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※　毛細管電泳對環境污染物及藥物分離之研究 III　※

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☑出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

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第一部份　毛細管電泳對環境污染物之分離研究

1 A、計劃緣由與目的

Chloropyridines, chlorophenols 及 triazines 皆為常見之環境污染物。對環境造成之污染實在不可忽視，必須加以監測。因此對此類污染物之之分離及分析方法的探討是個相當重要的課題。

此研究計劃擬以毛細管區帶電泳法(CZE)及微胞電動法(MEKC)分別對此類污染物分離做最佳化條件及電泳遷移行為之探討，並且對分析物與界面活性劑微胞之結合常數，遷移順序，以及分離機制做更進一步的測定與瞭解。分離參數對偵測極限至及解析度之影響以及應用新近發展的 sample stacking 技巧亦將加以探討，期能在 UV 偵測法上降低偵測極限(至 10^{-8}~10^{-9}M)。由於分析物與界面活性劑之間有相當程度的交互作用力，因此可利用電泳法加以測定並探討界面活性劑以及添加β-環糊精時在電泳緩衝液中臨界微胞濃度(CMC)之變化情形，以便能進一步瞭解在微胞化過程中及 CD-CZE 及 CD-MEKC 之分離機制差異之奧秘。

1 B、結果與討論

本年度之研究工作著重於以 sweeping 法探討 s-triazines 之線上濃縮及濃縮機制。此部份之研究工作進行順利，部分研究結果已加整理並向 *J. Chromatogr. A* 提出發表。詳見附件（1）。

第二部份　毛細管電泳對抗生素藥物分離之研究

2 A、計劃緣由與目的

抗生素如 cephalosporins 及 tetracyclines 為臨床上常用的藥物，因此，發展快速而有效的檢驗方法在藥物分析化學上是個重要的研究課題。此研究計劃在於發展以 CZE 及 MEKC 方法分離 cephalosporins 及 tetracyclines 並探討其電泳遷移行為；瞭解分離機制及遷移順序之源由。

2 B、結果與討論

本年度之研究工作著重於以 acetate buffer 在 pH 5.0-9.0 下添加 SDS 或 SDS+Brij 35 為界面活性劑，利用 MEKC 方法研究 tetracyclines 的分離及電泳遷移行為同時，也以 TTAB, CTAB 等陽離子界面活性劑利用 MEKC 方法研究 cephalosporins 的分離及電泳遷移行為。此部份之研究工作進行順利，部分研究結果已整理並向 *J. Chromatogr. A* 提出發表。詳見附件(2)。但 cephalosporins 的研究結果，目前仍在整理中。附件：研究論文二篇

附錄一

Migration Behavior and Separation of Tetracycline Antibiotics by Micellar Electrokinetic Chromatography

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Abstract

The migration behavior and separation of six tetracyclines (TCs) were investigated by micellar electrokinetic chromatography (MEKC) in the pH range 5.0-9.0 using ammonium acetate buffer with the addition of sodium dodecyl sulfate (SDS). Mixed SDS-Brij35, sodium cholate (SC) and tetradecyltrimethyl-ammonium bromide (TTAB) were also used as surfactants. The influences of surfactant concentration and buffer pH on the separation of TCs were examined and the separations of TCs were optimized. Complete separation of six TCs was achieved within 8 min with 15 mM ammonium acetate buffer containing 20 mM SDS, with or without the addition of Brij 35 (0.135 %, w/v), at pH 6.5 using a fused silica capillary (42 cm x 75 μm I. D.) at 15 kV. In general, good linear correlations of the logarithm of migration factor (log k') versus the logarithm of octanol-water partition coefficient (log P_{ow}) in these micellar systems, except for the TTAB-MEKC system, were obtained. The results indicate that the migration of TCs in MEKC is mainly based on hydrophobic interactions. However, hydrogen bonding interactions also play a significant role in influencing the chemical selectivity of TCs. In addition, the micelle-water partition coefficients (P_{mw}) of TCs, which are pH-dependent in the SDS-MEKC micellar system, were reported.

Introduction

Tetracycline antibiotics are extensively used to control bacterical infections in both humans and animals. In addition to therapeutical use, these drugs are widely used as a feed supplement in animal husbandry. The structures of tetracyclines (TCs) studied are schematically shown in Fig. 1. These compounds possess multiple functional groups with acid-base properties: the first acid dissociation constant of tetracylines, with a pKa value of approximately 3.3, is attributed to the hydroxyl group of the tricarbonyl system in ring A; the second acid dissociation constant, with a pKa value of approximately 7.6, is associated with the hydroxyl group of the dicarbonyl system between rings B and C; the third, with a pKa value of approximately 9.7, is assigned to the amino group of the dimethylamino moiety in ring A; the fourth, with a pKa value in the range 10.7-12.0, is associated with the phenolic hydroxyl group in ring D [1,2]. Accordingly, most TCs exhibit amphoteric character with an isoelectric point between 4 and 6. Thus, TCs exist in cationic form at more acidic pH values, in anionic form at more alkaline pH values, and in zwitterionic form at a pH near the isoelectric point, and depending on the pH of the buffer, can be separated as cations at low pH point as zwitterions near neutral pH, or as anions at an alkaline pH.

Tetracycline degrades to anhydrotetracycline (ATC) in acidic media at pH< 2 [1]. TC and ATC epimerize to form 4-epitetracycline (ETC) and 4-epianhydrotetracycline (EATC), respectively, at pH 2-6 [1,3]. Likewise, other tetracyclines, such as oxytetracycline (OTC) and demeclocycline (DMC), also form degradation products [4]. Since commercially available TCs may contain some significant amounts of degradation products and EATC is shown to be renal toxic, the separation

and monitoring of TCs and their impurities have drawn much attention.

High-performance liquid chromatography (HPLC) has been employed for separating and analyzing various TC mixtures over the past decades [5-11], despite the difficulties associated with the peak tailing and low efficiency due to interaction with the residual silanol groups on silica-base packing material. In recent years, capillary electrophoresis has gained increased importance as a powerful analytical tool [12-17]. This is due to its many advantageous features such as extremely high efficiency, high resolution, short analysis time and small consumption of sample and solvent volume in comparison with HPLC.

Tetracyline and its degradation products were efficiently separated by capillary zone electrophoresis (CZE) using either a phosphate buffer (20 mM) at pH 3.9 [18] or a basic carbonate buffer (80 mM) at pH 9.0 [19], with the addition of disodium ethylenediamine-tetraacetate (EDTA, 1-5 mM) as a complexing agent. The analysis of DMC and its major impurities was investigated by adding Triton X-100 (0.35 %, v/v) to a phosphate running buffer (50 mM) containing EDTA (1 mM) at pH 11.50 [4]. The electrophoretic behavior of seven tetracycline antibiotics, including TC, OTC, chlortetracycline (CTC), doxycylines (DOC), DMC, methacycline (MC) and minocycline (MNC) has been characterized by capillary electrophoresis (CE) using a phosphate buffer solution in the pH range 4-11 [20]. The optimum conditions for separating a mixture of TCs determined were buffer pH, 7.5; buffer concentration, 4.3 mM; and ionic strength, 18.2 mM. However, complete separation of these TCs was not achievable under these conditions [20]. Effective separation of various TCs, with the exception of DOC and MC, was readily achieved by CZE using a background electrolyte composed of citric acid (30 mM), β -analine (24.5 mM) and methanol (40 %, v/v) at pH 3.0 [21]. Moreover, the separations of impurities and degradation products of various TCs by

CE and capillary electrochromatography (CEC) were compared [21]. The complete separation of four commercially available TCs (TC, OTC, DOC and CTC) by non-aqueous capillary electrophoresis using methanol-acetonitrile (50:50, v/v) electrophoresis medium with the addition of different electrolyte was also demonstrated [22].

On the other hand, reports on the separation of tetracyclines by micellar electrokinetic capillary chromatography (MEKC) were few. Croubels et al. [23] analyzed TC, OTC, CTC and the degradation products of TC by adding non-ionic surfactants such as Triton X-100 (0.05 %) and Brij 35 (0.017 %) to a phosphate buffer solution at pH 2.2 to improve the separation. However complete separation of these tetracyclines is not achievable with the use of phosphate buffer as a background electrolyte. A method was developed for the determination of four tetracyclines, including TC, OTC, CTC and DOC, in bovine milk, serum and urine using a borate -phosphate buffer solution containing sodium dodecyl sulfate, 10 mM at pH 8.5 [24] with limits of detection down to a few ppb.

In this paper, we report the results of the separation of six tetracyclines by MEKC using an ammonium acetate buffer containing three different structural types of anionic surfactant and a cationic surfactant as micelles. The influences of buffer pH in the pH range 5.0-9.0 (for SDS only) and surfactant concentration, as well as the choice of background electrolyte, on the separation of TCs are examined. The partition coefficients of tetracyclines to SDS micelles are evaluated and the migration order of tetracyclines was discussed based on quantitative structure-retention relationships (QSRR).

Experimental

Chemicals and reagents

Six tetracyclines, including tetracycline, oxytetracycline, chlortetracycline, doxycycline , minocycline and demeclocycline, sodium dodecyl sulfate and sodium cholate and Sudan Ⅲ were obtained from Sigma (St. Louis, MO, USA). Polyoxyethylene (23) dodecyl ether (Brij 35), tetradecyl-trimethylammonium bromide (TTAB), ammonium acetate, and quinine hydrochloride were obtained from Aldrich (Milwaukee, WI, USA), TCI (Tokyo, Japan), Showa (Kyoto, Japan) and Kanto (Tokyo, Japan), respectively. All other chemicals were of analytical-reagent grade. Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA) .

Standard stock solutions of six tetracyclines in aqueous solution at a concentration of 1000 ppm were prepared. When needed, various concentrations of sample solution ranging from 10 -50 ppm were diluted from stock solution. Various proportions of acetic acid (1 M) or trisodium phosphate (1 M) were added to the ammonium acetate solution to reach a desired value in the range 5.0-9.0. All solutions were filtered through a membrane filter (0.22- μ m) before use.

Apparatus

Separations were made with a capillary electrophoresis system described previously [25]. The capillary dimensions were 43 cm $\times$ 75 μ m, I.D. The UV detection position is 7.0 cm from the cathodic end. Sample injection was done in a hydrodynamic mode during 1 second. The CE system was interfaced with a microcomputer and printer with software CE 1000 1.05A. For pH measurements, a pH meter (Suntex Model

SP-701, Taipei, Taiwan) was employed with a precision of ± 0.01 pH unit.

Electrophoretic procedure

When a new capillary was used, the capillary was washed using a standard sequence described previously [25]. To ensure reproducibility, all experiments were performed at 25℃ and measurements were run at least in triplicate. The capillary was prewashed with running buffer for 2 min before each injection and postwashed with deionized water at 25℃ for 5 min, followed with sodium hydroxide solution (0.1 M) at 60℃ for 5 min, and then with deionized water at 25℃ for 5 min to maintain proper reproducibility for run-to-run injections. The detection wavelength was set at 265 nm.

Calculations

Electrophoretic mobility

The electrophoretic mobility of analytes was calculated from the observed migration times with the equation:

$$\mu_{ep} = \mu - \mu_{eo} = \frac{L_d L_t}{V}\left(\frac{1}{t_m} - \frac{1}{t_{eo}}\right) \qquad (1)$$

where μ_{ep} is the electrophoretic mobility of the analyte tested, μ is the apparent mobility, μ_{eo} is the electroosmotic mobility, t_m is the migration time measured directly from the electropherogram, t_{eo} is the migration time for an uncharged solute (DMSO as neutral marker), L_t is the total length of capillary, L_d is the length of capillary between injection and detection, and V is the applied voltage.

Migration factor

The migration factor of analytes (k') was calculated from the observed migration times with the equation:

$$k' = \frac{t_m - t_o}{t_o(1 - \frac{t_m}{t_{mc}})} \qquad (2)$$

where t_o is the migration time in the absence of micelles, and t_{mc} is the migration time of micelles,

or

$$k' = \frac{(\mu_{ep} - \mu_o)}{(\mu_{mc} - \mu_{ep})} \qquad (3)$$

where μ_{ep}, μ_o, and μ_{mc} are the electrophoretic mobility of a solute, the electrophoretic mobility of a solute in the absence of micelles, and the electrophoretic mobility of micelle, respectively, calculated from the corresponding migration times.

