# Electrokinetic Chromatography for Drug Analysis. Separation and Determination of Cefpiramide in Human Plasma

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**Electrokinetic chromatography using a fused silica capillary and sodium dodecyl sulfate (SDS) solution has been applied to the separation and determination of cefpiramide (CPM) in human plasma with the use of antipyrine (AP) as an internal standard. A plasma sample was introduced into the capillary by siphoning. The calibration plot for CPM in plasma sample showed good linearity in the concentration range over 10 to 300 μg/ml. This method has advantages over usual high performance liquid chromatography (HPLC) in that it needs only a very small volume (< 10 nl) of plasma without pretreatment, and an extremely high separation efficiency (10 times or much higher plate number than usual HPLC) is obtained. The addition of SDS to the supporting electrolyte solution enabled (1) rapid release of protein-bound drug which allowed the total concentration to be determined, (2) reproducible results to be obtained by suppressing adsorption of protein onto the fused silica capillary and (3) rapid separation of drug from proteins by selective retardation of protein peaks.**

**Keywords** electrokinetic chromatography; capillary electrophoresis; SDS micelle; determination; cefpiramide; human plasma; capillary chromatography; β-lactam antibiotics

Electrokinetic chromatography, first introduced by Terabe *et al.*,[1] is a kind of free zone electrophoresis combined with chromatographic partition. It uses an open tubular capillary and an ionic surfactant solution with a concentration above the critical micellar concentration (CMC). The technology of this method is essentially based on the capillary zone electrophoresis (CZE) developed by Mikkers *et al.*,[2] Jorgenson and Lukacs[3] and Tsuda *et al.*[4] In CZE the charged solutes are separated based on their differential electrophoretic migration. When a high voltage is applied across an open tubular capillary filled with supporting electrolyte solution, a flow of the solution is induced in the capillary, directed from cathode to anode, when a fused silica capillary is used. This flow is known as an electroosmotic flow. When sodium dodecyl sulfate (SDS) at a concentration above the CMC is present in the supporting electrolyte solution for CZE, the micelles of SDS can serve as a pseudo stationary phase and the differential partitioning of solutes between the hydrophobic interior of the micelles and the aqueous phase participates in the separation of the solutes in cooperation with electroosmotic flow and electrophoretic migration of the micelles. Since the former flow is usually faster than the latter in a fused silica capillary, this method allows samples with no electric charge to be finally eluted out of the anodic end of the capillary with retention times between those of entirely solubilized substance (longest retention time) and insolubilized substance (shortest retention time). Naturally, since the charged solutes experience an electrophoretic effect in addition to solubilization in micelles, their retention times are not necessarily restricted within the above mentioned region. Since the electroosmosis produces an uniform profile of flow velocity (*i.e.* plug flow), sharp separation with an extremely high theoretical plate number up to some hundred thousands of plates can be attained in a relatively short period of time. Experimental studies have been made of the factors controlling electroosmotic flow,[5,6] the use of various surfactants,[7-10] extension of elution range[11] and gradient elution.[12] The applicability of electrokinetic chromatography has been demonstrated to the separation of substituted benzenes,[13] phenylthiohydantion amino acids,[14] chlorinated phenols,[15] metabolites of vitamin $B_6$,[16] purines,[17] amines,[18] phenolic compounds,[19] oligonucleotides,[20] antipyretic analgesic[21] and nucleic acid constituents,[22] while CZE was applied to the analysis of human urine[23] and proteins,[24] and the determination of drugs[25,26] in plasma after pretreatment. However neither CZE nor electrokinetic chromatography has been used for clinical purposes. Further investigations are necessary to make this method fit for clinical uses such as monitoring of drug levels in biological samples. This paper aims to investigate and solve the problems which occur when electrokinetic chromatography is applied to pharmaceutical analysis, with reference to separation and determination of cefpiramide in human plasma.

