Ching-Erh Lin
Wei-Ssu Liao
Kuo-Hsing Chen
Wann-Yin Lin

Department of Chemistry,
National Taiwan University,
Taipei, Taiwan

Influence of pH on electrophoretic behavior of phenothiazines and determination of pK_a values by capillary zone electrophoresis

The influence of buffer pH on the electrophoretic behavior of 13 structurally related phenothiazines and determination of pK_a values by capillary zone electrophoresis (CZE) were investigated. The results indicate that phenothiazines with a piperazine substituent behave quite differently from those with substituents having an aliphatic side chain or a piperidine moiety over the pH range studied. To separate these phenothiazines, it is preferable to select buffer pH in the range of 2.5–3.5. The pK_a values of phenothiazines with three different types of substituents attached at the 10-position of the phenothiazine ring were determined. The determination of pK_a values of phenothiazines allows us to rationalize the influence of buffer pH on the migration behavior of these compounds in CZE.

Keywords: Capillary zone electrophoresis / Phenothiazines / pK_a determination
DOI 10.1002/elps.200305559

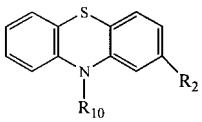
1 Introduction

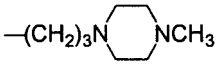
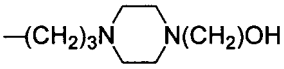
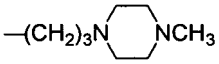
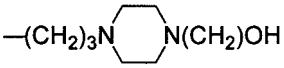
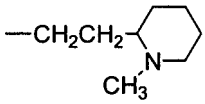
In the separation of charged analytes by capillary zone electrophoresis (CZE), buffer pH is a key parameter because it determines the extent of the ionization of analytes [1, 2]. For ionizable analytes with closely related structures, manipulation of buffer pH alone may not be able to achieve an effective separation. In this case, addition of cyclodextrins (CDs) or other electrolyte modifiers to an electrophoretic system is often considered to affect the selectivity. For better understanding of the interaction between the analytes and CDs, the binding constants of analytes to CDs are evaluated. However, when an ionizable analyte can simultaneously exist in two ionic forms in an electrophoretic system at a particular buffer pH, the evaluation of binding constants from the effective electrophoretic mobility of an ionizable analyte becomes complicated and difficult. Under these circumstances, a good knowledge of the pK_a values of phenothiazines is required [3].

Phenothiazines are a family of basic drugs with different substituents attached at the 2-position (R_2) and 10-position (R_{10}) of the phenothiazine ring. The R_{10} substituent is an alkyl piperazine group, a piperidine moiety, or an aliphatic side chain containing an amino group [3]. The structures of the 13 phenothiazines studied are depicted in Fig 1. These compounds are used as antipsychotic and neuroleptic agents or antihistamines. Applications of the CZE technique to the separation or enantioseparation of phenothiazines have previously been demonstrated [3, 5, 6, 8–15]. However, almost all of the work was performed at a particular buffer pH [6, 8–15]. Effective electrophoretic mobility of phenothiazines was measured only at four different buffer pH in the anionic mode with a reversed electroosmotic flow [5]. In our previous paper [3], the influence of buffer pH on the separation and migration behavior of phenothiazines was examined in a pH range limited between 2.5 and 5.5. Obviously, the influence of buffer pH on the separation and electrophoretic behavior of phenothiazine is rather incompletely explored.

Depending on the R_{10} substituent, there are two to three pK_a values for each individual phenothiazine derivative. In addition to the pK_a value of phenothiazine associating with the protonation-deprotonation at the nitrogen atom of the phenothiazine ring, one or two more pK_a values are related to the three different types of the R_{10} substituent, *i.e.*, one for phenothiazines with an aliphatic side chain containing an amino group or a piperidine moiety and two for phenothiazines with an alkyl piperazine group. In fact, despite of different types of the R_{10} substituent, only a single pK_a value for each individual phenothiazine, with the exception of fluphenazine and prochlorperazine, was found previously [5, 6, 16]. The pK_a values of phenothiazines with an alkyl piperazine group reported are in the range of 7.80–8.10, whereas those of phenothiazines with an aliphatic side chain containing an