In this work, quinine hydrochloride was used as a micelle marker for SDS and SDS-Brij 35, whereas Sudan Ⅲ was used for SC and TTAB.

Partition coefficient The migration factor (K') in MEKC is directly proportional to the micelle concentration through the following equation [26, 27]:

$$k' = P_{mw} \nu \{[S]\text{-cmc}\} \qquad (4)$$

where P_{mw} is the partition coefficient of solutes between the aqueous and micellar phases, ν is the molar volume of the surfactant, and [S] is the total surfactant concentration. With SDS as an anionic surfactant, ν is equal to 0.2483 $Lmol^{-1}$ [28].

Result and Discussion

Choice of background electrolyte

As reported previously [23], complete separation of tetracyclines is difficult to achieve using a phosphate buffer containing different types of surfactants, such as SDS, cetyltrimethylammonium bromide (CTAB) and bile salt. In this work, for separating tetracyclines at a pH near their isoelectric points, ammonium acetate was selected as a running buffer. The optimum concentration of ammonium acetate buffer was determined to be 15 mM.

Influence of SDS concentration

Fig. 2 shows the variation of the electrophoretic mobility of tetracyclines as a function of SDS concentration at pH 6.0. The electrophoretic mobility of TCs (migrating toward anode) increases with SDS concentration. The electroosmotic flow decreases with increasing concentration of SDS, but the mobility of micelle is almost constant at varied concentration of SDS. Consequently, the migration windows become broader with increasing SDS concentration. The resolution of tetracyclines increases with increasing concentration of SDS, and the migration time also increases. So the optimum SDS concentration was determined to be about 15-20 mM.

Influence of buffer pH

Fig. 3 shows the variation of the electrophoretic mobility as a function of buffer pH in the range 5.0-9.0. The electrophoretic mobility of each individual tetracycline (migrating toward anode) decreased, but the electroosmoatic flow increased, as the pH of the buffer increased. The

variation in the electrophoretic mobility as a function of pH for tetracyclines having the hydroxyl group as the R_2 substituent, such as OTC, TC, DMC and CTC is considerably smaller than those of tetracyclines with a hydrogen atom as the R_2 substituent, such as DOC and MNC, thus leading the electrophoretic mobility curves of DOC and CTC to cross over at a pH of about 5.3. Fig. 4 shows the electropherograms of tetracyclines obtained in the SDS micellar system at pH 5.0 and 6.5. The reversal of the migration order of DOC and CTC was observed at these two pH values and a complete separation of six tetracyclines was achieved within 8 min at pH 6.5 with the migration following the order OTC< TC < DMC< DOC< CTC< MNC.

The marked decrease in the electrophoretic mobility of TCs with increasing buffer pH is believed partly due to the decrease in the mole fractions of positively charged species and neutral species and the increase in the mole fraction of negatively charged species, and partly due to the drastic decrease in the binding constants of TCs to micelles. This will be described in the later section on the partition coefficient.

Influence of concentration of other surfactants

Fig 5 shows the variation of the electrophoretic mobility of TCs in a mixed micellar system containing SDS (20 mM) and Brij 35 (up to 0.135 %, w/v) at pH 6.5. The electrophoretic mobility of TCs (migrating toward anode) decreased with increasing concentration of Brij 35. This is expected owing to the decrease in the anionic charges of the SDS micelles on addition of Brij 35 [29,30].

It is interesting that the variation in the electrophoretic mobility of TCs with the hydroxyl group as the R_2 substituent, such as OTC, TC, DMC and CTC, which are relatively less hydrophobic than DOC and MNC, is greater than those of TCs with a hydrogen atom as the R_2

substituent, such as the aforementioned DOC and MNC. Evidently, the addition of Brij 35 to the SDS system would decrease the electrostatic interactions between TCs and micelles, and the migration behavior of TCs in mixed micelles, as shown in Fig. 6, is understandable. The migration of TCs follows the order OTC<TC<DMC<CTC<DOC<MNC in the mixed SDS -Brij 35 micellar system.

Fig. 7 shows the variation of the electrophoretic mobility of TCs in the SC micellar system at pH 6.5. As in the case of the SDS system, the electrophoretic mobility of TCs (migrating toward anode) increased and the migration window became broader with increasing concentration of SC from 30 mM to 80 mM.

Complete separation of OTC and TC was achievable when the concentration of SC was raised above 70 mM. However, peaks of DMC and CTC are unresolvable even with the concentration of SC at 80 mM. To avoid any complication caused by Joule heating, separation with the concentration of SC greater than 80 mM was not attempted. Fig. 8 shows the electropherogram of TCs obtained in the SC micellar system at pH 6.5.

Complete separation of TCs with ammonium acetate buffer in the TTAB micellar system is difficult. Peaks of CTC, DOC and MNC are migrating together in the buffer system containing 10-30 mM TTAB. Peaks of CTC and DOC are not separable even with the addition of acetonitrile (10 %, v/v) or methanol (30 %, v/v). Fig. 9 shows the electropherogram of TCs obtained in the TTAB micellar system at pH 6.5.

Partition coefficient

Fig. 10 shows the plots of capacity factor (k') calculated from eq. (2) for various TCs versus SDS concentration. The partition coefficients of

tetracyclines between aqueous and micellar phases (P_{mw}) were determined according to eq. (4). Table 1 lists the partition coefficients (P_{mw}), capacity factors (k') and critical micellar concentrations (cmc) obtained for individual tetracycline in the SDS micellar system at pH 6.0. The average cmc value is 4.5 ± 0.2 mM. As expected, this is smaller than the literature value of 8.2 mM owing to the presence of buffer electrolyte.

Table 2 gives the partition coefficients (P_{mw}) evaluated at five different pH value. The partition coefficient (P_{mw}) of individual tetracycline, which is pH-dependent, decreases quite drastically with increasing pH of the buffer. This is expected because the charge density of tetracyclines becomes more negative as the pH of the buffer increases from 6.0 to 8.0.

Migration order and the correlation of migration factor versus octanol-water partition coefficient

It has been generally accepted that separation of neutral and charged solutes in MEKC is based on the differential partitioning of solutes between aqueous phase and micellar phase in which hydrophobic interaction is the sole underlying force that influences the migration behavior of hydrophobic solutes. However, large differences in the migration behavior of solutes in MEKC with different types of surfactants suggest that this general belief is not accurate for all MEKC systems [31,32]. Depending on the chemical nature of both solutes and micelles, various chemical interactions other than hydrophobic interactions, such as dipolar or hydrogen bonding interactions, may occur between them in the partitioning process [31-33]. These interactions influence the migration behavior of solutes with various functional groups to a different extent, thus resulting in the differentiation of the selectivity and the alteration of the migration order of solutes.

In order to shed light on the migration order of tetracyclines, quantitative structure-retention relationship (QSRR) that describe the correlations between migration factor and octanol-water partition coefficient, a hydrophobic parameter, were examined in various micellar systems to better understand the underlying chemical interactions that influence the migration and selectivity of tetracyclines. In this study, we selected the SDS, SDS-Brij 35, SC and TTAB micellar systems.

For a micellar system in MEKC in which hydrophobic interactions play a major role in influencing the migration and selectivity of solutes, one may expect a linear relationship between the logarithm of capacity factor (log k') and the logarithm of octanol-water partition coefficient (log P_{ow}) as [34-36]:

$$\log k' = a \log P_{ow} + b \qquad (5)$$

It has been generally accepted that the migration of solutes in MEKC would correlate best with P_{ow} if the micellar system used has a similar hydrogen bonding affinity as 1-octanol [31,32]. Based on the values of P_{ow} of tetracyclines reported in the literature [37, 38] and the capacity factor (k') of TCs calculated in this work, the plots of log k' vs. log P_{ow} in four different micellar system at pH 6.5, as shown in Fig. 11, were obtained. The correlation coefficient (r^2) for the SDS, SDS-Brij 35 and SC micellar systems are high. However, a poor correlation was observed for the TTAB system. Table 3 lists the results of a log k' vs. log P_{ow} linear regression.

As the TTAB micelles have the most hydrogen bond acceptor characteristics among the four micellar systems used in this study [31,32], it is not surprising that the overall trend of the migration behavior of TCs in MEKC with TTAB micelles does not correlate well with the values of

log P_{ow}, because tetracyclines also possess hydrogen bond acceptor characteristics. The reasons for the abnormally large electrophoretic mobility of DMC in the TTAB-MEKC system are not clearly known. As indicated in Table 3, the correlation is greatly improved when DMC is eliminated from sample solutes.

The hydrogen bond acceptor characteristics of SC are weaker than those of TTAB, but stronger than for 1-octanol. The existence of higher correlation for the SC system is attributed to a similar hydrogen bonding pattern between SC micelles and 1-octanol. This result is consistent with the findings obtained by Yang et al.[32, 39].

The correlation of log k' vs. log P_{ow} in the SDS-MEKC system is not as good as for the SC system. This is attributed to the lower similarity in the hydrogen bonding pattern between SDS micelles and 1-octanol than that between SC micelles and 1-octanol. Moreover, the existence of congenerity problems in the SDS-MEKC system may cause the situation to be even worse [31, 32]. However, as SDS micelles are stronger hydrogen bond donors than 1-octanol, they exhibit more selective interactions towards tetracyclines which have hydrogen bond acceptor characteristics.

The addition of Brij 35 to the SDS micellar system resulted in higher correlation. This is expected because the anionic charges located at the surface of the SDS micelles are shielded on addition of Brij 35, which has a long polyoxyethylene chain, thus resulting in the decrease in the anionic charges of SDS micelles [40]. Therefore, excellent correlation of log k' vs. log P_{ow} in the mixed SDS-Brij 35 micellar system (with $r^2 > 0.942$) can be obtained.

As large differences in the migration behavior and selectivity for TCs in MEKC with different types of surfactants were observed and good linear correlations of log k' vs. log P_{ow} were obtained, the results reveal

that the migration of TCs depends primarily on the extent of micellar solubilization based on hydrophobic interactions; however, hydrogen bonding interactions may play an important role in these micellar system.

Conclusion

Complete separation of six tetracyclines was achieved using ammonium acetate buffer with the addition of SDS or mixed SDS-Brij35 at pH 6.5 with an applied voltage of 15 kV. The results of present studies indicate that the migration of TCs in MEKC is primarily based on hydrophobic interaction. However, electrostatic interaction, which is primarily due to hydrogen bonding interaction, may play a significant role in influencing the selectivity of TCs, thus leading to the alteration of the migration order of TCs in MEKC with different structural types of surfactants.

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References

1. L. A. Mitscher, *The Chemistry of the Tetracycline Antibiotics (Medicinal Research Series, vol 9)*, Marcel Dekker, New York, 1978.
2. N. E. Rigler, S. P. Bag, D. E. Leyden, J. L. Sudmeier, and C. N. Reilley, *Anal. Chem.*, 37 (1965) 872.
3. N. H. Khan, P. Wera, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.* 13 (1990) 1351.
4. Y. M. Li, A. V. Schepdael, E. Roets and J. Hoogmartens, *J. Chromatogr. A*, 740 (1996) 119.
5. S. A. Barker and C. C. Walker, *J. Chromatogr.,* 624 (1992) 195.
6. R. W. Fedeniuk, S. Ramamurthi and A. R. McCurdy, *J. Chromatogr. B.*, 677 (1996) 291.
7. H. Oka, Y. Ikai, N. Kawamura, K. Uno and M. Yamada, *J. Chromatogr.*, 393 (1987) 285.
8. H. Oka, Y. Ikai, N. Kawamura, K. Uno and M. Yamada, *J. Chromatogr.*, 400 (1987) 253.
9. H. Oka, Y. Ikai, N. Kawamura, K. Uno and M. Yamada, *J. Chromatogr.*, 411 (1987) 313.
10. W. Nadong, J. Thuranira, K. Vermeulen, E. Roets and J. Hoogmartens, J. Liq. Chromatogr., 15 (1992) 2529.
11. W. Nadong, K. Verresen, E. Roets and J. Hoogmartens, J. Chromatogr., 586 (1991) 61.
12. S. F. Y. Li, *Capillary Electrophoresis: Principles, and Practice and applications,* Elsevier, Amsterdam, 1993.
13. N. A. Guzman (Editor), Capillary Electrophoresis Technology, Marcel Dekker, New York, 1993.
14. P. Camilleri (Editor), *Capillary Electrophoresi: Theory*

and Practice, CRC Press, Boca Raton, FL., 1993.