## Experimental

**Reagents** Sodium dodecyl sulfate (SDS), disodium hydrogen phosphate and sodium dihydrogen phosphate of analytical reagent grade were obtained from Wako Pure Chemicals (Osaka, Japan) and used as received. Cefpiramide (CPM) was obtained from Sumitomo Pharm. Co. (Osaka). Water was purified with a Milli-Q system (Nihon Millipore Kogyo K. K., Yonezawa, Japan). CPM and antipyrine (AP) for clinical use were used without further purification. Human plasma was prepared from fresh human blood in a usual manner.

**Apparatus** A schematic illustration of the apparatus which we have designed, constructed and used in this study is shown in Fig. 1. High voltage was applied to the electrodes using a DC power supplier delivering 3—24 kV (model HSR24-P, Matsusada Precision Device Co., Shiga, Japan). Separation was performed in a fused silica capillary tube (75 cm × 50 μm i.d., Scientific Glass Engineering Inc., Australia). The buffer solution at each end of the capillary was contained in a small glass vessel capped with a silicon rubber stopper having two small bore holes, one for the insertion of the capillary and the other for the platinum wire electrode. About a 2 mm length of the capillary 20 cm distant from the anodic end was made transparent by carefully burning away the polymer coating. This part was used as an on-column cell for ultraviolet (UV)-detection. The detection was achieved by measuring UV absorption at 280 nm (UVIDEC 100-III, Jasco, Tokyko, Japan). Operaion was carried out at 37 °C in a temperature controlled chamber.

**Samples** Sample solutions were prepared by dissolving known amounts of CPM and AP in human plasma (plasma sample) or in phosphate buffer solution (buffer sample). The concentrations are given in the text. The phosphate buffer solutions were the same as those used as the supporting electrolyte solution. These sample solutions were passed through 0.45 μm pore size membrane filter prior to use.

**Procedure** A fused silica capillary tube was filled with phosphate buffer

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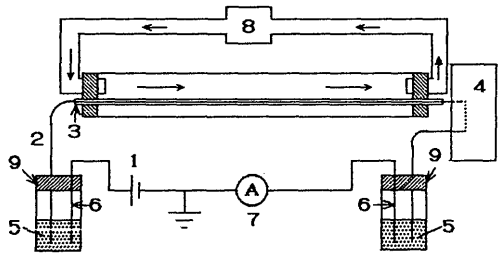


Fig. 1. Schematic Diagram of Electrokinetic Chromatograph

1, regulated high-voltage DC power supplier; 2, fused silica capillary; 3, polyethylene tube; 4, UV detector; 5, supporting electrolyte solution; 6, platinum electrode; 7, micro ampere meter; 8, thermostat; 9, silicon rubber cap.

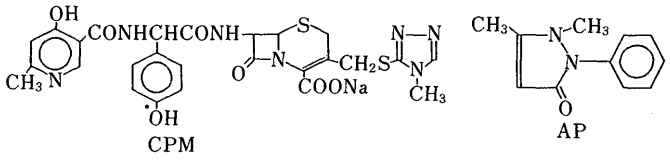


Fig. 2. Chemical Structures of Cefpiramide (CPM) and Antipyrine (AP)

solution (pH 5.0—9.0, ionic strength 0.01—0.1) by using a 50-$\mu$l microsyringe, and both ends of the capillary were dipped into the phosphate buffer solution whose surfaces were kept at the same level. The introduction of sample solution into the capillary was achieved by siphoning. The siphoning was done manually by transferring one end of the capillary into the sample solution whose level was raised 5 cm higher than the other. After siphoning for an appropriate period of time, the end of the capillary was quickly returned to the buffer solution and a high voltage (+15 kV at the sample introduction side and 0 V at the end of detection side) was applied.