Correspondence: Prof. C. E. Lin, National Taiwan University, Department of Chemistry, 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan
E-mail: celin@ccms.ntu.edu.tw
Fax: +886-2-23636359



Substituent	Phenothiazines	R_2	R_{10}
Piperazine	Trifluoperazine (**1**)	CF_3	
	Fluphenazine (**2**)	CF_3	
	Prochlorperazine (**4**)	Cl	
	Perphenazine (**5**)	Cl	
Aliphatic side chain	Promethazine[a] (**3**)	H	$-CH_2CH(CH_3)N(CH_3)_2$
	Ethopropazine[a] (**6**)	H	$-CH_2CH(CH_3)N(C_2H_5)_2$
	Promazine (**7**)	H	$-(CH_2)_3N(CH_3)_2$
	Triflupromazine (**8**)	CF_3	$-(CH_2)_3N(CH_3)_2$
	Acetopromazine (**9**)	$COCH_3$	$-(CH_2)_3N(CH_3)_2$
	Trimeprazine[a] (**10**)	H	$-CH_2CH(CH_3)CH_2N(CH_3)_2$
	Chlorpromazine (**11**)	Cl	$-(CH_2)_3N(CH_3)_2$
	Methotrimeprazine[a] (**12**)	OCH_3	$-CH_2CH(CH_3)CH_2N(CH_3)_2$
Piperidine moiety	Thioridazinen (**13**)	SCH_3	

a) Compounds possessing a chiral center

Figure 1. Structures of the 13 phenothiazines studied.

amino group are in the range of 9.00–9.58; and the pK_a value of thioridazine is 9.50. The first pK_a values reported for fluphenazine and prochlorperazine are 3.9 and 3.78, respectively [16]. With reference to the two pK_a values of piperazine [7] and of fluphenazine and prochlorperazine [16], the pK_a values reported for phenothiazines with a piperazine substituent in the range of 7.8–8.1 should be referred to the second protonated species of the piperazine moiety. Apparently, the pK_a values reported in the literature are inadequate, particularly for phenothiazines with an alkyl piperazine group. Moreover, it should be pointed out that as described in our previous study on the separation and migration behavior of structurally closed related phenotjiazines in cyclodextrin-modified CZE [3], the evaluation of the binding constants of phenothiazines with an alkyl piperazine substituent at pH 3.0 is hampered without knowing the pK_a value for the first protonated species of the piperazine moiety.

As capillary electrophoresis is a convenient method for precise pK_a determination [1, 2, 17–24], this technique can be applied to determine the pK_a values of phenothiazines. In this work, the electrophoretic mobility of phenothiazines as a function of buffer pH in the range of 2.5–11.5 is measured. The influence of pH on the migration behavior of phenothiazines in CZE is explored and the pK_a values of phenothiazines are determined.

2 Materials and methods

2.1 Apparatus

All CE separations were performed on a Beckman P/ACE System MDQ equipped with a photodiode array detector for absorbance measurements at 240 nm (Beckman Coulter, Fullerton, CA, USA). Uncoated fused-silica capil-

laries purchased from Polymicro Technologies (Phoenix, AZ, USA) were used. The dimensions of the capillary were 60.2 cm × 50 μm ID. 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

Thirteen 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°C. 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°C. 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 70 μA. 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:

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

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 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

3.1 Influence of buffer pH on the separation and migration behavior of phenothiazines

Figure 2 shows the variations of the electrophoretic mobility of phenothiazines as a function of buffer pH in the range 2.5–11.5 using a phosphate background electrolyte (40 mM). As can be seen, the electrophoretic mobility of phenothiazines with an alkyl piperazine substituent decreases drastically in the pH range 2.5–9.5, while the electrophoretic mobility of phenothiazines with substitu-