15. D. Coleman (Editor), *Directory of Capillary Electrophoresis*, Elsevier, Amsterdam, 1994.
16. J. P. Landers (Editor), *Handbook of Capillary Electrophoresis*, CRC Press, Boca Raton, FL, 1994.
17. H. Shintani and J. Polonsky (Editor), *Handbook of Capillary Electrophoresis Applications,* Blackie Academic &Professional, London, 1997.
18. C.-X. Zhang, Z.-P. Sun, D.-K. Ling and Y.-J. Zhang, *J. Chromatogr.*, 627 (1992) 281.
19. A. V. Schepdael, J. Saevels, X. Lepoudre, R. Kibaya, N. Z. Gang, E. Roets and J. Hoogmartens, *J. High Resol. Chromatogr.*, 18 (1995) 695.
20. M. F. M. Tavares and V. L. McGuffin, *J. Chromatogr. A*, 686 (1994) 129.
21. J. J. Pesek and M. T. Matyska, *J. Chromatogr. A*, 736 (1996) 313.
22. J. Tjornelund and S. H. Hansen, *J. Chromatogr. A*, 737 (1996) 291.
23. S. Croubels, W. Baeyens, C. Dewaele and C. V. Peteghem, *J. Chromatogr. A*, 673 (1994) 267.
24. C. L. Chen, X. Gu, *J. AOAC Int.,* 78 (1995), 1369.
25. C. E. Lin, W. C. Lin, and W. C. Chiou, *J. Chromatogra. A*, 722 (1996) 333.
26. S. Terabe, K. Otsuka, K. I chikawa, A. Tsuchiya, and T. Ando, *Anal. Chem.,* 56 (1984) 111.
27. M. G. Khaledi, S. C. Smith and J. K. Strasters., *Anal. Chem.,* 63 (1991) 1820 .
28. D. W. Armstrong and G. Y. Stine, *J. Am. Chem. Soc.*, 105 (1983) 2962.
29. S. Terabe, K. Otsuka, T. Ando, *Anal. Chem.*, 57 (1985) 834.
30. S. Terabe, H. Ozaki, Y. Ishihama, Bunseki Kagaku, 42 (1993) 859.

31. S. Yang and M. G. Khaledi, *Anal. Chem.*, 67 (1995) 499.

32. S. Yang, J. G. Bumgarner, L. F. R. Kruk and M. G. Khaledi, *J. Chromatogr. A*, 721 (1996) 323.

33. S. Yang and M. G. Khaledi, *J. Chromatogr. A*, 692 (1995) 301.

34.C. Hansch and A. Leo, Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley Interscience, New York, 1979.

35. C. Hansch, in E. Aries (Editor), Drug Design, Vol 1, Academic Press, New York, 1979, p. 271.

36. R. Kaliszan, Quantitative Structure-Chromatographic Retention Relationships, Wiley Interscience, New York, 1987.

37. J. L. Colaizzi and P. R. Klink, *J. Pharm. Sci.*, 58 (1969) 1184.

38. G. Miller, H. Smith, W. Rock and S. Hedberg, *J. Pharm. Sci.*, 66 (1977) 88.

39.S. Yang, J. G. Bumgarner, and M. G. Khaledi, *J. Chromatogr. A*, 738 (1996) 265.

40. Y. Ishihama, Y. Oda, K. Uchikawa, and N. Asakawa, *Chem. Pharm. Bull.,* 42 (1994) 1525.

Captions of Figures

Fig. 1. Structures of the tetracyclines studied.

Fig. 2 Variation of the electrophoretic mobility of tetracyclines as a function of SDS concentration using ammonium acetate buffer (15 mM) at pH 6.0. Capillary: 43 cm x 50 μm, I.D., Other operating conditions: 15 kV, 25℃. Curve identification: ( ) OTC, (▲) TC, (ð) DMC, (Δ) DOC, (●) CTC, (o) MNC; the numbers denote the analytes shown in Fig. 1.

Fig. 3 Variatrion of the electrophoretic mobility of tetracyclines as a function of pH in the range 5.0-9.0 using ammonium acetate buffer (15 mM) containing SDS (20 mM). Other operating conditions and peak identification are the same as for Fig. 2.

Fig. 4 Electropherograms of tetracyclines obtained with ammonium acetate buffer (15 mM) containing SDS (20 mM) at varied pH: (A) 5.0, (B) 6.5. Other operating conditions and peak identification are the same as for Fig. 2.

Fig. 5 Variation of the electrophoretic mobility of tetracyclines in a mixed micellar system containing SDS (20 mM) and Brij 35 (up to 0.135 %, w/v) with ammonium acetate buffer (15 mM) at pH 6.5. Other operating conditions and peak identification are the same as for Fig. 2.

Fig. 6 Electropherogram of tetracyclines obtained with ammonium acetate buffer (15 mM) containing SDS (20 mM) and Brij 35 (0.135 %, w/v) at pH 6.5. Other operating conditions and peak identification are the same as for Fig. 2.

Fig. 7 Variation of the electrophoretic mobility of tetracyclines as a function of SC concentration using ammonium acetate buffer (15 mM) at pH 6.5. Applied voltage: 12 kV. Other operating conditions and peak identification are the same as for Fig. 2.

Fig. 8 Electropherogram of tetracyclines obtained with ammonium acetate buffer (15 mM) containing SC (80 mM) at pH 6.5. Other operating conditions and peak identification are the same as for Fig. 7.

Fig. 9 Electropherogram of tetracyclines obtained with ammonium acetate buffer (30 mM) containing TTAB (30 mM) at pH 6.5. Applied voltage: -10 kV. Other operating conditions and peak identification are the same as for Fig. 2.

Fig. 10 Plots of migration factor (k') of tetracyclines as a function of SDS concentration at pH 6.0.

Fig. 11 Correlations of log k' versus log P_{ow} for tetracylines in four different micellar systems at pH 6.5: (A), SDS (20 mM); (B), SDS (20 mM)-Brij (0.135 %, w/v); (C), SC (80 mM); (D), TTAB (30 mM).

附錄二

On-line Concentration of s-Triazine Herbicides in Micellar Electrokinetic Chromatography Using a Cationic Surfactant

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Key words: On-line concentration; sample stacking; sweeping; s-triazines; herbicides

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ABSTRACT

On-line concentration of neutral species of s-triazine herbicides in micellar electrokinetic chromatography using tetradecylammonium bromide (TTAB) as a cationic surfactant was investigated. Factors affecting the stacking of analytes were examined. The results indicate that the stacking efficiency is markedly improved with addition of phosphate buffer in the sample matrix. It was found that, depending on the nature of the analytes, the most effective stacking of these analytes occurs when the ratio of the conductivity of buffer electrolyte to that of sample matrix is in the range 1.4-1.2, with sample matrix containing phosphate buffer. Micelle concentration in the separation buffer is also a crucial factor to enhance the stacking efficiency and detection sensitivity of analytes. Moreover, the stacking efficiency of each individual analyte depends on its binding constant to TTAB micelles. The concentration effect is primarily based on sweeping mechanism which is operated in a normal stacking mode with reversed electrode polarity in the presence of reversed electroosmotic flow. As a result of concentration enhancement, the detection limits of these herbicides can reach about 9-15 *n*g/mL with UV detection.

1. INTRODUCTION

Capillary electrophoresis (CE) has been proven to be a powerful analytical tool for separating charged species of diverse samples, due to many of its advantageous features such as extremely high column efficiency, rapid analysis and small volumes of sample consumption in comparison with HPLC. For separating neutral analytes, micellar electrokinetic chromatography (MEKC) is the method of choice. However, the very limited optical path length, due to small inner diameter of the capillary (100-25 μm) and low sample volume injected (*n*L), make the detection of low-concentration samples with a UV detector difficult or even impossible without sample preconcentration.

To achieve more sensitive detection, fluorescence detection, particularly laser-induced fluorescence detection [1, 2] may be used. Unfortunately, this type of detection is not generally applicable to environmental analysis because only a few compounds have native fluorescence and most analytes need to be derivatized with an appropriate fluorescent tag. In addition, only a limited number of laser sources are available.

Alternatively, on-line sample concentration by either field-amplified sample stacking [3-16] or isotachophoretic sample stacking [17-19] can be employed for enhancing detectability in capillary electrophoresis. For field-amplified sample stacking, upon the introduction of a long plug of low-conductivity sample solution into the capillary previously filled with a high-conductivity buffer electrolyte, sample stacking occurs at the concentration boundary between the low conductivity of sample zone and the high conductivity of separation zone when a high voltage is applied across the capillary. For neutral analytes,

effective mobility necessary for stacking is provided by charged micelles in MEKC. Stacking with reversed migration micelles by large-volume sample injection may give enrichment factors of more than 500 [16-18]. Recently, head-column field-amplified sample stacking in binary system capillary electrophoresis has been demonstrated to provide over 1000-fold sensitivity enhancement [20, 21]. Moreover, Terabe and his coworkers [22-29] have reported that the stacking of neutral or ionic analytes in MEKC could be achieved based on sweeping technique. Depending on the nature of sample analytes, this technique can provide up to a 5000-fold concentration enhancement [22] or even approaching a million-fold sensitivity increase by applying cation-selective exhaustive injection and sweeping technique [28]. All these efforts make the detection of environmental analytical samples by UV absorption at trace concentration levels becoming possible.

s-Triazines are important selective pre- and post-emergence herbicides used widely for the control of broadleaf and grassy weeds [30]. These herbicides may contaminate drinking water sources. Six triazine herbicides, including prometryn and terbutryn are on the priority list in European Union drinking water guideline [31]. Simetryn and the two triazine herbicides aforementioned are on the priority list of pesticides in the USA national pesticide survey for a monitoring program on pesticides [31, 32]. Because of their extensive use, relatively high persistence and toxicity in environmental matrices [31-33], s-triazines are of great environmental concern. Thus, the development of new analytical methods is desirable.

Several papers on the separation of s-triazines by MEKC have been reported [34-39]. The analysis of simazine and atrazine in samples of river water [35] and the determination of four chloro- and three

methylthio-s-triazines in water [36] were conducted using sodium dodecyl sulfate (SDS) as an anionic surfactant. The separation of three chloro- and two methylthio-s-triazines was performed by partial filling micellar electrokinetic chromatography using SDS micelles [37]. The separation of prometon, prometryn and propazine was investigated using anionic octylglucoside-borate micelles at alkaline pH [34, 38]. Recently, we reported the separation of thirteen s-triazines, including five chloro-, three methoxy- and five alkylthio-s-triazines, in MEKC using a cationic surfactant [39].

In this study, four methylthio-s-triazines are selected as test compounds. We demonstrate the influences of the concentration of both sample matrix and separation buffer on the stacking efficiency and detection sensitivity of these analytes by sweeping technique using a cationic surfactant. The concentration enhancement of neutral species of these s-triazines at pH 6.0 is studied and the limits of detection are determined.

2. EXPERIMENTAL

2.1. Chemicals and reagents

Four methylthio-s-triazines, including simetryn, ametryn, prometryn and terbutryn, were purchased from Supelco (Bellefonte, PA, USA). Tetradecyltrimethylammonium bromide (TTAB) was acquired from Tokyo Kasei Kogyo (TCI, Tokyo, Japan). All other chemicals were of analytical-reagent grade. Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA)

Standard solutions of s-triazines were prepared at a concentration of 10 μg/mL in a solution containing 4 % (v/v) acetonitrile. Further dilution of sample solution with deionized water down to 0.05 μg/mL was carried out in the determination of the limits of detection. The pH of the buffer and sample solutions was adjusted by mixing various proportions of a certain concentration of trisodium phosphate with the same concentration of phosphoric acid solutions to attain pH 6.0. All solutions were filtered through a membrane filter (0.22 μm) before use.