## Results and Discussion

Figure 3 illustrates the observed velocity of standard CPM ($V_{CPM}$), AP ($V_{AP}$) and acetone ($V_{EO}$) as a function of SDS concentration in the phosphate buffer solution (pH 8.0, ionic strength 0.05), where acetone was used to indicate the velocity of electroosmotic flow, because it is not solubilized in the SDS micelle. $V_{CPM}$ and $V_{EO}$ were slightly decreased by addition of 10 mM SDS (CMC of SDS in 0.02 M phosphate buffer solution was expected to be about 5 mM[27]) and were almost unchanged with further increase up to 30 mM. In contrast, AP moved as fast as acetone when SDS was not present in the phosphate buffer (*i.e.* in the usual CZE mode) and $V_{AP}$ was rapidly decreased to a level lower than $V_{CPM}$ by addition of 30 mM SDS. These results indicate that CPM was hardly solubilized in the SDS micelle because CPM had a negative charge under these conditions (pH 8.0) due to dissociation of the carboxylic acid group ($pK_a = 2.3$) and suffered from electrostatic repulsion. Thus, it tended to migrate by electrophoresis toward the cathodic end of the capillary. On the other hand, since AP is expected to be neutral under these conditions, it was much more solubilized than CPM in the SDS micelles, which also tended to migrate toward the cathodic terminal. Thus the difference between $V_{CPM}$ and $V_{AP}$ depended on the balance between the following two effects; the degree of solubilization of AP in the micelles and the relative velocity of electrophoretic migration of SDS micelles and CPM anion. Naturally, a lower concentration of SDS produced less solubilization, which results in a larger $V_{AP}$ value, and electrophoretic migration of the micelle was expected to be faster than that of CPM anion. Further, it is

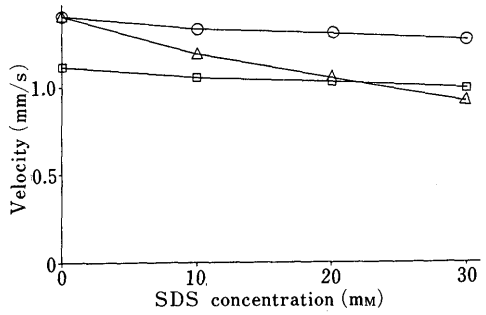


Fig. 3. Effect of SDS Concentration on Migration Velocity

(□), CPM; (△), AP; (○), acetone; ionic strength, 0.05 (phosphate buffer solution, pH 8); total applied voltage, 15 kV; total tube length, 750 mm (effective length 550 mm); detection wavelength, 280 nm; temperature, 37 °C; sample introduction time, 15 s.

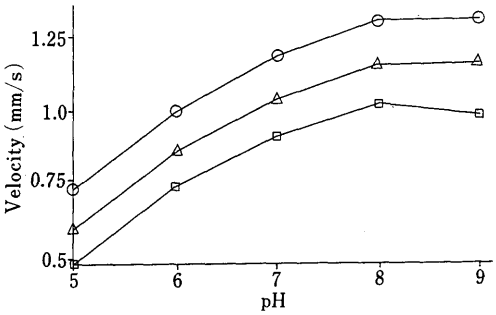


Fig. 4. Relationship between pH of Supporting Electrolyte Solution and Migration Velocity

(□), CPM; (△), AP; (○), acetone; phosphate buffer solution containing 10 mM SDS. Other conditions are the same as those in Fig. 3.

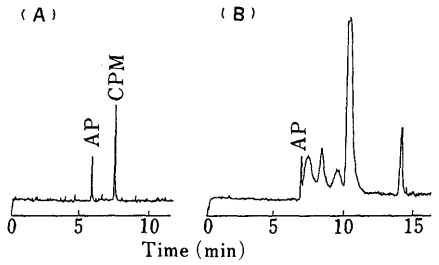


Fig. 5. Electrokinetic Separation of Cefpiramide and Antipyrine

(A) In buffer sample and (B) in plasma sample. Phosphate buffer solution (pH 8) without SDS. Other conditions are the same as those in Fig. 3.

speculated from the result in Fig. 3 that the former effect was stronger than the latter in the region of SDS concentration below 22 mM, while the reverse is true above 22 mM.

Figure 4 shows the pH dependence of $V_{CPM}$, $V_{AP}$ and $V_{EO}$ in the phosphate buffer solution (I = 0.05) containing 10 mM SDS. The migration velocities were increased in parallel with each other with increasing pH value. This may be the result of increasing dissociation of residual silanol groups on the surface of the fused silica capillary. A slight decrease in $V_{CPM}$ at pH 9 may be ascribed to the partial dissociation of phenolic hydroxyl groups on the lateral chain.