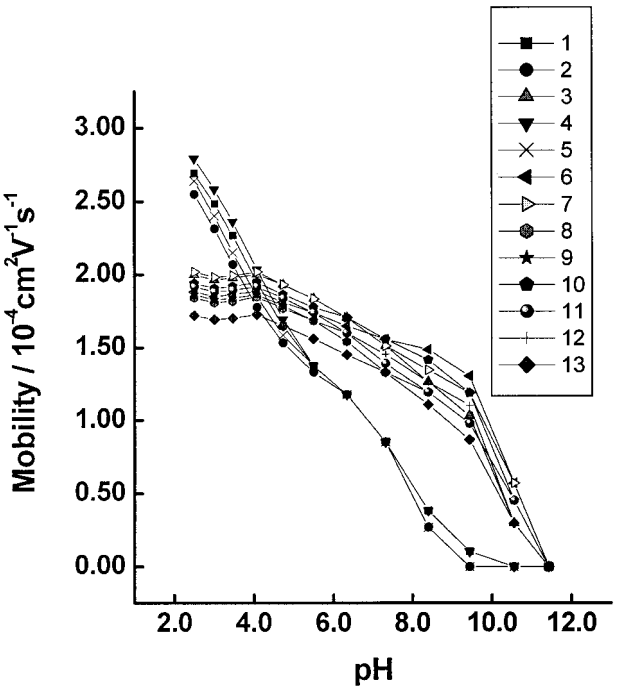


Figure 2. Variations of the electrophoretic mobility of phenothiazines as a function of buffer pH in the range of 2.5–5.5 using a phosphate buffer (40 mM). Capillary, 60.2 cm × 50 μm, ID; sample concentration, 10 μg/mL; detection wavelength, 240 nm; other operating conditions, 20 kV, 25°C. Curve identification: 1, trifluoperazine; 2, fluphenazine; 3, promethazine; 4, prochlorperazine; 5, perphenazine; 6, ethopropazine; 7, promazine; 8, triflupromazine; 9, acetopromazine; 10, trimeprazine; 11, chlorpromazine; 12, methotrimeprazine; 13, thioridazine. *Compounds possessing a chiral center.

ents having a piperidine moiety and an aliphatic side chain remains almost constantly in the pH range 2.5–4.0, decreases gradually in the range 4.0–9.5, then decreases drastically in the range 9.5–11.5. The separability of phenothiazines becomes worse at pH above 3.5 because phenothiazines with piperazine substituents may comigrate with phenothiazines with substituents having an aliphatic side chain at a pH in the range of 3.8–5.0 or merge together at a pH in the range of 5.5–7.5. Therefore, to separate these phenothiazines, it is preferable to selecte buffer pH in the range 2.5–3.5. In fact, complete separation of the 13 phenothiazines, together with the enantiomers of ethopropazine and trimeprazine, could be successfully achieved with addition of hydroxypropyl-β-CD (HP-β-CD) at 0.5 mM in a phosphate buffer at pH 3.0 [3]. The results also clearly indicate that phenothiazines with piperazine substituents behave quite differently from those with substituents having an aliphatic side chain and a piperidine moiety. The migration behavior of phenothiazines can be predicted, once the pK_a values and limiting electrophoretic mobility are known.

3.2 Determination of pK_a values of phenothiazines

The mobility curves of phenothiazines shown in Fig. 2 allow us to determine the pK_a values for each individual phenothiazine. The trial pK_a values of these phenothiazines can be determined from the inflection points of the electrophoretic mobility curves, provided that the curves (or the segments of the curves) are not too straight to determine the inflection point. As demonstrated previously [25], a simulated mobility curve which is best-fitted to the experimental mobility curve is first obtained through the utilization of Sigmoidal Fit of Microcal Origin software (Version 6.0) so that small differences in the electrophoretic mobility ($\Delta\mu$) for every small increment of buffer pH (ΔpH) in the pH range considered are calculated. The first or second derivatives of $\Delta\mu$ with respect to ΔpH is then plotted against buffer pH for each mobility curve and the pH value of the inflection point corresponding to the trial pK_a value is determined.

