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Analysis of a dilute sample by capillary zone electrophoresis with isotachophoretic preconcentration

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ABSTRACT

A capillary zone electrophoresis (CZE) apparatus utilizing on-line isotachophoretic (ITP) preseparation was constructed and it was shown that the dependence of the migration time in CZE on sample concentration was avoided by ITP preconcentration. The concentration and volume of the test mixture injected by using a microsyringe were in the range of 166-1.66 μM and OS-50 μl, respectively. The ratio of the sample volume to the separation column volume (27 μl) was in the range 1.9-185%. Although the migration time in the CZE stage did not change when ITP preconcentration was utilized, if the latter was not used the migration time was prolonged up to 400% non-linearly to the above ratio. The utility of ITP-CZE for the determination of trace components in a matrix was also demonstrated.

INTRODUCTION

Ideal zone electrophoretic migration occurs in a homogeneous separation field filled with a background electrolyte solution, where the concentration of a sample should be negligibly small in comparison with the concentration of the background electrolyte (possibly less than a few per cent). This requirement can be easily perturbed by overloading a concentrated or a dilute sample solution. Especially the overloading of a dilute sample with low conductivity may cause the separation behaviour to deteriorate, because the dilute sample present in a separation column may reduce the migration current under constant-voltage operation; although this current reduction is gradually recovered, the fundamental qualitative index of migration time may be seriously affected. The dilute sample should be preconcentrated to avoid this situation.

Isotachophoresis (ITP) is the most suitable preconcentration method for capillary zone electrophoresis (CZE). Combined ITP-CZE has already been demonstrated as the capillary version of Ornstein-Davis disc electrophoresis in the initial stage of the development of CZE by Mikkers [1]. Hjertén *et al.* [2] have shown some applications for biological samples and suggested the importance of the technique in the future. Recently, Kaniansky and Marak [3] and Foret *et al.* [4] showed that the low relative sensitivity of CZE was improved by using isotachophoretic preconcentration. Some applications using this coupled technique have been reported emphasizing its high relative sensitivity [2–5].

Migration time is a qualitative index in CZE. If the operational conditions remain the same, the migration time should not depend on the nature of the sample solution, such as the concentration of sample components. In this work, the analytical advantage of ITP-CZE utilizing microsyringe injection was investigated, especial-

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ly with regard to the improvement of the consistency of the migration time for a test mixture with various concentrations.

For the evaluation of overloading effects of a dilute sample in CZE, the ratio of the sample volume to the separation column volume can be used as one of practical measures. As the sample volume is obtained most easily and accurately when microsyringe injection is used, a septum-type injector was used with a coupled column made with relatively wide-bore tubes (0.5 and 0.25 mm I.D.). This allows the precise evaluation of the overloading effect in CZE, although the separability may be poorer in comparison with that of a CZE analyser with a separation capillary tube of cu. 50 μm I.D.

EXPERIMENTAL

Apparatus and operation

Fig. 1A shows a schematic diagram of the ITP-CZE analyser. It consists of two electrophoretic analysers, of which part of their separation tubes is common. The analyser had four electrode compartments (EL1–EL4), EL1 and EL2 being for the ITP stage and EL3 and EL4 for the CZE stage.

The electric current path in the ITP stage was EL1–INJ–UV1–PG–V1–V2–EL2; the separation columns and electrodes in the INJ–UV1–PG-Vl-V2-EL2 path were filled with leading electrolyte and those of the EL1–INJ path with terminating electrolyte.

A sample solution was injected at the injection valve (INJ) by using a microsyringe. The sample injected was isotachophoretically separated through a separation tube (S1), which was a fused-silica capillary (FSC) (60 cm x 0.66 mm O.D. x 0.53 mm I.D.). Preseparated zones were monitored by using both an ultraviolet detector (UV1) and a potential gradient detector (PG). Detector UV1, operated at 470 nm, was constructed using a UV lamp and a power supply (a Hamamatsu Model L1626 deuterium lamp and a Hamamatsu Model C1518), a grating monochromator from a Hitachi Model 139 UV-Vis spectrophotometer and a photodiode (Hamamatsu Model S1226-8BQ silicone photodiode).