2.2. Apparatus

Electrophoretic experiments were performed using a Beckman Coulter (Fullerton, CA, USA) Model P/ACE MDQ capillary electrophoresis system, equipped with a diode array UV-visible detector and an automatic injection system. The CE system with MDQ softwares was interfaced with a microcomputer and a Hewlett Packard desklet 670C printer. The fused silica capillaries of 50 μm, I.D. were purchased from

Polymicro Technologies (phoenix, AZ, USA). The total length of capillary is 60 cm and the position of UV detector is 10 cm from the anodic end. For pH measurements, a pH meter (Suntex Model SP-701, Taipei, Taiwan) was employed with a precision of 0.01 pH unit. For conductivity measurements, a conductivity meter (Suntex SC-170, Taipei, Taiwan) calibrated with a 0.01 N KCl solution to a value of 1.413 mS/cm (at 25 ^{0}C) was used.

2.3. Electrophoretic procedure

When a new capillary was used, the capillary was washed 20 min with sodium hydroxide solution (1.0 M) at 25 ^{0}C, followed by sodium hydroxide solution (0.1 M) for 20 min and then deionized and purified water for another 20 min at 25 ^{0}C.

To ensure reproducibility, all experiments were performed at 25 ^{0}C, and measurements were run at least in triplicate. The capillary was prewashed with running buffer for 5 min before each injection and postwashed for 3 min with deionized water to maintain proper reproducibility for run-to-run injections. Sample injections were made in pressure injection mode at a pressure of 68.9 mbar (1psi) or indicated elsewhere. An applied voltage of -20 kV was selected in the electrophoretic separation. Detection was performed at 222 nm.

The stacking was performed by injecting sample solutions for a much longer time compared to the usual hydrodynamic injection. Sample solutions were introduced at the cathodic end of the capillary. Varied plug lengths of sample solution up to 18.1 % occupancy of the capillary were assessed by introducing sample solution for a varied injection time up to

105 s.

3. RESULTS and DISCUSSION

The addition of a cationic surfactant to the electrophoretic buffer may induce the reversal of the electroosmotic flow (EOF) in the electrophoretic separation. In fact, the EOF was reversed when the concentration of TTAB added in the phosphate buffer (70 mM) at pH 6.0 exceeded 0.2 mM [40]. In this study, on-line concentration of s-triazines was performed under the conditions of reversed EOF, as the concentrations of TTAB employed were much greater than the critical micelle concentration of TTAB which was determined to be 1.6 ± 0.2 mM at pH 6.0 [41].

3.1 Effect of sample matrix on stacking efficiency

In a previous report [39], thirteen s-triazines, including the four chloro-s-triazines and four methylthio-s-triazines selected in this work, were completely separated by MEKC in a phosphate buffer (70 mM) containing TTAB (15 mM) as a cationic surfactant at pH 6.0. For a large volume of sample injection, however, reoptimization of separation parameters, such as phosphate concentration in the sample matrix and micelle concentration in the separation buffer, in particular, is necessary in order to achieve an effective and efficient stacking.

To examine the influences of sample matrix on the stacking efficiency and detection sensitivity of sample analytes, sample analytes were dissolved in an aqueous solution containing varied concentrations of either phosphate buffer in the range 10~70 mM and 4% acetonitrile as well. Fig. 1 shows some typical electropherograms of four

methylthio-s-triazines obtained with sample solutions containing varied concentrations of phosphate buffer (0, 30, 50, 70 mM), while a separation buffer is composed of 40 mM phosphate buffer and 40 mM TTAB at pH 6.0. Sample analytes at a concentration of 10 μg/mL were injected for 30 s. As shown in Fig. 1A, the peaks of the four s-triazines obtained without addition of phosphate buffer in the sample matrix are rather broad and are poorly resolved. Apparently, the analytes are not in the efficient stacking conditions. The resolutions of peaks become even worse with a longer injection time. In contrast, as shown in Fig. 1B-D, with addition of phosphate buffer in the sample matrix, the peaks become sharpened and the peak height of these sample analytes increases with increasing phosphate concentration up to 50 mM. However, the peak height of these s-triazines decreases with further increasing the concentration of phosphate buffer in the sample matrix.

The conductivity values of sample matrices and those of buffer electrolytes at varied concentration were measured. The values of enhancement factor (γ) defined as the ratio of the conductivity of buffer electrolyte to that of sample matrix are indicated in Fig. 1. It should be noted that the γ value of the most effective stacking of analytes is in the range 1.4-1.2, instead of 1.0. Similar phenomena were observed for chloro-s-triazines as for methylthio-s-triazines and the ¥ value for most efficient stacking of these analytes was found to be 1.19 [42]. Evidently, the afore-mentioned results reveal that the stacking of analytes is primarily due to sweeping mechanism proposed by Quirino and Terabe [22], although, in this study, it is operated in a normal stacking mode with reversed electrode polarity in the presence of reversed electroosmotic flow. As the ¥ values of the most effective stacking are not closed to 1.0, the contribution of field-amplified sample stacking to the enhancement of

detection sensitivity may not be completely ignored.

3.2 Stacking of analytes by sweeping

Fig. 2 depicts the schematic stacking mechanism of a neutral analyte dissolved in a sample matrix containing phosphate buffer with a separation buffer containing a cationic surfactant. As illustrated in Fig. 2A, the capillary column is initially filled with a micellar background electrolyte (BGE). A sample zone containing nonmicellar sample matrix or simply water is injected hydrodynamically with pressure for a period much longer than usual. By application of voltage at negative polarity (Fig. 2B), the electroosmotic flow is directed toward the anode (as cationic micelles is adsorbed on the capillary wall) and the micelles migrate toward the cathode. During sweeping, the analytes stacked at the concentration boundary between the region of $[mc]_s$ and that of [mc] =0 in the sample zone, where $[mc]_s$ denotes the concentration of micelles in the sample zone after sweeping and [mc] =0 indicates that no micelles are present in the original sample zone. The separation is then achieved via MEKC (Fig. 2C).

3.3 Effect of micelle concentration

The stacking efficiency and detection sensitivity of these test analytes are greatly affected by TTAB concentration. The peak height of each individual analyte increases with increasing micelle concentration until reaching the maximum, then it decreases with further increasing micelle concentration. Fig. 3 shows the variations of the peak height of methylthio-s-triazines as a function of TTAB concentration. As can be seen, the optimal TTAB concentrations determined for terbutryn,

prometryn, ametryn and simetryn are about 30, 45, 50 and 70 mM, respectively, at the sample concentration of 10 μg/mL for a 30-s injection. In the present study, the optimal TTAB concentration for a simultaneous detection of methylthio-s-triazines is 40 mM for a 30-s injection and 60 mM for a 60-s or longer injection time. It should be noted that, with TTAB micelles at a concentration less than 20 mM, a satisfactory stacking of terbutryn for a 30-s injection is difficult.

3.4 Stacking efficiency versus sample plug length

The stacking efficiencies in terms of peak height (SE_{height}) for a neutral analyte is defined as the peak of the analyte for varied lengths of sample plug (H_{stack}) divided by the peak height of the corresponding analyte obtained for 1-s injection (H_{1s}). As a certain minimum injection time is required with Beckman A/PCE MDQ system for a particular injection pressure, H_{1s} is defined as the stacking efficient of a minimal injection time (H_{min}) divided by the minimal injection time. For example, with an injection pressure of 1 psi, the minimum injection time is 3.5 s. Then H_{1s} is equivalent to $H_{3.5s}$ divided by 3.5.

For pressure injections, the length of sample plug in a capillary is directly proportional to the product of the injection pressure and injection time. Thus a 30-s injection of sample solution with a pressure of 1 psi corresponds to a sample plug length of 4.23 cm, which is 6.04 % occupancy of the capillary.

Fig. 4 shows the effect of sample plug length on the SE_{height} using a mixture of four methylthio-s-triazines at a concentration of 1.0 μg/mL. As illustrated, the stacking efficiency of these test analytes increases

linearly with increasing injection time up to about 70, 60, 35, and 20 s for terbutryn, prometryn, ametryn and simetryn, respectively, then increase gradually with further increasing injection time. Consequently, the maximum detection sensitivity of terbutryn, prometryn, ametryn, and simetryn are obtained with a duration of injection time of 70, 60, 35, and 20 s, respectively.

To demonstrate the stacking efficiency and the enhancement of detection sensitivity, Fig. 5 shows a typical electrophoreogram of the four methylthio-s-triazines obtained for a 30-s sample injection together with an electrophoreogram obtained for a 2.5-s injection under the optimal condition of usual injection time for comparison. Evidently, by the application of sweeping technique, the detection sensitivity of analytes can be greatly enhanced.

3.5 Peak width versus binding constant

It is of interest to note that, under the effective stacking conditions, the peak widths of these s-triazines increase in the order: simetryn > ametryn > prometryn > terbutryn. As the magnitudes of binding constants of methylthio-s-triazines to TTAB micelles also increase in the same order as for the peak width of the analytes [39], the dependence of the stacking of these analytes on their binding constants is evident. As a matter of fact, it is observed that the stronger the interaction between the analytes and the micelles, the narrower the peak width. As the binding constant of a sample analyte is linearly related to its retention factor, the result is qualitatively consistent with the finding obtained by Quirino and Terabe [22].

3.6 Detection limits and reproducibility

The limits of detection (LOD) at a signal to noise ratio (S/N) is equally to 3, as well as the reproducibility of migration times and peak heights, for these s-triazines were determined. The migration times of these analytes were quite reproducible, with relative standard deviations (RSD) varying in the range 0.8-1.0 % (n=8). The variations of the peak height with RSD less than 10.5 % were obtained. The values of LOD determined for these methylthio-s-triazines with sample concentration in the range of 1000-50 *n*g/mL for a 30-s injection time are ranging from 9 *n*g/mL for simetryn to 15 *n*g/mL for terbutryn. Table 1 gives the data of analysis for these four analytes.

4. CONCLUSION

On-line concentration of neutral species of s-triazine herbicides in MEKC using a cationic surfactant is demonstrated. The stacking efficiency of analytes can be greatly enhanced by sweeping with addition of buffer electrolyte in the sample matrix and with an appropriate micelle concentration in the separation buffer. Reoptimization of separation parameters is necessary for a large-volume sample injection. For analytes with considerably different binding constants to the micelles, the optimal micelle concentration for an efficient stacking may be different from one analyte to the other.

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REFERENCES

[1] L. Hernandez, N. Joshi, P. Verdeguer, N.A. Guzman, *in Capillary Electrophoresis Technology,* N.A. Guzman (Ed), Marcel Dekker, New York, NY, 1993, pp. 605-614.

[2] D.B. Craig, C.Y. Wong, N.J. Dovichi, *Anal. Chem.,* 68 (1996) 69.

[3] R.L. Chien, D.S. Burgi, *J. Chromatogr.*, 559 (1991) 141.

[4] R.L. Chien, D.S. Burgi, *J. Chromatogr.*, 559 (1991) 153.

[5] D.S. Burgi, R.L. Chien, *Anal. Chem.*, 63 (1991) 2042.

[6] R.L. Chien, D.S. Burgi, *Anal. Chem.*, 64 (1992) 489A.

[7] D.S. Burgi, R.L. Chien, *J. Microcolumn Sep.*, 3 (1991) 199.

[8] D.S. Burgi, *Anal. Chem.*, 65 (1993) 3726.

[9] Z. Liu, P. Sam, S.R. Sirimanne, P.C. McClure, J. Grainger, D.G. Patterson, *J. Chromatogr.*, 673 (1994) 125.

[10] K.R. Nielsen, J.P. Foley, *J. Chromatogr. A*, 686 (1994) 283.

[11] J.P. Quirino, S. Terabe, *J. Chromatogr. A,* 781 (1997) 119.

[12] J.P. Quirino, S. Terabe, *J. Chromatogr. A,* 791 (1997) 255.

[13] J.P. Quirino, S. Terabe, *Anal. Chem.,* 70 (1998) 149.

[14] J.P. Quirino, S. Terabe, *Anal. Chem.,* 70 (1998) 1893.