Figure 5 shows the separation profiles of buffer sample (Fig. 5(A)) and plasma sample (Fig. 5 (B)), both containing 100 $\mu$g/ml CPM and 100 $\mu$g/ml AP, where SDS was not present in the supporting electrolyte solution (phosphate

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buffer, pH 8.0, I=0.05). The actual volumes of these samples introduced into the capillary were not exactly the same because of the difference in their viscosity, although the siphoning was done for the same period of time (15 s). AP and CPM in the buffer sample were sharply separated within 8 min, but those in plasma sample overlapped completely with protein peaks and the peak of CPM was not observed. These results could be ascribed not only to the similarity in the apparent migration velocity (most plasma proteins and CPM have negative charge at pH 8), but also to the slow release of drug from the plasma protein (especially for CPM) and also to the decreased velocity of electroosmotic flow due to adsorption of protein on the surface of the fused silica capillary. This was confirmed by the facts that the elution of AP and CPM was retarded when buffer sample was applied after repeated introductions of plasma sample, and that such a phenomenon was not observed after the capillary was washed with 1 N NaOH solution. Thus complete separation of the AP and CPM peaks from the protein peaks was not attained by varying the operating conditions unless SDS was added. Figure 6 shows the chromatograms of the same samples as those in Fig. 5, where 10 mM SDS was added to the buffer solution used in the operation presented in Fig. 5, and acetone was included as the marker of electroosmotic flow velocity. As expected from the results in Fig. 4, the addition of 10 mM SDS resulted in less mutual separation and in slow elution of CPM and AP. However the separation of CPM and AP from plasma protein (Fig. 5 (B)) was markedly improved as a result of selectively retarded elution of the protein peaks. This is possibly because the plasma proteins solubilized by SDS had strong negative charge and therefore tended to migrate by electrophoresis toward the cathodic end of the capillary faster than in the case of CZE shown in Fig. 4. To avoid the consequent lengthened analysis time, the elution was discontinued by stopping the supply of high voltage just after the emergence of the CPM peak, and the plasma proteins were washed out of the capillary by injecting 1 N NaOH or phosphate buffer solution using a 50-$\mu$l microsyringe. This procedure shortened the analysis time to less than 10 min and resulted in better reproducibility in separation and quantitation, as mentioned later.

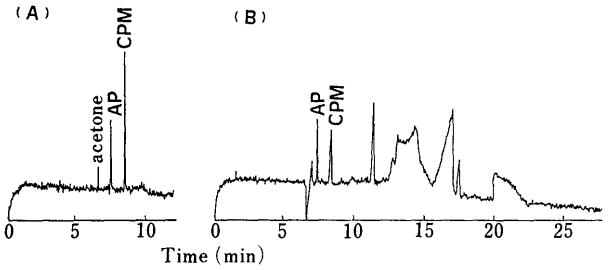


Fig. 6. Electrokinetic Separation of Cefpiramide and Antipyrine

(A) In buffer sample and (B) in plasma sample. Phosphate buffer solution (pH 8) containing 10 mM SDS. Other conditions are the same as those in Fig. 3.

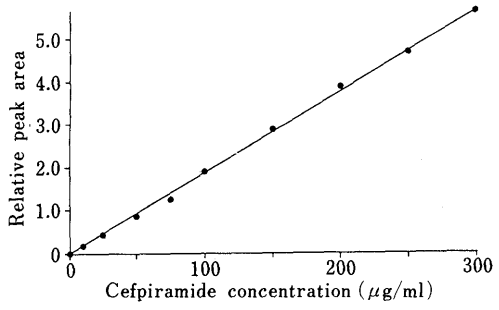


Fig. 7. Calibration Plot for CPM

Relative peak area, CPM/AP, was plotted *vs.* concentration of CPM spiked in human plasma. Other conditions are the same as those in Fig. 6.