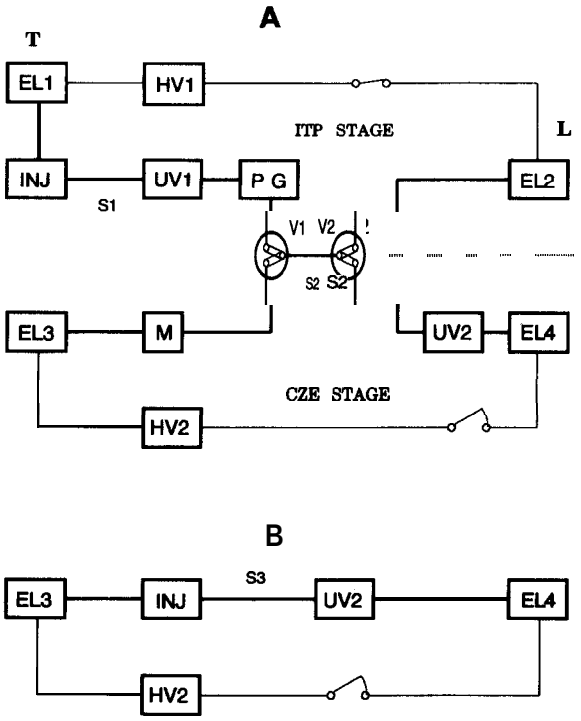

Fig. 1. Schematic diagrams of (A) the ITP-CZE analyser and (B) the CZE analyser. EL1 = Terminating electrolyte compartment; EL2 = leading electrolyte compartment; EL3 and EL4 = background electrolyte compartments for CZE; S1 = separation column for ITP stage; S2 = separation column interfacing ITP and CZE stages; S3 = separation column for CZE stage; INJ = sample injection valve; V1 and V2 = valves for changing current path; UV1 and UV2 = ultraviolet detectors; PG = potential gradient detector; M = membrane to prevent electrolyte flow with a bypass valve; HV1 and HV2 = high-voltage power supplies.

UV1 and V1 were connected by a PTFE tube (40 cm x 1 mm O.D. x 0.5 mm I.D.).

A constant current of 100 μA was supplied to EL1 and EL2 and it took cu. 1 h before the sample migrated into the separation column S2, which is the interface of the ITP and CZE stages. The separation column S2 was a PTFE tube (7 cm x 1 mm O.D. x 0.5 mm I.D.). Two rotary valves (V1 and V2) (Model MPV-FR6, GL Sciences, Tokyo, Japan) were used to change the migration path of the sample from the ITP to the CZE stage.

After the isotachophoretic zones had migrated into the separation column S2, the migration current was cut off and valves V1 and V2 were

turned to change the electric current path from EL1–INJ–UV1–PG–V1–V2–EL2 to EL3–M–V1–V2–UV2–EL4. The amount of sample supplied in this stage can be limited by adjusting the length of S2. The timing of the change of the electrophoretic mode was determined from the migration of an appropriate dye.

The current path for the CZE stage was EL3–M-Vl-V2-UV2-EL4. The stacked sample zones migrated toward the other ultraviolet detector (UV2) forming separated zones. Detector UV2 was a GL Sciences Model 502UA UV–Vis detector operated at 230 nm.

Electrode compartments EL3 and EL4 were filled with background electrolyte. M is a semipermeable membrane device to prevent electrolyte flow which might occur during valve operation (V1 and V2). Device M was equipped with a bypass valve for filling background electrolyte. The separation column (S3) consisted of two FSC tubes (5 cm × 0.66 mm O.D. x0.53 mm I.D. and 22 cm x 0.33 mm O.D. x 0.25 mm I.D.) connected in series. Two FSC tubes were connected using a reducing union connector.

In order to compare the separation behaviour, the same apparatus was also used as a simple CZE analyser as shown in Fig. 1B. The sample was also injected by using a microsyringe from the injection port (INJ). The separation column between INJ and UV2 was essentially the same as S3 which was used in the CZE stage. The total volume of the tubes connecting EL3 and EL4 was 300 μl and the volume of the separation column between INJ and UV was 27 μl, including the volume of the injection valve (5 μl).

While the sample solution was injected, the drain valve at EL3 (not shown in Fig. 1B) was open. Therefore, the effective length of the separation column S3 was kept constant in all migration experiments. Detector UV2 was operated at 230 nm. When a constant voltage of 14 kV was supplied to EL3 and EL4, the migration current was 25 μA.