[15] J.P. Quirino, K. Otsuka, S. Terabe, *J. Chromatogr. B,* 714 (1998) 29.

[16] J.P. Quirino, S. Terabe, *J. Chromatogr. A,* 798 (1998) 251.

[17] P. Gebauer, W. Thormann, P. Bocek, *J. Chromatogr.,* 608 (1992) 47.

[18] C. Schwer, F.J. Lottspeich, *J. Chromatogr.*, 623 (1992) 345.

[19] T.J. Thompson, F. Foret, P. Vouros, B.L. Karger, *Anal. Chem.,* 65 (1993) 900.

[20] C.X. Zhang, W. Thormann, *Anal. Chem.,* 68 (1998) 2523.

[21] C.X. Zhang, W. Thormann, *Anal. Chem.*, 70 (1998) 540.

[22] J.P. Quirino, S. Terabe, *Science,* 282 (1998) 465.

[23] J.P. Quirino, S. Terabe, K.Otsuka, J.B. Vincent, G. Vigh, *J. Chromatogr. A*, 838 (1999) 3.

[24] J.P. Quirino, S. Terabe, *J. Chromatogr. A*, 850 (1999) 339.

[25] J.P. Quirino, S. Terabe, *Anal. Chem.*, 71 (1999) 1638.

[26] J.P. Quirino, S. Terabe, *J. Chromatogr. A*, 856 (1999) 465.

[27] J.P. Quirino, S. Terabe, *Electrophoresis*, 21 (2000) 355.

[28] J.P. Quirino, S. Terabe, *Anal. Chem.*, 72 (2000) 1023.

[29] J.P. Quirino, S. Terabe, P. Bocek, *Anal. Chem.*, 72 (2000) 1934.

[30] F.A. Gunther (Editor), Residue Reviews, Springer-Verlag, Heidelberg, Germany, 1970,Vol. 32.

[31] D. Barcelo, *J. Chromatogr. A,* 643 (1993) 117.

[32] J.R. Dean, G. Wade, I.J. Barnabas, *J. Chromatogr. A,* 733 (1996) 295.

[33] V. Pacakova, K. Stulik, J. Jiskra, *J. Chromatogr. A,* 754 (1996) 17.

[34] J.T. Smith, W. Nashabeh, Z. El Rassi, *Anal. Chem.,* 66 (1994) 1119.

[35] C. Desiderio, S. Fanali, *Electrophoresis,* 13 (1992) 698.

[36] R.C. Martinez, E.R. Gonzalo, A.I.M. Dominguez, J.D. Alvarez, J.H. Mendez, *J. Chromatogr. A,* 733 (1996) 349.

[37] J. Cai, Z. El Rassi, *J. Chromatogr.*, 608 (1992) 31.

[38] W.M. Nelson, C.S. Lee, *Anal. Chem.*, 68 (1996) 3265.

[39] C.E. Lin, C.C. Hsueh, T.Z. Wang, T.C. Chiu, Y.C. Chen, (1999) *J. Chromatogr. A,* 835, 197.

[40] C.E. Lin, T.Z. Wang, C.C. Hsueh, Y.C. Liu, *J. Chromatogr. A,* 878 (1999) 137.

[41] C.E. Lin, T.Z. Wang, T.C. Chiou, C.C. Hsueh, *J. High Resol. Chromatogr.,* 22 (1999) 265.

[42] C.E. Lin, T.Y. Yang, Y.C. Liu, T.Z. Wang, (in preparation)

S/N=3) and reproducibility of methylthio-s-triazines for 60-s injection time.[a]

Simetryn	Ametryn	Prometryn	Terbytryn
y=2.2324x –0.0159	y=2.3910x –0.0232	y=2.0220x –0.0294	y=1.9795x +0.0488
0.9998	0.9999	0.9991	0.9987
13	9	14	15
1.01	1.00	0.80	0.83
8.7	9.1	9.4	10.5

ate buffer containing less than 5% CH_3CN.
sphate buffer containing 40 mM TTAB at pH 6.0

Figure Captions

Fig. 1 Effect of sample matrix on the stacking efficiency and detection sensitivity of methylthio-s-triazines. Sample matrix：(A), water; (B), 30 mM phosphate buffer; (C), 50 mM phosphate buffer; (D), 70 mM phosphate buffer. Separation buffer, 40 mM TTAB in 40 mM phosphate buffer at pH 6.0; injection pressure, 1 psi; injection time, 30 s; capillary, 70 cm × 50 μm I.D; applied voltage, -20 kV, detection wavelength, 222 nm; temperature, 25℃; sample concentration, 10 μg/ml, sample dissolved in a sample matrix containing 4 % acetonitrile solution. Peak identification, 1 = simetryn, 2 = ametryn, 3 = prometryn, 4 = terbutryn.

Fig. 2 Schematic diagram of a stacking mechanism by sweeping using a cationic surfactant (with sample matrix containing phosphate buffer).

Fig. 3 The variation of peak heights of sample analytes as a function of TTAB concentration. The electrophoretic conditions are the same as for Fig. 1C.

Fig. 4 Plots of SE_{height} versus injection time with a mixture of four methylthio-s-triazines at sample concentration of 1.0 μg/mL. Electrophoretic conditions are the same as for Fig. 1C, except sample concentration.

Fig. 5 Detection sensitivity of analytes measured under two different separation conditions: (A) without sample stacking (2.5-s injection with an injection pressure of 0.4 psi; sample concentration, 1.0 μg/mL); (B) with sweeping-stacking (30-s injection with an injection pressure of 1 psi; sample concentration, 1.0 μg/mL) Other operating conditions are the same as for Fig. 1C.

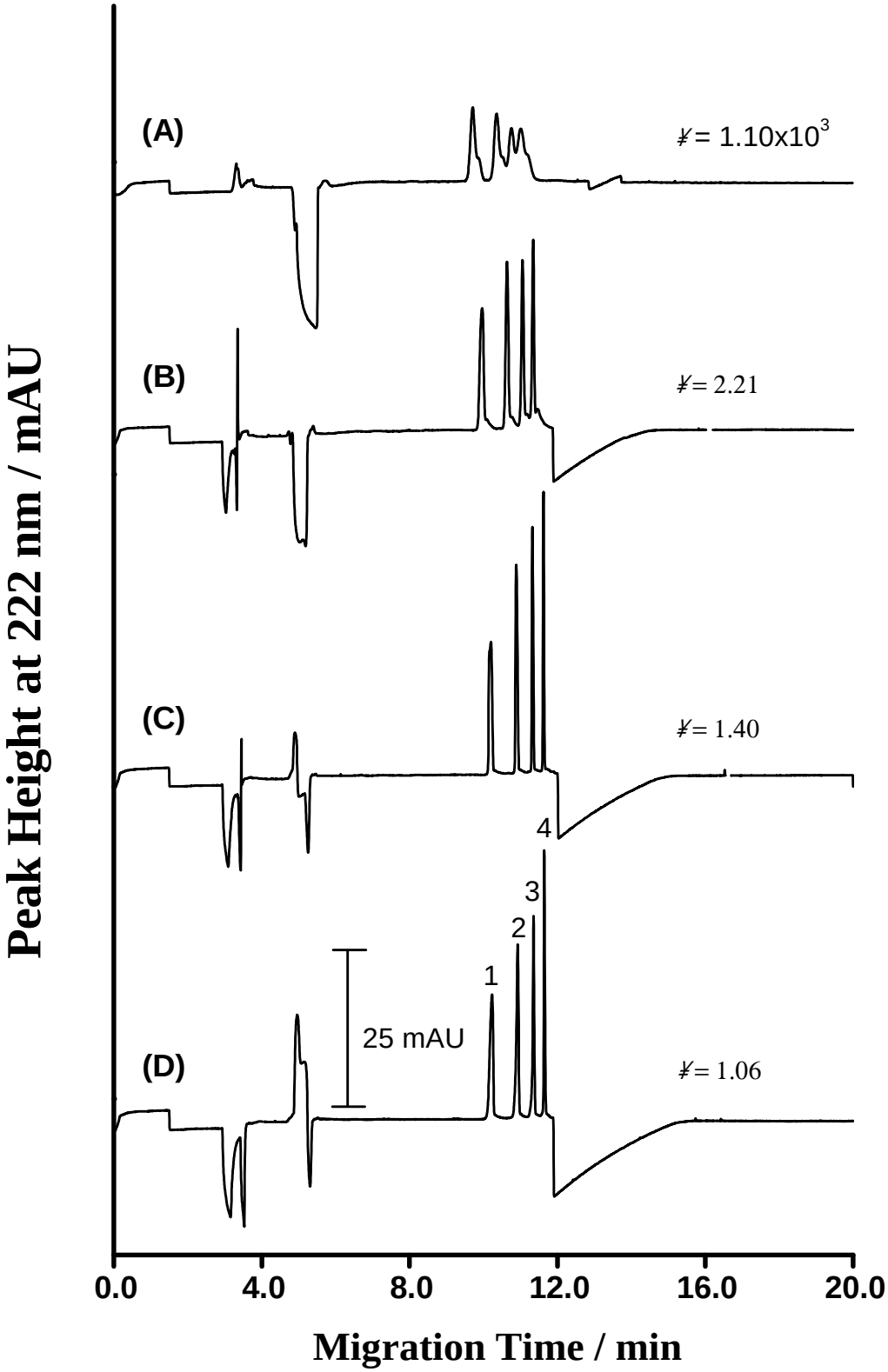
(A)
¥ = 1.10x10^3
(B)
¥ = 2.21
(C)
¥ = 1.40
(D)
¥ = 1.06
1
2
3
4
25 mAU
Peak Height at 222 nm / mAU
0.0
4.0
8.0
12.0
16.0
20.0
Migration Time / min

Fig. 1

A. Sample injection

B. Stacking of analytes

C. Separation after sweeping

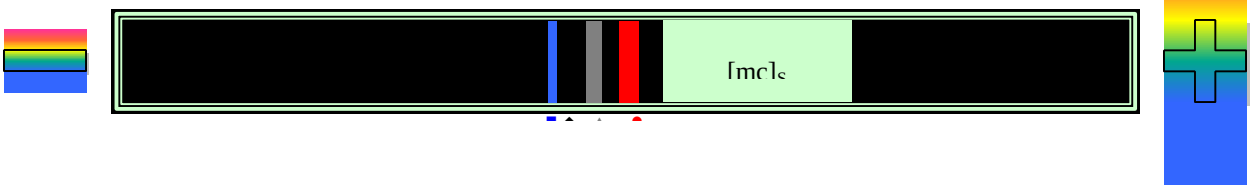

Fig. 2

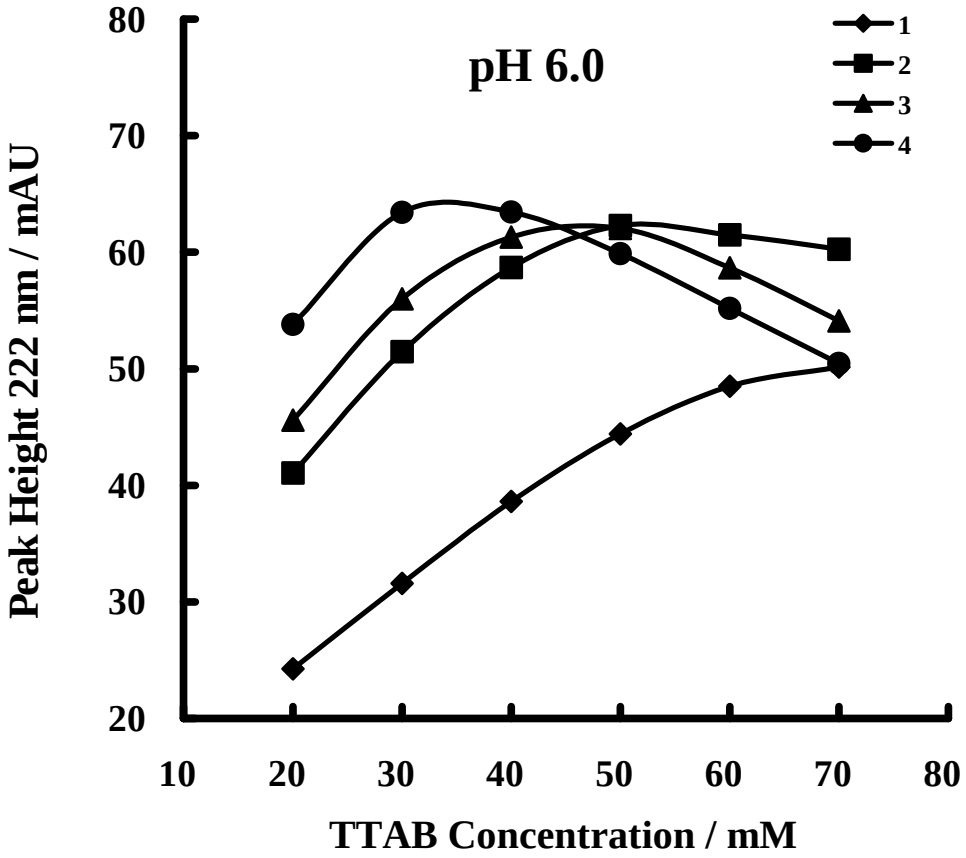
pH 6.0
1
2
3
4
Peak Height 222 nm / mAU
80
70
60
50
40
30
20
10
20
30
40
50
60
70
80
TTAB Concentration / mM