The calibration line for CPM in plasma sample is shown in Fig. 7, where AP was used as an internal standard. Good linearity (correlation coefficient 99.8%) was obtained over the concentration range of CPM from 10 to 300 $\mu$g/ml, which covers the plasma levels usually encountered in clinical chemotherapy. This calibration plot was almost identical with that obtained from the buffer sample containing CPM and AP in the same concentration range as above. This means that CPM and AP bound to plasma protein were completely released during separation in the capillary and, therefore, total amounts of bound and unbound CPM could be determined by the present method. The limit of detection for CPM was 5 $\mu$g/ml at $S/N=3$.

The reproducibility of electroosmotic flow velocity, migration velocity and relative peak area (R.P.A.) of CPM and AP are shown in Table I, where "cleaning solution" means

TABLE I. Reproducibility Data with (A) and without (B) SDS

(A)

| Sample | Cleaning solution | $V_{EO}$ mean (mm/s) | C.V. (%) | $V_{AP}$ mean (mm/s) | C.V. (%) | $V_{CPM}$ mean (mm/s) | C.V. (%) | R.P.A. mean | C.V. (%) |
|---|---|---|---|---|---|---|---|---|---|
| Buffer sample | 1 N NaOH | 1.32 | 0.8 | 1.16 | 0.9 | 1.02 | 0.9 | 1.94 | 3.3 |
| | Nothing[a] | 1.31 | 0.3 | 1.16 | 0.3 | 1.03 | 0.3 | 1.93 | 4.3 |
| Plasma sample | 1 N NaOH | 1.33 | 0.8 | 1.18 | 1.2 | 1.03 | 1.1 | 1.95 | 3.8 |
| | Buffer[b] | 1.33 | 0.3 | 1.18 | 0.5 | 1.04 | 0.5 | 1.89 | 3.3 |

(B)

| Sample | Cleaning solution | $V_{EO}$ mean (mm/s) | C.V. (%) |
|---|---|---|---|
| Buffer sample | Nothing[a] | 1.39 | 0.6 |
| Plasma sample | 1 N NaOH | 1.25 | 1.7 |
| | Buffer | 1.02 | 18.0 |

Reproducibility in velocity of electroosmotic flow ($V_{EO}$), migration velocity of cefpiramide ($V_{CPM}$) and antipyrine ($V_{AP}$), and their relative peak area (R.P.A.) obtained by seven repeated injections: phosphate buffer solution (pH 8) containing 10 mM SDS (A) and without SDS (B). Other conditions are the same as those in Fig. 3. *a*) No washing. *b*) Containing 10 mM SDS. C.V., coefficient of variation.

TABLE II. Theoretical Plate Numbers

| Sample | $N_{AP}$ SDS 10 mM mean (/m) | C.V. (%) | $N_{CPM}$ SDS 10 mM mean (/m) | C.V. (%) | SDS 50 mM mean (/m) | C.V. (%) |
|---|---|---|---|---|---|---|
| Buffer sample | $2.1\times10^5$ | 1.8 | $3.1\times10^5$ | 3.0 | $3.5\times10^5$ | 3.5 |
| Plasma sample | $2.0\times10^5$ | 3.5 | $9.6\times10^4$ | 4.2 | $2.6\times10^5$ | 6.0 |

Comparison of the theoretical plate numbers: phosphate buffer solution (pH 8) containing 10 or 50 mM SDS. Other conditions are the same as those in Fig. 3.

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TABLE III. Effect of Injection Volume of Buffer Sample

| Injection time (s) | $V_{AP}$ mean (mm/s) | C.V. (%) | $V_{CPM}$ mean (mm/s) | C.V. (%) | R.P.A. mean | C.V. (%) | $N_{AP}$ mean (/m) | C.V. (%) | $N_{CPM}$ mean (/m) | C.V. (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 15 | 1.16 | 0.3 | 1.03 | 0.3 | 1.93 | 4.3 | $2.1 \times 10^5$ | 1.8 | $3.1 \times 10^5$ | 3.0 |
| 30 | 1.18 | 0.5 | 1.03 | 0.4 | 1.88 | 1.4 | $1.7 \times 10^5$ | 5.0 | $2.5 \times 10^5$ | 2.9 |

Other conditions are the same as those in Fig. 6.