The determination of pK_a values of phenothiazines is actually based on the dependence of the effective electrophoretic mobility of an analyte on buffer pH. The effective electrophoretic mobility (μ_{eff}) of phenothiazines, excluding the ones with piperazine substituents, can be described by the following equation [23]:

$$\mu_{eff} = \frac{[H_3O^+]^2\mu_{BH_2^{2+}} + K_{a1}[H_3O^+]\mu_{BH^+}}{[H_3O^+]^2 + K_{a1}[H_3O^+] + K_{a1}K_{a2}} \quad (2)$$

where μ_{BH^+} and $\mu_{BH_2^{2+}}$ are the electrophoretic mobility of the first and second protonated species of an analyte, respectively. The two pK_a values and the two limiting mobilities of each individual phenothiazines are then determined by adjusting the trial values of these four parameters and curve-fitting the experimental mobility data with the predicted mobility curve as a function of buffer pH through the utilization of Microcal Origin software until

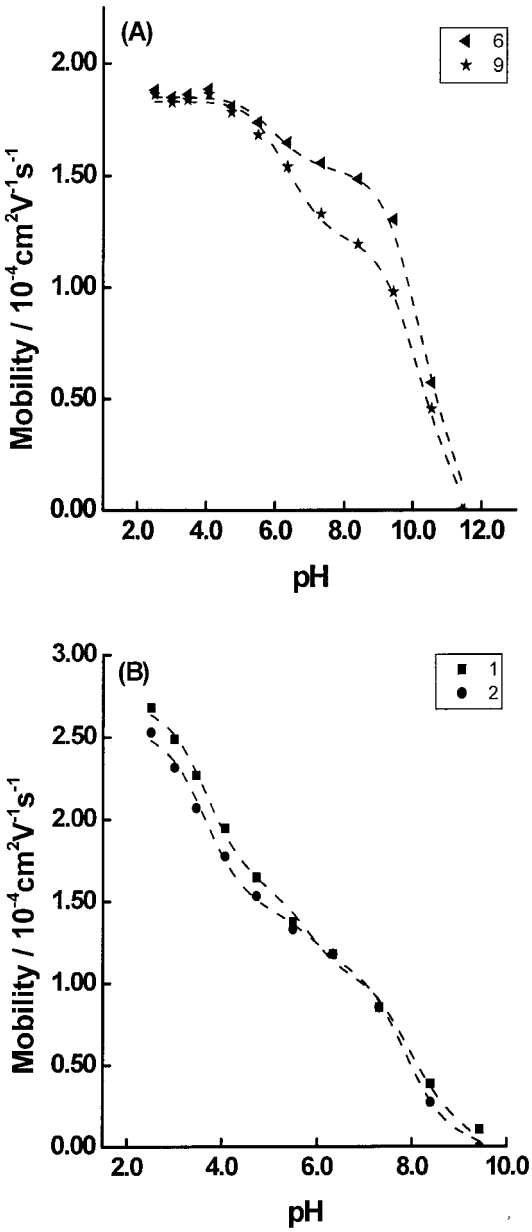


Figure 3. Agreement between the predicted mobility curves (represented by dashed lines) and observed mobility curves (shown by data points) for (A) phenothiazines with substituents having aliphatic side chain: (◀) ethopropazine and (★) acetopromazine, and (B) phenothiazines with piperazine substituents: (■) trifluoperazine and (●) fluphenazine.

Table 1. pK_a values and limiting mobility data of phenothiazines with aliphatic side chain and piperidine substituents

Phenothiazines		Literature values[a)]	pK_a values		Limiting mobility[b)]	
		pK_{a2}	pK_{a2}	pK_{a1}	μ_{BH^+}	$\mu_{BH_2^{2+}}$
Promethazine	(**3**)	9.1	10.00	6.50	1.33	1.96
Ethopropazine	(**6**)	9.6	10.30	6.00	1.52	1.85
Promazine	(**7**)	9.4	10.30	6.35	1.38	1.98
Triflupromazine	(**8**)	9.2	10.22	6.45	1.21	1.81
Acetopromazine	(**9**)	9.30	10.22	6.35	1.21	1.83
Trimeprazine	(**10**)	9.00	10.12	6.32	1.47	1.91
Chlorpromazine	(**11**)	9.30	10.10	6.40	1.26	1.87
Methotrimeprazine	(**12**)	9.2	10.05	6.50	1.32	1.80
Thioridazine	(**13**)	9.50	9.75	6.10	1.26	1.69

a) Literature values obtained from [5, 6, 16]
b) Mobility in $10^{-4}cm^2V^{-1}s^{-1}$

the best fit is obtained. For illustration, Fig. 3A shows the best fit of mobility curves for ethopropazine (**6**) and acetopromazine (**9**). As can be seen, the agreement between the predicted and observed mobility curves is very good. The pK_a values and limiting mobilities determined for these phenothiazines with substituents having a piperidine moiety or an aliphatic side chain are listed in Table 1.