The injection valve used was that of a Shimadzu IP-1B isotachophoretic analyser. The potential gradient detector (PGD) and constant-current power supply used for the ITP stage (HV1) were those for a Shimadzu IP-2A instrument. The constant-voltage power supply used for the CZE stage (HV2) was a Matsusada Precision Devices (Kusatsu, Japan) Model HER-30P0.5-LW.

Data from the UV detectors were acquired by using an NEC (Tokyo, Japan) PC9801VX microcomputer (80286-80287, clock 10 MHz) and were processed for the evaluation of migration times and peak areas. All experiments were carried out at 25°C.

Chemicals and operational electrolyte systems

The chemicals used for the preparation of test mixtures were p-chlorobenzoic acid, *m*-chlorobenzoic acid, 2,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, picric acid and 4,5-dihydroxy-3-(*p*-sulphophenylazo)-2,7-naphthalenedisulphonic acid (SPADNS). The sodium salt of SPADNS was purchased from Dojin (Kumamoto, Japan). The other chemicals were extra-pure grade reagents obtained from Tokyo Kasei (Tokyo, Japan). They were dissolved in high-purity deionized water obtained by using an ion exchanger (Model PURIC-R; Japan Organo, Tokyo, Japan). The specific resistance of the water used was $18.3 \cdot 10^6$ R/cm.

Table I shows the operational electrolyte systems used. Hydroxypropylcellulose (HPC, 0.1%) was added to the leading and terminating electrolytes to suppress electroendosmosis. The viscosity of a 2% aqueous solution of HPC was 1000-4000 cP at 20°C. pH measurements were

TABLE I

ELECTROLYTE SYSTEM USED IN ITP-CZE SEPARATION

The background electrolyte for CZE was the same as the terminating electrolyte for the ITP stage. HPC = Hydroxypropylcellulose.

Component	Leading electrolyte	Terminating electrolyte
Electrolyte solution	10 m*M* HCl	10 m*M* succinic acid
pH buffer	β-Alanine	β-Alanine
pH	3.5	3.9
Additive	0.1% HPC	0.1% HPC

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

RESULTS AND DISCUSSION

Migration time of a dilute sample

Fig. 2 shows the pH dependence of the effective mobility simulated for the components of the test mixtures and also that of succinic acid, which was the anionic component of the background electrolyte used. The ionic strength assumed for the solution was 0.01. For the simulation of the effective mobility, the absolute mobilities and thermodynamic constants [6] were corrected to the values at the above ionic strength by using Onsager's equation and the Debye–Hückel equation [7], respectively.

Fig. 2 indicates that the separation was straightforward in the pH range 3.5-4. In these experiments, the pH of the leading electrolyte for ITP was 3.5 and that of the background electrolyte for CZE was 3.9.

When electroendosmosis is negligibly small, the migration time (t) is simply expressed as

$$t = l/v = l/(\bar{m}E) \quad (1)$$

where l is the length of the separation column, v the migration velocity of a separand, $\bar{m}$ the effective mobility, and E the potential gradient.

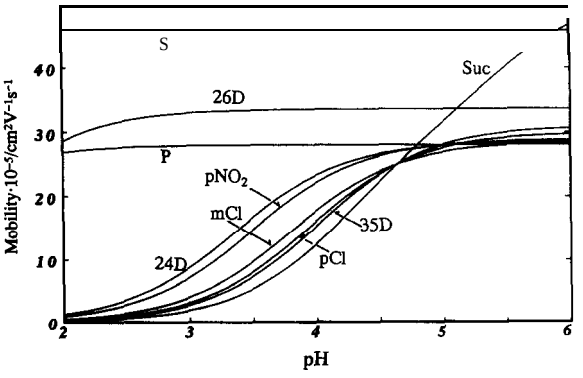

Fig. 2. Dependence of effective mobility on the pH of the background electrolyte solution. The samples are *p*-chlorobenzoic acid (pCl), m-chlorobenzoic acid (mCl), 2,4-dihydroxybenzoic acid (24D), 2,6-dihydroxybenzoic acid (26D), 3,5-dihydroxybenzoic acid (35D), p-nitrobenzoic acid (pNO_2), picric acid (P), SPADNS (S) and succinic acid (Suc). Ionic strength = 0.01.