the solution used for removing plasma proteins from the capillary just after elution of the CPM peak. As found in Table I (A), where 10 mM SDS was present in the phosphate buffer (pH 8.0), reproducible results for $V_{EO}$, $V_{AP}$, $V_{CPM}$ and R.P.A. values were obtained following the repeated introductions ($n=7$) of plasma samples, when either 1 N NaOH or phosphate buffer solution containing 10 mM SDS was used as a cleaning solution. Naturally, the buffer sample gave reproducible results even when the capillary was not cleaned. In contrast, as found in Table I (B), where SDS was not contained in the supporting electrolyte solution, $V_{EO}$ values were appreciably decreased by the introduction of a plasma sample even when the capillary was cleaned with 1 N NaOH or phosphate buffer solution (pH 8.0). These results indicate that SDS, by solubilizing plasma proteins, prevented their adsorption on the capillary.

Table II shows the theoretical plate numbers observed with CPM and AP ($N_{CPM}$, $N_{AP}$) following introductions of buffer sample and plasma sample, where the phosphate buffer solution (pH 8.0) containing 10 or 50 mM SDS was used as both the cleaning solution and the supporting electrolyte solution. The $N_{CPM}$ was reduced to about one third (from $3.1 \times 10^5$ to $9.6 \times 10^4$) by the introduction of a plasma sample when 10 mM SDS was contained in the phosphate buffer solution, although the migration velocities ($V_{CPM}$, $V_{AP}$) and R.P.A. were slightly affected as shown in Table I. However, the reduction of $N_{CPM}$ was improvd by increasing the SDS concentration to 50 mM. In contrast, $N_{AP}$ did not suffer from such a difference in the nature of samples. These results suggest that higher protein binding of CPM[28] than AP[29] caused slower release of CPM from protein, which resulted in the broadening of the CPM peak. Thus, it is considered that increasing the concentration of SDS accelerated the release of drug from plasma protein during migration in the supporting electrolyte solution.

Table III shows the effect of sample volume on the reproducibility of migration velocity, relative peak area and theoretical plate number, where the sample volume was changed by siphoning the buffer sample for 15 and 30 s. The actual volume of sample introduced, though not measured, was expected to be of the order of $10^{-9}$ l, while the total volume of the electrolyte solution effective for the migration was about $10^{-6}$ l. The doubling of the siphoning time did not affect $V_{AP}$, $V_{CPM}$ and relative peak area, but appreciably decreased $N_{AP}$ and $N_{CPM}$ values as expected from the previous reports.[30] Thus, it was preferable to control the injected amount of sample by changing the concentration rather than the volume of sample solution.

In conclusion, electrokinetic chromatography has advantages over conventional high performance liquid chromatography (HPLC) in that it allows direct introduction of a very small volume (<10 nl) of plasma sample without pretreatments such as deproteinization and extraction, and that extremely high separation efficiency (10 times or much higher plate number than usual HPLC) can be obtained in a relatively short period of time. The addition of SDS to the supporting electrolyte solution enabled (1) rapid release of protein-bound drug which allowed the total concentration to be determined, (2) reproducible results to be obtained by suppressing adsorption of protein onto the fused silica capillary, and (3) rapid and sharp separation of drug from proteins by selective retardation of the elution of protein peaks.

Recently, microinjectors using electroosmotic and electrophoretic migration,[31,32] automatic siphoning[33,34] and rotary valve[35] methods have been devised for the introduction of a small volume of sample solution for CZE. However, these devices still seem to be inadequate for the quantitative introduction of <100 nl volume of viscous samples containing a wide variety of components, such as the plasma used in the present study. Instead, the use of an internal standard gave reproducible results for the quantitation of CPM concentration down to 5 μg/ml with a coefficient of variation of <4%. For the general use of electrokinetic chromatography for clinical purposes such as monitoring of drug levels in biological fluids, it will be necessary to use a microautosampler which allows quantitative and reproducible introduction of <10 nl of viscous samples, a column switching device for the small-bore capillary and highly sensitive detectors such as fluorescence,[36] electrochemical[37,38] and conductivity[39] detectors.

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