The pK_a values determined for the protonated species resulting from the protonation at the nitrogen atom of the phenothiazine ring are in the range of 6.00–6.50, while those from the alkyl side chain are in the range of 10.00–10.30 and that from a piperidine group is 9.75. The pK_a values evaluated by the CZE method for phenothiazines with an alkyl side chain are reasonable, although they are greater than those reported in the literature by 0.7–1.1 pH unit. Since the literature values are not clear regarding the method and experimental conditions used, we are not in a position to make any further comments.

Similarly, for phenothiazines with piperazine substituents, the effective electrophoretic mobility can be described by the equation:

$$\mu_{eff} = \frac{[H_3O^+]^3\mu_{BH_3^{3+}} + K_{a1}[H_3O^+]^2\mu_{BH_2^{2+}} + K_{a1}K_{a2}[H_3O^+]\mu_{BH^+}}{[H_3O^+]^3 + K_{a1}[H_3O^+]^2 + K_{a1}K_{a2}[H_3O^+] + K_{a1}K_{a2}K_{a3}} \quad (3)$$

where $\mu_{BH_3^{3+}}$ is the electrophoretic mobility of the third protonated species of an analyte and K_{a1}, K_{a2}, and K_{a3} represent the three dissociation constants of the conjugated acids. The three pK_a values and three limiting mobilities of each individual phenothiazine with a piperazine substituent are then determined by adjusting the trial values of these six parameters and curve-fitting the experimental mobility data with the predicted mobility curve as a function of buffer pH through the utilization of Microcal Origin software until the best fit is obtained. For illustration, Fig. 3B shows the best fit of mobility curves for trifluoperazine (**1**) and fluphenazine (**2**). As can be seen, the agreement between the predicted and observed mobility curves is very good. The pK_a values and limiting mobilities determined for these phenothiazines with piperazine substituents are listed in Table 2.

Table 2. pK_a values and limiting mobility data of phenothiazines with piperazine substituents

Phenothiazines		Literature values[a)]		pK_a values			Limiting mobility[b)]		
		pK_{a3}	pK_{a1}	pK_{a3}	pK_{a2}	pK_{a1}	μ_{BH^+}	$\mu_{BH_2^{2+}}$	$\mu_{BH_3^{3+}}$
Trifluoperazine	(**1**)	8.1	–	8.15	5.85	3.68	1.00	1.60	2.70
Fluphenazine	(**2**)	8.1	3.9	7.93	5.95	3.65	1.07	1.45	2.55
Prochlorperazine	(**4**)	8.10	3.78	8.10	5.90	3.86	1.00	1.54	2.74
Perphenazine	(**5**)	7.8	–	7.90	5.90	3.60	1.05	1.54	2.65

a) Literature values obtained from [5, 6, 16]
b) Mobility in $10^{-4}cm^2V^{-1}s^{-1}$

The pK_a values determined for the protonated species resulting from the protonation at the nitrogen atom of the phenothiazine ring are in the range of 5.85–5.95. The two pK_a values evaluated for phenothiazines originating from the piperazine moiety are in the ranges of 3.60–3.86 and 7.90–8.15. The RSD values of the pK_a values determined for these phenothiazines are in the range of 2.5–4.5% (n = 4). The pK_{a3} values evaluated in this work are highly agreeable with those reported in the literature. Based on the fact that the two pK_a values reported are 5.33 and 9.73 for piperazine [7], 3.9 and 8.1 for fluphenazine, and 3.78 and 8.1 for prochlorperazine [16] and the differences in these two pK_a values are in the range of 4.2–4.4, we believe that the pK_a values determined in this work are quite reasonable because the differences in the pK_{a1} and pK_{a3} values determined for phenothiazines with piperazine substituents are in the range of 4.3–4.5.

