus: 90 fractions were obtained in 2.5 h using two different electrolyte systems, 1 and 2 in Table I (ES1 and ES2). Three adjacent fractions were mixed to increase the volume for convenience, and the pH values of the solutions were measured. Fig. 4 shows the results of pH measurement under three conditions: (A) no migration current and the electrolyte system unidirectional (ES2), (B) migration current 15 mA and ES2 was used, and (C) migration current 15 mA and the electrolyte system bidirectional (ES1). As shown in Fig. 4B, the observed pH profile was simple when a unidirectional electrolyte system was used. Fractions 45–55 corresponded to the cationic terminating zone of S0163TbS0180T-alanine and the average pH was 3.9, in good agreement with the simulated value of 3.83. There was no pH change in the fractions from 1 to 40.

On the other hand, when the bidirectional electrolyte system was used, as shown in Fig. 4C, another zone was seen from fractions 25 to 40. It was definitely assigned to the developed terminating zone of acetic acid. The observed pH value (4.4) was again in good agreement with the simulated pH value (4.38), confirming bidirectional isotachophoretic migration.

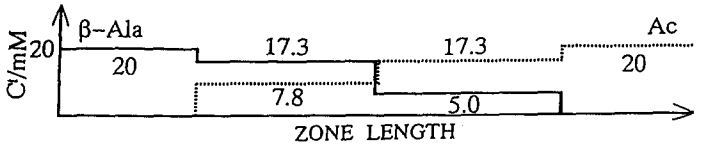
Fig. 5 shows the migration process of picric acid and astrazone pink with the bidirectional electrolyte system (3 in Table I) observed with the use of the position-scanning UV detection system. Each component was injected into the separation tube individually to prevent precipitation by ion pairing. They were pushed towards the centre of the separation capillary by the flow of the anolyte. Appar-

(A)Before migration

(B)Steady state configuration and pH (Blank run)



(C)Total concentration of acetic acid(Ac) and β-alanine



(D)Potential gradient ratio R_E

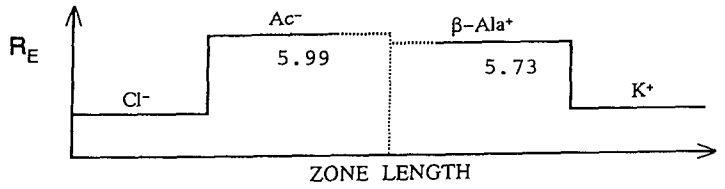


Fig. 3. Configuration of a bidirectional electrolyte system and simulation at the steady state.

ently from Fig. 5, the boundary velocity of rectangular zones was constant after 500 s, confirming isotachophoretic migration in both directions. The initial velocity change was due to mixing of samples with the operational electrolytes.

The boundary velocity was then measured exactly for three-component samples. Fig. 6 shows the observed transient isotachopherograms for a three-component mixture of SPADNS, MCA and PA. The amount of each sample was 60 nmol. The boundaries between the leading and the SPADNS zones in Fig. 6 were rearranged at the same abscissa position. Table II summarizes the boundary velocities observed with unidirectional and bidirectional electrolyte systems. For cations, only the steady-state boundaries are shown, because a mixed zone between the leading ion and toluidine blue was formed. Apparently from Table II and Fig. 6, the boundary velocity of the separated zones and the resolution time of each mixed zone agree well with each other, confirming that the separated zones migrated isotachophoretically and there was no significant difference between the migration behaviours of a bidirectional and a unidirectional system.

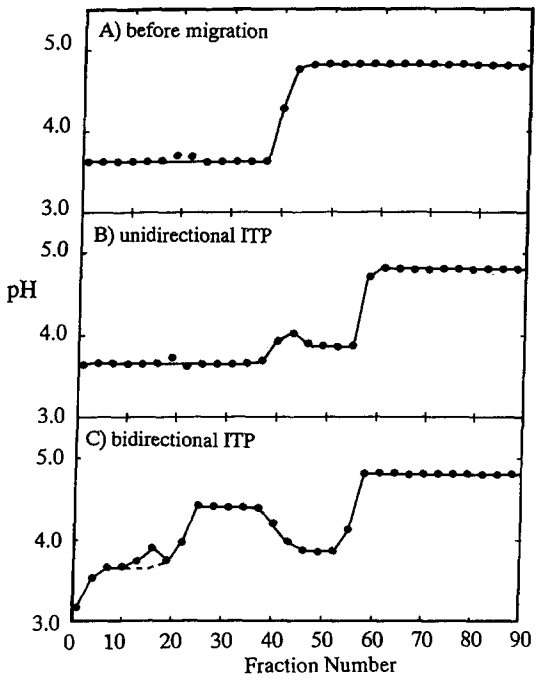


Fig. 4. pH of fractions obtained by the use of a free-flow isotachophoretic apparatus. (A) No migration current and electrolyte system unidirectional (electrolyte system 2 in Table I); (B) migration current = 15 mA and electrolyte system 2 was used; (C) migration current = 15 mA and electrolyte system bidirectional (electrolyte system 1). Cathode, right-hand side; anode, left-hand side.