From eqn. 1, a high reproducibility of the migration time in CZE is obtained when both the effective mobility of the separands and the potential gradient of the separation field are kept constant. Even if the background electrolyte was carefully chosen, these conditions might be perturbed by overloading of the sample solution: the overloaded dilute sample solution may change the ionic strength and/or the pH of the background electrolyte and affect the effective mobility of the separands. Moreover, the dilute solution may reduce the migration current. This will lower the potential gradient of the separation column and cause a delay of the migration time.

This aspect was studied for a six-component mixture of p-chlorobenzoic acid (pCl), *m*-chlorobenzoic acid (mCl), 2,4-dihydroxybenzoic acid (24D), 3,5-dihydroxybenzoic acid (35D), picric acid (P) and SPADNS (S). The concentration and volume of the test mixtures were varied in the ranges 166-1.66 μM and 0.5-50 μl respectively; the total molar amount of the separands was kept constant at 500 pmol. The sample volume injected was 1.9-185% of the volume of the separation column (27 μl) and 0.17–17% of the total volume of the tubes connecting the electrodes (300 μl). The migration voltage applied was 14 kV and the current was 25 μA when the sample volume was small.

Fig. 3 shows the observed migration time vs. the volume injected. An increase in the sample concentration or the conductivity of the sample solution caused a delay of the migration time: with a change in sample volume from 0.5 to 50 μl, the migration time was prolonged non-linearly. For example, the migration time of SPADNS changed from 865 to 3657 s and that of pCl changed from 3326 to 10722 s. Although the migration current was 25 μA when the sample volume was 0.5 μl, it decreased to 5 μA at the initial stage of migration when the sample volume was as large as 50 μl. The reduced migration current recovered to the normal value of 25 μA very slowly.

This delay may lead to misassignment of the separands if the migration time is the only criterion considered in the qualitative analysis. The only way to avoid such a delay caused by

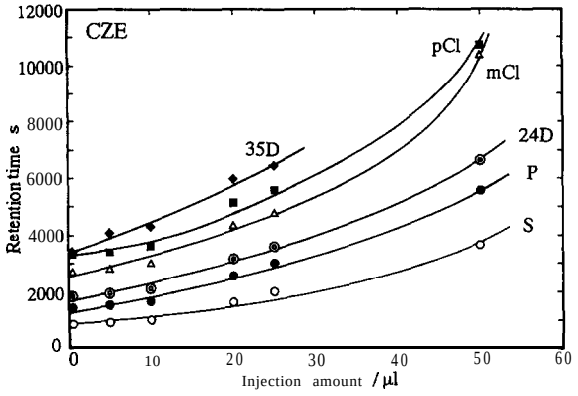

Fig. 3. Migration time vs. sample volume in CZE. The samples are p-chlorobenzoic acid (pCl), *m*-chlorobenzoic acid (mCl), 2,4-dihydroxybenzoic acid (24D), 3,5-dihydroxybenzoic acid (35D), picric acid (P), and SPADNS (S). The total amount of sample injected was kept at 500 pmol, the applied high voltage was 14 kV, the volume of the separation tube was 27 μl and the total volume of the tubes connecting the electrodes was 300 μl.

sample overloading is to introduce a concentrated 'sample. Taking into account the convenience of on-line preconcentration, isotachophoretic pretreatment is the best approach to combine with CZE. Fig. 4 shows the migration time of the same sample in the CZE stage after the ITP pretreatment. The migration time was almost constant for all the samples of different concentrations, confirming the utility of ITP preconcentration.

The conductivity of the sample can be varied

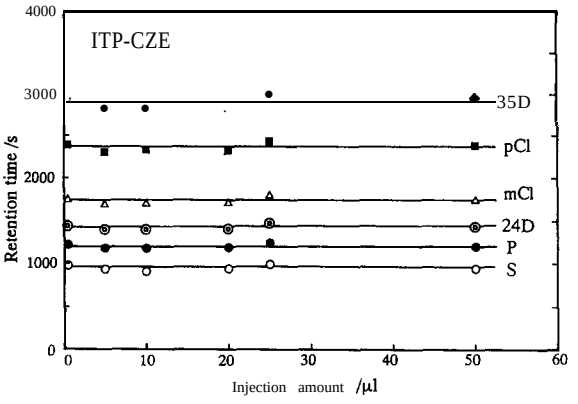

Fig. 4. Migration time vs. sample volume in ITP-CZE. Samples as in Fig. 3. The migration current in ITP preconcentration was 100 μA.

not only by variations in the concentration of the sample but also by the pH of the sample solution. It should be noted that the migration time in a CZE analysis is closely related to the properties of the sample solution even if the analyte components are the same. In commercial CZE analysers using hydrostatic techniques for sampling, a similar situation will occur although not so drastic as in the present experiments. It should be noted that the ratio of the sample volume to the separation column volume will easily reach the unsafe region when the separation column is short.