In conclusion, precise pK_a values of phenothiazines were determined by applying CZE through the analysis of mobility curves with the aid of computer simulation. The determination of pK_a values of phenothiazines allows us to rationalize the influence of buffer pH on the migration behavior of these compounds in CZE and to evaluate the binding constants of phenothiazines with a piperazine group to CDs in the case of enantioseparation at low pH.

We thank the National Science Council of ROC in Taiwan for financial support.

Received October 14, 2002

4 References

[1] Smith, S. C., Khaledi, M. G., *Anal. Chem.* 1993, *65*, 193–198.

[2] Lin, C. E., Lin, W. C., Chiou, W. C., *J. Chromatogr. A* 1995, *705*, 325–333.

[3] Chen, K. H., Lin, C. E., Liao, W. S., Lin, W. Y., Hsiao, Y. Y., *J. Chromatogr. A* 2002, *979*, 399–408.

[4] Delgado, J. N., Remers, W. A. (Eds.),*Organic Medicinal and Pharmaceutical Chemistry*, Lippincott-Raven Publishers, Philadelphia, PA 1998.

[5] Muijselaar, P. G. H. M., Claessens, H. A., Cramers, C. A., *J. Chromatogr. A* 1996, *735*, 395–402.

[6] De Boer, T., Bijma, R., Ensing, K., *J. Capil. Electrophor.* 1998, *5*,65–71.

[7] Harris, D. C.,*Exploring Chemical Analysis*, Freeman and Company, New York 2001.

[8] Lin, C. E., Chen, K. H., *J. Chromatogr. A* 2001, *930*, 155–163.

[9] Wang, F., Khaledi, M. G., *Anal. Chem*. 1996, *68*, 3460–3467.

[10] Chankvetadze, B., Kartozia, I., Burjanadze, N., Bergenthal, D., Luftmann, H., Blaschke, G., *Chromatographia* 2001, *53*, S290–S295.

[11] Wang, R., Lu, X., Xin, H., Wu, M., *Chromatographia* 2000, *51*, 29–36.

[12] Wang, R. Y., Lu, X. N., Wu, M. J., Wang, E. K., *J. Chromatogr. B* 1999, *721*, 327–332.

[13] Busch, S., Kraak, J. C., Poppe, H., *J. Chromatogr.* 1993, *635*, 119–126.

[14] Zhang, X. X., Hong, F., Chang, W. B., Ci, Y. X., Ye, Y. H., *Anal. Chim. Acta* 1999, *392*, 175–181.

[15] Aumatell, A., Wells, R. J., *J. Chromatogr. B* 1995, *669*, 331–344.

[16] Hansch, C., Sammes, P. G., Taylor, T. B. (Eds.), *Comprehensive Medicinal Chemistry*, Pergamon Press, Oxford 1990.

[17] Beckers, J. L., Everaerts, F. M., Ackermans, M. T., *J. Chromatogr.* 1991, *537*, 407–428.

[18] Gluck, S. J., Cleveland Jr., J. A., *J. Chromatogr. A* 1994, *680*, 43–48.

[19] Cai, J., Smith, J. T., El Rassi, Z., *J. High Resolut. Chromatogr.* 1992, *15*, 30–32.

[20] Cleveland Jr., J. A., Benko, M. H., Gluck, S. J., Walbrohl, Y. M., *J. Chromatogr. A* 1993, *652*, 301–308.

[21] Lin, C. E., Chang, C. C., Lin, W. C., Lin, E. C., *J. Chromatogr. A* 1996, *753*, 133–138.

[22] Lin, C. E., Chang, C. C., Lin, W. C., *J. Chromatogr. A* 1997, *768*, 105–112.

[23] Lin, C. E., Chen, Y. T., *J. Chromatogr. A* 2000, *871*, 357–366.

[24] Barbosa, J., Barron, D., Jimenez-Lozano, E., *J. Chromatogr. A* 1999, *839*, 183–192.

[25] Lin, C. E., Chen, H. W., Lin, E. C., Lin, K. S., Huang, H. C., *J. Chromatogr. A* 2000, *879*, 197–210.