Analysis of a test mixture

Lastly, isotachopherograms of anionic and cationic components in a sample were obtained simultaneously using a dual detection system (two potential gradient detectors) to demonstrate the analytical utility. Fig. 7 shows the observed isotachopherograms of an equimolar six-component mixture with electrolyte system 1. The ordinate scale of each detection system was not normalized. The total sample amount was 120 nmol and the migration current was 100 μA. The time-based zone lengths were measured varying the sample amount. The calibration lines evaluated for the sample components are shown in Fig. 8. The linearity is good, as usual, suggesting that bidirectional isotachophoresis is useful in practical analyses using conventional detectors such as the potential gradient detector.

In conclusion, we have confirmed bidirectional isotachophoretic separation by direct measurement of the pH of separated zones and the boundary velocity using a position-scanning detector. Bidirectional isotachophoresis is possible when the anolyte is a leading electrolyte for anions and the catholyte is a leading electrolyte for cations, and the pH-buffering ions in each electrolyte also play the role of the terminating ions. The one demerit of bidirectional isotachophoresis is that a dual detection sys-

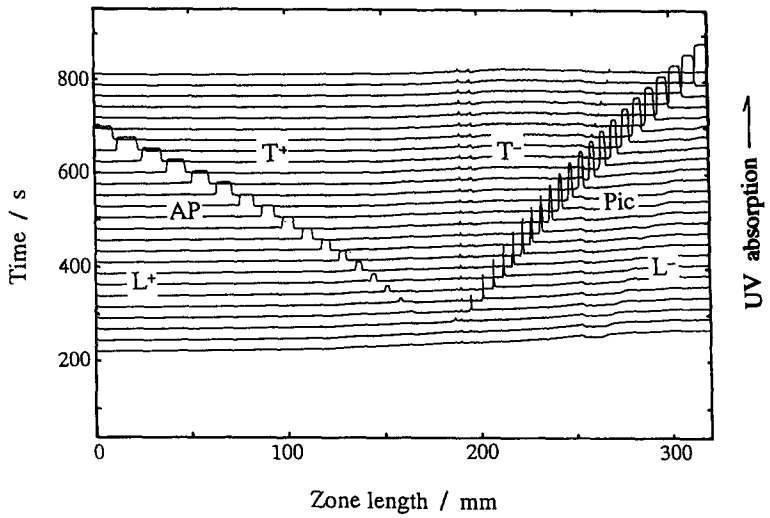


Fig. 5. Bidirectional isotachophoresis of picric acid and astrazone pink observed with the use of a position-scanning UV detection system. Electrolyte system 3 in Table I was used. Migration current, 50 μA.

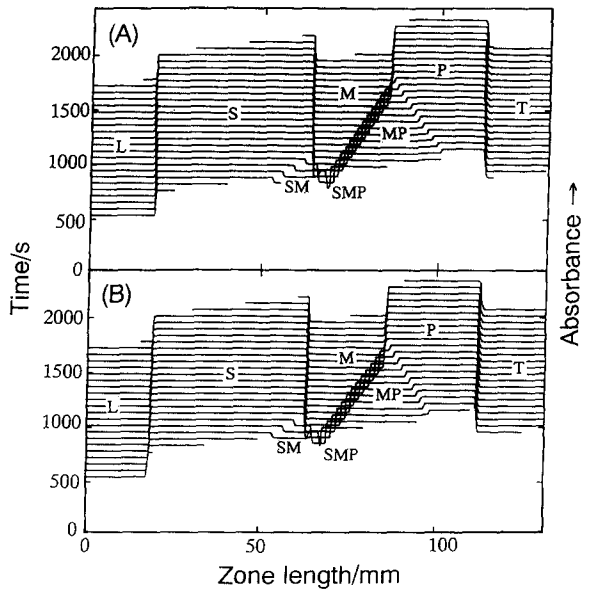


Fig. 6. Transient isotachopherogram of SPADNS (S), monochloroacetic acid (M) and picric acid (P) obtained with the use of a position-scanning UV spectrophotometric detector. The amount of each sample was 60 nmol. (A) Unidirectional electrolyte system; (B) bidirectional system (see Table I). Migration current, 50 μA.

TABLE II

BOUNDARY VELOCITIES OBSERVED BY THE USE OF A POSITION-SCANNING UV DETECTION SYSTEM

Electrolyte system No. 3; migration current, 50 μA. L = leading; S = SPADNS; M = monochloroacetic acid; P = picric acid; B = toluidine blue; R = Tris; A = astrazone pink; SM = mixed zone of SPADNS and monochloroacetic acid; MP = mixed zone of monochloroacetic acid and picric acid; SMP = mixed zone of SPADNS, monochloroacetic acid (M) and picric acid.