Analysis of minor components in a matrix component

In addition to the overloading of a dilute sample, another problem in a CZE analysis is the possible error in the determination of minor components in a matrix. If the sample is dilute, the error becomes worse. Combined ITP-CZE may reduce the possible error because of preseparation and preconcentration by ITP.

Fig. 5 shows the isotachopherogram of the test mixture [SPADNS-picric acid–2,4-dihydroxybenzoic acid-m-chlorobenzoic acid–*p*-chlorobenzoic acid (100:1:1:1:1)]. The concentration of SPADNS was 7.14 m*M* and that of the others was 0.0714 m*M*. A 5-μl sample was injected, which contains 52 nmol of the components, the amount of the matrix SPADNS being 50 nmol. In this instance, ITP trace was not useful for the determination of the minor components, because they were highly concentrated and the zones

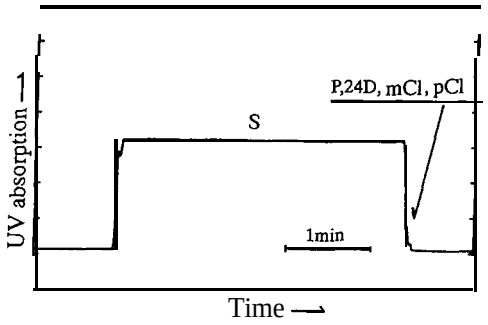

Fig. 5. Isotachopherogram of a test mixture [SPADNS-picric acid-2,4-dihydroxybenzoic acid-m-chlorobenzoic acid–*p*-chlorobenzoic acid (100:1:1:1:1)]. Total amount = 52 nmol; λ = 470 nm.

become very short. For example, the concentration simulated for picric acid at the isotachophoretic steady state was 6.3 m*M* and it was concentrated 88 times more than that in the test mixture.

The isotachophoretically separated zones were subsequently analysed by CZE. Fig. 6 shows the result obtained in the CZE stage, where most of the matrix component SPADNS was discarded when the migration mode was changed from ITP to CZE. A good separation was observed.

The on-line combination of ITP and CZE may therefore be the most convenient and practical solution for the determination of trace components in a sample. ITP pretreatment permits a 100-10 OOO-fold preconcentration of dilute components. There is no doubt that ITP preseparation makes an ideal sample solution for a CZE analysis in principle, although the purity of the reagents used for the electrolyte systems becomes problematic, as pointed out by Foret et *al.* [4]. In addition, in analyses for minor components, the complete separation of such components in the ITP stage is essential to obtain accurate analytical results. It should be noted that the isotachophoretic mixed zones between the minor components and the matrix are apt to be overlooked, because the qualitative information for the zones resembled closely that of the pure matrix zone [8].

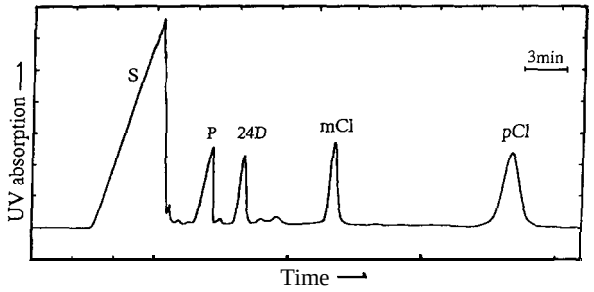

Fig. 6. Electropherogram of a SPADNS (S)–picric acid (P)–2,4-dihydroxybenzoic acid (24D)–*m*-chlorobenzoic acid (mCl)–*p*-chlorobenzoic acid (pCl) (100:1:1:1:1) test mixture obtained in the CZE mode after ITP pretreatment. λ = 230 nm; migration voltage = 14 kV; current = 25 μA; recording started after 10 min.

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