Fig. 3

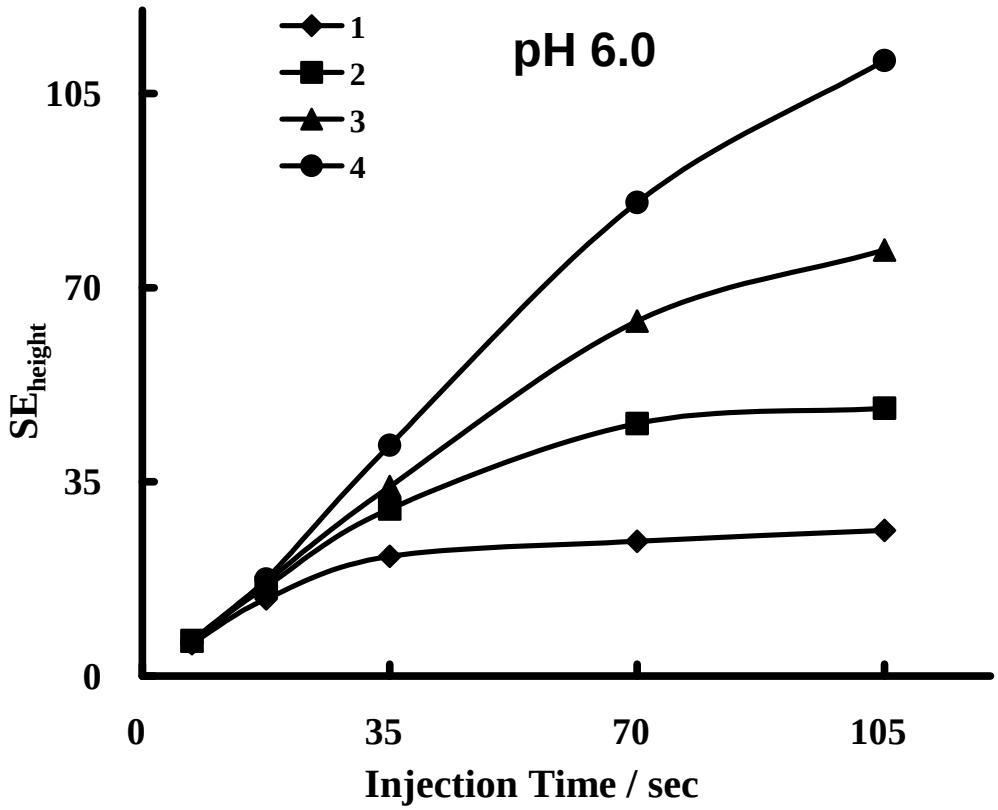

Fig. 4

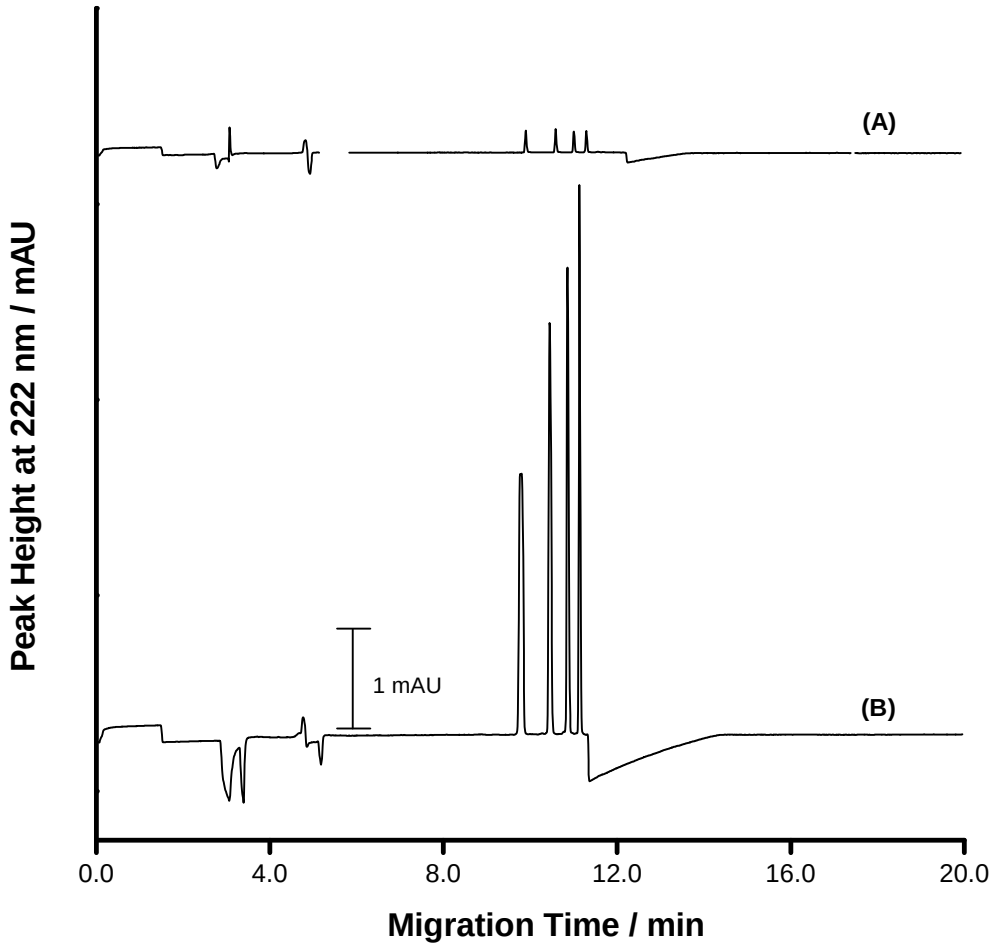
Peak Height at 222 nm / mAU
(A)
1 mAU
(B)
0.0
4.0
8.0
12.0
16.0
20.0
Migration Time / min

Fig. 5

附錄三

出席國際學術會議報告書 (國科會補助)

90 年 11 月 20 日

報告人姓名	林敬二	服務機關名稱及職務	國立台灣大學 化學系教授
會議期間及地點	90 年 9 月 11-14 日 日本，京都市	國科會 研究計劃	NSC 90-2113-M-002-059
會議名稱	(中文) 高效能液相分離國際研討會議— HPLC Kyoto (英文) International Symposium on High Performance Liquid Phase Separations HPLC Kyoto		
發表論文題目	(中文) 利用環糊精為對掌選擇試劑以毛細管區帶電泳法進行 Phenothiazines 的對掌分離 (英文) Enantioseparation of phenothiazines in capillary zone electrophoresis using cyclodextrins as chiral selectors		

報告內容

一、參加會議經過：

高效液相分離國際研討會於日本京都市舉行。會期為 9 月 11-14 日，2001 年，由日本京都纖維工藝大學(Kyoto Institute of Technology)的 N. Tanaka 教授及姬路工藝大學(Himeji Institute of Technology)的 S. Terabe 教授籌辦。此次研討會主要由日本的層析科學學會為主辦單位。

此次研討會共有口頭論文 66 篇及壁報論文 160 篇共二百餘篇的發表。參加人數約有二百多人。大會於 9 月 11 日下午揭幕。首先由美國東北大學的 B. L. Karger 教授講述利用 LC、CE、MS 等技術對高解析度蛋白基因體分離之新進發展，接著由日本東京大學的 Y. Sakaki 教授及美國 Oak Ridge 國家實驗室的 I. M. Ransey 博士等二位國際重量級的大師做專題演講，展開為期四天的學術活動。晚上舉行酒會招待。此次大會專題演講及學術研討分主題及二個場地進行研討，研討氣氛相當熱絡。此次研討會的主題包括蛋白質因體、藥物及生醫、新管柱技術、靜相示性、對掌分離、新靜相、微分離技術、微晶片、單細胞分析。研討會所邀請及參加的國際知名學者不少，聆聽他們的研究結果，對毛細管電泳分離的現況及未來研究趨勢，會更能掌握。此研討會雖比 HPLC 國際研討會的規模小一點，且受美國 911 事件影

響，使得參加人數頓顯更少，但研討會內容仍具有其特色及價值。

在此研討會中，筆者受邀做專題報告— 論文題目為：Enantioseparation of phenothiazines in capillary zone electrophoresis using cyclodextrins as chiral selectors，甚獲同一研究領域的學者所重視。此論文將發表刊登於 J. Chromatogr. A 中。此研討會另外也邀請暨南大學應化系主任楊重熙教授做一專題報告。筆者並受邀為 Session chairman 主持 Chiral separation 的 session。

二、與會心得：

此次參加毛細管電分離技術國際研討會，除了能多了解此研究領域進展的現況，吸收新知識，交換研究心得之外，並能多認識一些國際知名學者。此次研討會所發表的論文有不少值得觀摩學習，並能啟發研究構想，實在獲益不少。台灣學者除了筆者與楊教授外，另外還有中國醫藥研究所的蔡東湖教授及暨南大學應化系的何佳安教授參加，但參加人數還是太少，應該多多參加。

三、考察參觀活動：除參觀儀器展外，大會另外安排京都市郊多處神社及廟宇。

四、攜回資料：論文摘要乙冊。

五、建議：

參加國際學術研討會，不僅參加者能增廣見聞與學識，也可讓國際人士瞭解台灣學者的研究成果，應多多鼓勵參加。

Enantioseparation of Phenothiazines in Capillary Zone Electrophoresis Using Cyclodextrins as Chiral Selectors

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Keywords: Phenothiazines, cyclodextrins, enantioseparation, antipsychotics, inclusion complexes, capillary zone electrophoresis

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ABSTRACT

In this study, enantioseparations of five phenothiazines, including promethazine, ethopropazine, trimeprazine, methotrimeprazine, and thioridazine, in cyclodextrin (CD) -modified capillary zone electrophoresis were investigated using a phosphate buffer (40 mM) at pH 3.0. We focussed on the separation of phenothiazines with the use of CDs at low concentrations. Three different CDs, including β-CD, hydroxypropyl-β-CD (HP-β-CD) and γ-CD, were chosen as chiral selectors. The results indicate that effective enantioseparation of phenothiazines, except for methotrimeprazine, is simultaneously achievable with addition of γ-CD at a concentration of 2.5-6.0 mM. The enantiomers of ethopropazine and trimeprazine are effectively separated with addition of HP-β-CD at low concentrations, in the range 0.4-6.0 mM, whereas those of promethazine and trimeprazine are baseline resolved with β-CD at much lower concentrations (0.02-3.0 mM) than with HP-β-CD. The results also confirm that the separation window is greatly enlarged at low CD concentrations. Moreover, drastic variations of the electrophoretic mobility of phenothiazines as a function of CD concentration reveal that phenothiazines interact very strongly with CDs in the order γ-CD < HP-β-CD < β-CD.