Boundary	Velocity (mm/s)		Difference (%)
	Unidirectional	Bidirectional	
Anions			
L/S	0.254	0.253	−0.4
S/M	0.254	0.253	−0.4
M/P	0.253	0.252	−0.4
P/T	0.254	0.253	−0.4
S/SM	0.203	0.201	−1.0
SM/M	0.291	0.292	0.3
M/MP	0.232	0.231	−0.4
MP/P	0.274	0.272	−0.7
SM/SMP	0.185	0.181	−2.2
SMP/MP	0.314	0.314	0.0
Cations			
L/B	0.315	0.319	1.3
B/R	0.314	0.318	1.3
R/A	0.315	0.319	1.3
A/T	0.315	0.319	1.3

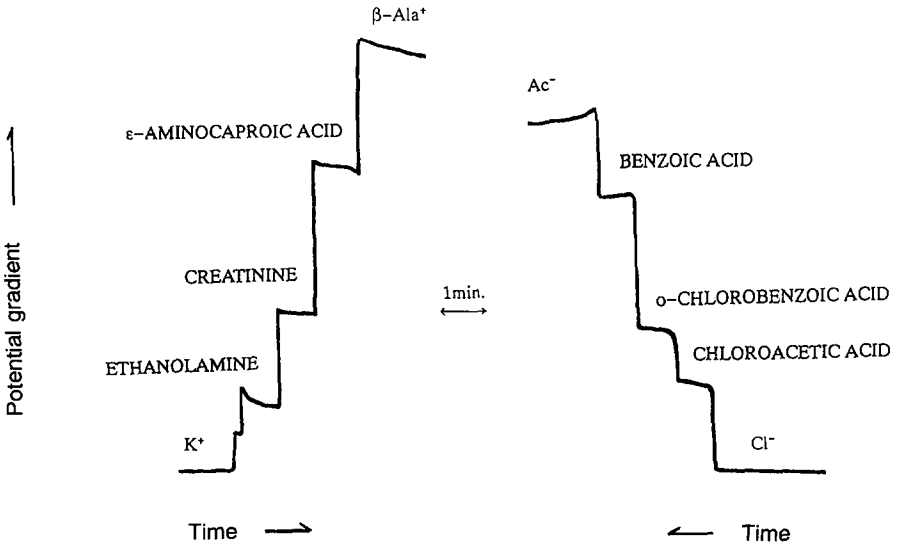


Fig. 7. Observed isotachopherograms from an equimolar six-component mixture of ethanolamine, creatinine, ε-aminocaproic acid, monochloroacetic acid, picric acid, *o*-chlorobenzoic acid and benzoic acid using electrolyte system 1. The total sample amount was 120 nmol. Migration current, 100 μA.

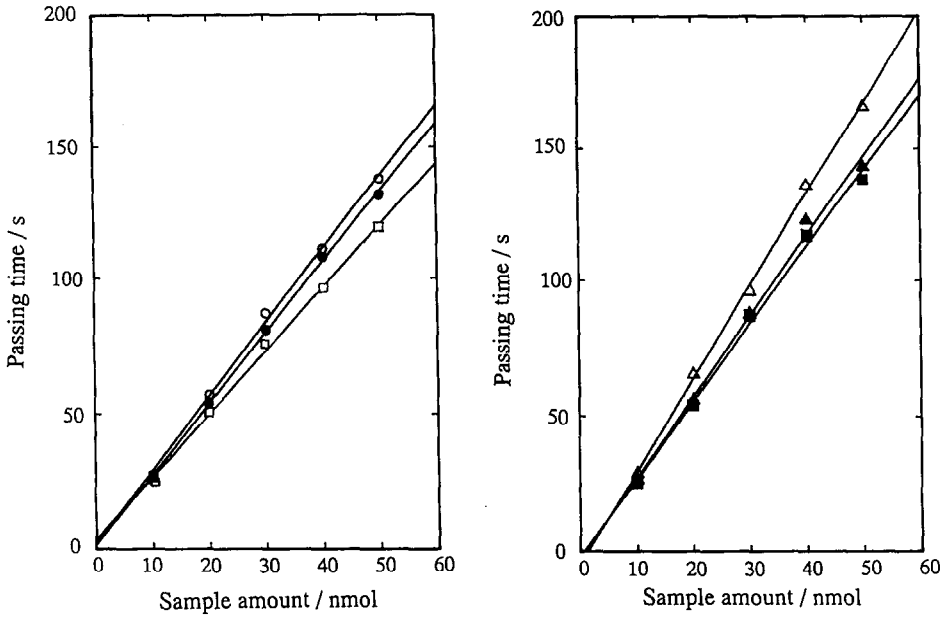


Fig. 8. Calibration line for a six-component mixture of (▲) ethanolamine, (■) creatinine, (△) ε-aminocaproic acid, (□) monochloroacetic acid, (○) *o*-chlorobenzoic acid and (●) benzoic acid with electrolyte system 1. Migration current, 100 μA.

tem is necessary. Bidirectional isotachophoresis can be utilized in practice by the use of two leading electrolytes for anionic and cationic analyses in the pH range 3–11 as usual, although one must take care of the pH difference of the anolyte and the catholyte, as discussed. The possible combination of the electrolyte systems and the predicted migration behaviour will be reported in due course.

ACKNOWLEDGEMENTS

The authors express their thanks to Power Reactor and Nuclear Fuel Development (Tokyo, Japan) for providing the free-flow apparatus for this study.

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