1. Introduction

Phenothiazines are generally used as antipsychotic and neuroleptic agents. Potentially useful phenothiazine derivatives have different substituents attached at the 2-postion (R_2) and 10-position of the phenothiazine ring (R_{10}) with an alkyl piperazine group or an aliphatic side chain containing an amino group [1]. Among them, promethazine, ethopropazine, trimeprazine, methotrimeprazine, and thioridazine possess a chiral center. Fig. 1 depicts the structures of phenothiazines studied.

During the past two decades, separation of enantiomers of phenothiazines has usually been performed by high performance liquid chromatography (HPLC) [2-7]. In recent years, the applications of capillary electrophoresis (CE) to the chiral separation and/or determination of pharmaceutical compounds have become popular and have attracted the attention of many researchers [8-14]. This is due to the many advantageous features of CE, such as its extremely high efficiency, high resolution, rapid analysis and small consumption of sample, in comparison with HPLC [15-17].

The applications of the CE technique to the separation of phenothiazines have previously been demonstrated using either micellar electrokinetic chromatography [19, 20] or capillary zone electrophoresis (CZE) [20-24]. However, only three articles were reported in the literature regarding the applications of the CE technique to the enantioseparation and/or determination of phenothiazines [22-24]. The enantiomers of promethazine were separated by CZE with albumin as

chiral selector using a phosphate buffer at pH 7.6 [22, 23]. Enantioseparation of trimeprazine has been demonstrated with addition of HP-β-CD (8-22 mM) to a phosphate buffer (100 mM) at pH 2.5 and at 15.5℃ [24]. Thus, the understanding of the enantioseparation of phenothiazines in CZE is rather incomplete or even lacking. Obviously, a systematic and more thorough investigation on the chiral separation of phenothiazines is desirable. In this report, three different CDs, including β-CD, HP-β-CD and γ-CD, were selected as chiral selectors with an emphasis on the separation of five phenothiazines using CDs at low concentrations. The variations of the electrophoretic mobility of phenothiazines as a function of CD concentration at pH 3.0 were examined. Here we present the results of the investigation.

2. Experimental

2.1. Apparatus

All CE separation was performed on a Beckman P/ACE System MDQ with a photodiode array detector for absorbance measurements at 240 nm (Beckman Coulter, Fullerton, CA, USA). Uncoated fused-silica capillaries purchased from Polymicro Technologies (Phoenix, AZ, USA) were used. The dimensions of the capillary were 60.2 cm × 50 μm I.D. The effective length of the capillary was 50 cm from the injection end of the capillary. The CE system was interfaced with a microcomputer and a laser printer. System Gold software of Beckman was used for data acquisition. For pH measurements, a pH meter (Suntex Model SP-701, Taipei, Taiwan) was employed with a precision of ± 0.01 pH unit.

2.2 Chemicals and reagents

Five phenothiazines were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA. USA).

Standard solutions of phenothiazines at a concentration of 10 μg/mL were prepared by dissolving analytes in an aqueous solution. The pH of a phosphate buffer was adjusted to the desired pH value by mixing various proportions of a certain concentration of trisodiumphosphate solution with the same concentration of phosphoric acid. All buffer solutions, freshly prepared weekly and stored in a refrigerator before use,

were filtered through a membrane filter (0.22 μm).

2.3. Electrophoretic procedure

When a new capillary was used, the capillary was washed 30 min with 1.0 M NaOH solution, followed by 30 min with deionized water at 25℃. Before each injection, the capillary was prewashed for 3 min with running buffer and postwashed for 3 min with deionized water, 3 min with 0.1 M NaOH, and 5 min with deionized water to maintain proper reproducibility of run-to-run injections. Sample injections were done in a hydrodynamic mode over 5 s under a pressure of 1.0 psi at 25℃. The measurements were run at least in triplicate to ensure reproducibility. An applied voltage of 20 kV for phosphate buffer was selected to keep the total current less than 100 μA in order to avoid experimental complications resulting from Joule heating. The detection wavelength was set at 240 nm. Peak identification was conducted by spiking with the analyte to be identified. Mesityl oxide was used as neutral marker. The relative standard deviation of migration time is less than 0.6 % (n=5).

2.4. Mobility calculations

The electrophoretic mobility of analytes was calculated from the observed migration times with the equation:

$$\mu_{ep} = \mu - \mu_{eo} = \frac{L_d L_t}{V}\left(\frac{1}{t_m} - \frac{1}{t_{eo}}\right)$$

where μ_{ep} is the electrophoretic mobility of the analyte tested, μ is the

apparent mobility, μ_{eo} is the electroosmotic mobility, t_m is the migration time measured directly from the electropherogram, t_{eo} is the migration time for an unchanged solute, L_t is the total length of capillary, L_d is the length of capillary between injection and detection, and V is the applied voltage.

3. Results and discussion

It has been demonstrated that the addition of HP-β-CD to the electrophoretic system can enlarge the separation window so that better separability and greater selectivity of phenothiazines can be obtained [24]. Accordingly, the extent of the separation window, which is governed by the concentration of CD, depends on the extent of the complexation between phenothiazines and CDs.

3.1 Enantioseparation of phenothiazines with addition of HP- -CD

The variation of the electrophoretic mobility of phenothiazine enantiomers as a function of HP-β-CD concentration in the range 0-8 mM using a phosphate buffer (40 mM) at pH 3.0 was investigated. The electrophoretic mobility of each individual phenothiazine was found to decrease drastically with increasing HP-β-CD concentration, especially in the region 0-3 mM. This phenomenon reveals that phenothiazines interact strongly with HP-β-CD. The greater the extent of the variation of electrophoretic mobility, the stronger the interaction between phenothiazines and HP-β-CD. Thus, the interaction between the five phenothiazines and HP-β-CD increases in the order promethazine < ethopropazine < trimeprazine < methotrimeprazine < thioridazine. Since trimeprazine migrates faster than ethopropazine toward the cathode in CZE separation in the absence of HP-β-CD at pH 3.0, the reversal of the migration order of trimeprazine and ethopropazine occurs upon the addition of HP-β-CD to the background electrolyte at pH 3.0.

Effective enantioseparation of ethopropazine and trimeprazine was achieved with addition of HP-β-CD at a concentration in the range 0.4-8.0 mM and 0.1-6.0 mM, respectively, in a phosphate buffer at pH 3.0. However, the enantiomers of thioridazine could only be resolved with HP-β-CD at a concentration less than 0.2 mM. For illustration, Figs. 2A-F show some typical electropherograms of these phenothiazines with HP-β-CD at 0.1, 0.4, 1.2, 2.5, 6.0 and 8.0 mM, respectively. The enantiomers of trimeprazine and thioridazine were baseline separated with addition of 0.1 mM HP-β-CD (Fig. 2A); complete enantioseparation of ethopropazine was achieved with HP-β-CD at 0.4 mM (Fig. 2B). Under the circumstances of baseline separation of the two enantiomers, the enantioselectivity of trimeprazine increased with increasing HP-β-CD concentration from 0.1 to 1.2 mM (Fig. 2C), but decreased with further increasing HP-β-CD concentration from 1.2 to 6.0 mM (Fig. 2E). The peaks between the two enantiomers of trimeprazine were not resolvable at all when HP-β-CD concentration exceeded 15 mM [25]. Similarly, the enantioselectivity of ethopropazine also increased with increasing HP-β-CD concentration from 0.1 to 1.2 mM, but to a lesser extent than that of trimeprazine; it then decreased with further increasing HP-β-CD concentration from 1.2 to 8.0 mM, while keeping the two enantiomers of ethopropazine baseline separated (Fig. 2F). On the other hand, the two enantiomers of promethazine were barely resolved with addition of HP-β-CD at a concentration in the range 1.6-4.0 mM, although the separability was slightly improved at about 2.5 mM (Fig. 2D). However, as observed previously by Boer et al. with HP-β-CD at concentrations greater than 8 mM [24], no enantioseparation of methotrimeprazine could

be achieved with HP-β-CD at any concentration in the range 0-8.0 mM.

The separation window is greatly enlarged with addition of HP-β-CD, especially at low HP-β-CD concentrations (0-3 mM). Thus, for better enantioseparability and greater enantioselectivity of phenothiazines, the use of HP-β-CD at low concentrations (< 8 mM) is more advantageous than at the relatively higher concentrations employed by Boer et al. [24]. The optimal concentration ranges of HP-β-CD for enantioseparation of ethopropazine and trimeprazine were found to be 0.4-8.0 mM and 0.1-6.0 mM, respectively.

With HP-β-CD as a chiral selector, chiral recognition of the enantiomers of ethopropazine and trimeprazine is apparently related to an appropriate arrangement of the chain lengths between the aliphatic side chain of phenothiazines (chiral selectrands) and the hydroxypropyl group of HP-β-CD (chiral selector). This arrangement may involve hydrogen bonding interaction between the tertiary amino group of phenothiazines and the hydroxypropyl group of HP-β-CD located at the rim of the CD cavity. Similar arguments are also applicable to chiral recognition of promethazine and trimeprazine with the use of β-CD, which will be discussed shortly in Section 3.2.

By comparing the structure of methotrimeprazine with that of trimeprazine, we believe that enantioseparation of methotrimeprazine with HP-β-CD is hindered when the 2-position of the phenothiazine ring contains a methoxy group. Apparently, hydrogen bonding interaction between the aliphatic side chain (R_{10}) of methotrimeprazine and the

hydroxylpropyl group of HP-β-CD located at the rim of the CD cavity is greatly reduced.

3.2 Enantioseparation of phenothiazines with addition of -CD

The variation of the electrophoretic mobility of phenothiazine enantiomers as a function of β-CD concentration in the range 0-6 mM under the same electrophoretic conditions as in the case of HP-β-CD was examined. The variation of the electrophoretic mobility of phenothiazines was similar to that when HP-β-CD was used. More effective enantioseparation of promethazine and trimeprazine, but with no enantioseparation of ethopropazine, was noted. The enantiomers of thioridazine could be resolved with β-CD only in a very limited concentration range (< 0.3 mM). Obviously, stronger interaction of each individual phenothiazine with β-CD than with HP-β-CD is occurring. In fact, the binding constants of phenothiazines to β-CD are about 2.5- to 3-fold greater than those of phenothiazines to HP-β-CD [25].

Effective enantioseparation of promethazine and trimeprazine was achieved with addition of β-CD at a concentration in the range 0.3-2.0 mM and 0.02-2.0 mM, respectively. However, no enantioseparation of ethopropazine and methotrimeprazine could be achieved. The optimal concentration of β-CD for enantioseparation of trimeprazine was surprisingly small. In comparison with the results obtained with HP-β-CD, this result could not be rationalized simply based on the binding strength of trimeprazine with β-CD alone. Further investigation is needed.

For illustration, Figs. 3 A-F show some typical electropherograms of these phenothiazines with β-CD at 0.02, 0.05, 0.1, 0.3, 2.0, and 6.0 mM, respectively. The enantiomers of trimeprazine were baseline separated with addition of β-CD at a concentration as low as 0.02 mM (Fig. 3A). With β-CD at this concentration, ethopropazine and trimeprazine were not completely separated. However, as shown in the attachment of Fig. 3A, effective enantioseparation of trimeprazine was achieved when trimeprazine alone was injected into the capillary column. The enantioselectivity of trimeprazine, while keeping the two enantiomers baseline separated, increased with increasing β-CD concentration, similar to the case with HP-β-CD, reaching its maximum value at 0.3 mM (Fig. 3D), and then decreased with increasing β-CD concentration from 0.3 to 2.0 mM (Fig. 3E). Beyond 2.0 mM, the resolution of the peaks between the two enantiomers of trimeprazine decreased. The enantiomers of trimeprazine became unresolvable when the β-CD concentration exceeded 6.0 mM (Fig. 3F). On the other hand, the enantioseparation of thioridazine could be achieved with β-CD only in the concentration range 0.05-0.3 mM (Fig. 3B-D). The enantiomers of promethazine were partially resolved with β-CD at 0.1 mM (Fig. 3C); complete enantioseparation of promethazine was achieved with β-CD at 0.3 mM (Fig. 3D). Similarly, the enantioselectivity of promethazine increased with increasing β-CD concentration, but to a slightly lesser extent than that of trimeprazine. The enantioselectivity of promethazine reached its maximum value at about 1.0 mM, then decreased with increasing β-CD concentration from 1.0 to 2.0 mM, while keeping the two enantiomers of promethazine baseline separated (Fig. 3E).

The structures of inclusion complexes of β-CD with phenothiazine derivatives were theoretically examined using computer graphic modeling [3] and molecular dynamics calculation [26]. It was suggested that approximately one half of the phenothiazine ring was embedded in the cavity of β-CD and that the other half of the phenothiazine ring, together with the R_2 and R_{10} substituents, was located outside the cavity of β-CD [26]. Accordingly, a hydrogen bond might be formed between the nitrogen atom of the aliphatic side chain (R_{10}) and the hydroxyl group of β-CD located at the rim of β-CD cavity [3, 26]. Thus the results obtained in this work reveal that the hydrogen bonding interaction should play an important role in chiral discrimination of the enantiomers of phenothiazines. By comparing the structure of ethopropazine with those of promethazine and trimeprazine, it is thought that, owing to the bulky nature of tertiary diethylamino group in ethopropazine, the formation of a hydrogen bond between the aliphatic side chain containing an amino group in ethopropazine and the hydroxy group of β-CD located at the rim of the β-CD cavity is considerably reduced or even prevented, thus leading to the failure of chiral recognition of ethopropazine.

3.3 Enantioseparation of phenothiazines with addition of mixed CDs

As described in Section 3.2, effective enantioseparation of promethazine and trimeprazine was simultaneously achieved with addition of 0.3-6.0 mM β-CD to a phosphate buffer (40 mM) at pH 3.0. On the other hand, as described in Section 3.1, the enantiomers of ethopropazine and trimeprazine were effectively and simultaneously

separated with addition of 0.4-2.0 mM HP-β-CD to the same background electrolyte. Thus, we thought that complete enantioseparation of promethazine, ethopropazine and trimeprazine might be simultaneously achievable with addition of mixed CDs composed of β-CD and HP-β-CD. In fact, by fixing the β-CD concentration at 0.3 mM and varying the HP-β-CD concentration from 0.4 to 2.0 mM, the resolution of the two enantiomer peaks of ethopropazine can progressively be improved as the concentration of HP-β-CD increases. Complete enantioseparation of those three phenothiazines was successfully achieved with addition of mixed CDs composed of 0.3 mM β-CD and 1.4 mM HP-β-CD. Fig. 4 shows such an electropherogram of phenothiazines obtained.

3.4 Enantioseparation of phenothiazines with addition of ¥-CD

The variation of the electrophoretic mobility of phenothiazine enantiomers as a function of γ-CD concentration in the range 0-10 mM using the same phosphate buffer at pH 3.0 as in the case of HP-β-CD was studied. The variation of electrophoretic mobility of phenothiazines was similar to that with HP-β-CD, but not as drastic. Effective enantioseparation of promethazine, ethopropazine, trimeprazine, and thioridazine, but not of methotrimeprazine, was achieved. In comparison with the results obtained in Fig. 2, the less drastic change in the electrophoretic mobility as a function of γ-CD concentration reveals that the interaction of phenothiazine is comparatively weaker with γ-CD than with HP-β-CD. This is probably due to the loose fit of the size of phenothiazines to the cavity of γ-CD. The binding constants of

phenothiazines to β-CD are about 4.5- to 9-fold smaller than those of phenothiazines to HP-β-CD [25].

Effective enantioseparation of promethazine, ethopropazine, trimeprazine, and thioridazine was achieved with addition of γ-CD at a concentration in the range 2.5-10.0, 1.6-10.0, 2.5-6.0 and 0.1-10.0 mM, respectively. For illustration, Fig. 5A-E show some typical electropherograms of these five phenothiazines with addition of γ-CD at 0.5, 1.2, 1.6, 3.5, and 8.0 mM, respectively. With addition of 0.5 mM γ-CD, trimeprazine was separated from ethopropazine and the enantiomers of trimeprazine were barely resolved (Fig. 5A). The enantiomers of trimeprazine were baseline separated with addition of 1.6 mM γ-CD, whereas the enantiomers of promethazine and ethopropazine were not completely separated (Fig. 5C). It seemed that, unlike the cases of HP-β-CD and β-CD, the enantioselectivity of trimeprazine did not significantly increase with increasing γ-CD concentration from 1.6 to 8.0 mM while keeping the two enantiomers baseline separated (Fig. 5E). Beyond 8.0 mM of γ-CD, the resolution of the peaks of the two enantiomers of trimeprazine decreased. On the other hand, the peaks of the two enantiomers of promethazine and ethopropazine were barely resolved with 1.2 mM γ-CD (Fig. 5B); complete enantioseparation of promethazine and ethopropazine was achieved with 3.5 mM γ-CD (Fig. 5D). Similarly, the enantioselectivity of promethazine and ethopropazine increased slightly with increasing γ-CD concentration, reaching its maximum value at about 15.0 mM, and then decreased slightly with further increasing γ-CD concentration from 15.0 to 21.0 mM, while

keeping the two enantiomers of promethazine and ethopropazine baseline separated [25].

3.5 Effective enantioseparation

The concentration ranges of CDs for effective enantioseparation of those five phenothiazines, together with the observed optimal CD concentration ranges, are summarized in Table 1. As indicated, effective enantioseparation of promethazine, ethopropazine, trimeprazine, and thioridazine can be simultaneously achieved with γ-CD at a concentration in the range 2.5-6.0 mM. On the other hand, simultaneous and effective enantioseparation of promethazine, trimeprazine and thioridazine can be accomplished with β-CD only in a limited concentration range (0.1-0.3 mM).

For better understanding of the enantioseparation of each individual phenothiazine with various CDs, it is of interest to compare the observed and calculated optimal CD concentrations. The optimal CD concentrations ($C_{optimal}$) were calculated based on the equation $C_{optimal} = (K_1K_2)^{-1/2}$, where K_1 and K_2 are the binding constants of the two enantiomers of each individual phenothiazine. The optimal CD concentrations calculated for ethopropazine, trimeprazine and thioridazine with HP-β-CD were 1.4, 0.3 and 0.1 mM, respectively; those for promethazine, trimeprazine and thioridazine with β-CD were 0.8, 0.2 and 0.03 mM, respectively, and those for promethazine, ethopropazine, trimeprazine and thioridazine were 9.5, 5.8, 2.7 and 0.6 mM, respectively. The calculated optimal CD concentrations are in good agreement with the

observed values for these phenothiazines, except trimeprazine. The results clearly indicate that effective enantioseparation of phenothiazines occurs more readily with the use of CDs at concentrations less than 6 mM. This is particularly true for enantioseparation of trimeprazine and thioridazine with HP-β-CD and enantioseparation of promethazine, trimeprazine and thioridazine with β-CD.

4. Conclusion

Effective enantioseparation of promethazine, ethopropazine, trimeprazine and thioridazine in capillary zone electrophoresis was simultaneously achieved with cyclodextrins (CDs) as a chiral selector using a phosphate buffer at pH 3.0. Because phenothiazines interact strongly with CDs, the use of CDs at relatively low concentrations is sufficient to achieve effective enantioseparation. The results of the present investigation may also suggest that the separation of phenothiazine derivatives is advantageous using CDs as electrolyte modifiers.

Acknowledgement

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5. References

1. J. N. Delgado, W. A. Remers (Editors), Textbook of Organic Medicinal and Pharmaceutical Chemistry, Lippincott-Raven Publishers, Philadelphia, PA, 1998, p. 450.
2. S. Li, W. C. Purdy, *J. Pharm. Biomed. Anal.*, 49 (1991) 409.
3. S. Piperaki, A. Perakis, M. Parissi-Poulou, *J. Chromatogr. A*, 660 (1994) 339.
4. A. A. L. Van Overbeke, W. R. G. Baeyens, A. Beyaert, H. Y. Aboul-Enein, H. Oda, *J. Liq. Chromatogr.*, 20 (1997) 693.
5. E. Ameyibor, J. T. Stewart, *J. Liq. Chromatogr.*, 20 (1997) 855.
6. H. Makamba, V. Andrisano, R. Gotti, V. Cavrini, G. Felix, *J. Chromatogr. A.*, 818 (1998) 43.
7. D. T. Witte, R. A. de Aeeuw, B. F. H. Frenth, *J. High Resol. Chromatogr.*, 13 (1990) 569.
8. K. D. Altria, in H. Shintani, J. Polonsky (Editors), Handbook of Capillary Electrophoresis Applications, Blackie Academic & Professional, London, 1997, p. 334.
9. A. Aumatell, in H. Shintani, J. Polonsky (Editors), Handbook of Capillary Electrophoresis Applications, Blackie Academic & Professional, London, 1997, p. 345.
10. K. Otsuka, S. Terabe, in N. A. Guzman (Editor), Capillary Electrophoresis Technology, Marcel Dekker, New York, NY., 1993, p. 617.
11. G. N. Okafo, P. Camilleri, in P. Camilleri (Editor), Capillary

Electrophoresis, CRC press, Boca Raton, FL. 1993, p. 163.

12. G. Gubitz, M. G. Schmid, *Electrophoresis*, 21 (2000) 4112.
13. D. Wistuba, V. Schurig, *Electrophoresis*, 21 (2000) 4136.
14. S. Fanali, *J. Chromatogr. A*, 875 (2000) 89.
15. H. Shintani, J. Polonsky (Editors), Handbook of Capillary Electrophoresis Applications, Blackie Academic & Professional, London, 1997
16. N. A. Guzman (Editor), Capillary Electrophoresis Technology, Marcel Dekker, New York, NY., 1993
17. P. Camilleri (Editor), Capillary Electrophoresis, CRC press, Boca Raton, FL. 1993
18. M. G. Khaledi (Editor), High Performance Capillary Electrophoresis: theory, techniques and applications, John Wiley & Sons, New York, NY., 1998
19. A. Aumatell, R. J. Wells, *J. Chromatogr. B,* 669 (1995) 331.
20. P. G. H. M. Muijselaar, H. A. Claessens, C. A. Cramers, *J. Chromatogr. A,* 735 (1996) 395.
21. R. Y. Wang, X. N. Lu, M. J. Wu, E. K. Wang, *J. Chromatogr. B*, 721 (1999) 327.
22. S. Busch, J. C. Kraak, H. Poppe, *J. Chromatogr*. 635 (1993) 119.
23. X. X. Zhang, F. Hong, W. B. Chang, Y. X. Ci, Y. H. Ye, *Anal. Chim. Acta,* 392 (1999) 175.
24. T. de Boer, R. Bijma, K. Ensing, *J. Capillary Electrophor*., 5 (1998) 65.
25. K. H. Chen, C. E. Lin, (unpublished results).
26. Q. X. Guo, H. Y. Liu, X. Q. Ruan, X. Q. Zhang, Y. Y. Shi, Y. C. Liu, *J.*

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6. Figure Captions

Fig. 1. Structures of the phenothiazines studied.

Fig. 2. Electropherograms of the phenothiazines obtained with 40 mM phosphate buffer containing varied concentration of HP-β-CD at pH 3.0: (A) 0.1 mM, (B) 0.4 mM, (C) 1.2 mM, (D) 2.5 mM, (E) 6.0 mM, (F) 8.0 mM. Peak identification: 1=promethazine, 2=ethopropazine, 3=trimeprazine, 4=methotrimeprazine, 5=thioridazine.

Fig. 3. Electropherograms of the phenothiazines obtained with 40 mM phosphate buffer containing varied concentration of β-CD at pH 3.0 : (A) 0.02 mM, (B) 0.05 mM, (C) 0.1 mM, (D) 0.3 mM, (E) 2.0 mM, (F) 6.0 mM. Peak identification is the same as for Fig. 2.

Fig. 4. Electropherograms of phenothiazines obtained with 40 mM phosphate buffer containing a mixed CD composed of 0.3 mM β-CD and 1.4 mM HP-β-CD at pH 3.0. Peak identification is the same as for Fig. 2.

Fig. 5. Electropherograms of phenothiazines obtained with 40 mM phosphate buffer containing varied concentrations of γ-CD at pH 3.0 : (A) 0.5 mM, (B) 1.2 mM, (C) 1.6 mM, (D) 3.5 mM, and (E) 8.0 mM. Peak identification is the same as for Fig. 2.