Journal of Chromatography, 644 (1993) 175–182
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 114

Influence of column temperature and physico-chemical properties on the electrophoretic behaviour of polyglycine peptides in free-solution capillary electrophoresis

Nong Chen*, Lei Wang and Yukui Zhang

National Chromatographic Research & Analysis Centre, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116012 (China)

(First received October 8th, 1992; revised manuscript received March 24th, 1993)

ABSTRACT

The changes in electromigration time with column temperature for polyglycine peptides were found to be due to temperature-induced viscosity changes of water. A quantitative linear relationship between the logarithm of migration times (log t_m) and the reciprocal of column temperature ($1/T$) was derived under a constant electric field strength. The slope of the plot of log t_m *vs.* $1/T$ was directly related to the activation energy of diffusion (AED). It was also found that the effect of column temperature on migration times is much more significant under constant-voltage than under constant-current operation. Polyglycine peptides differing only in size were chosen as model molecules for the test of the mobility model. Systematic correlations between t_m and the number of glycine amino acids (n) were made. A linear relationship between t_m and $n^{0.5}$ was found for polyglycine peptides under different operating modes. It was observed that the extrapolations of the linear relationships between t_m and $n^{0.5}$ with different column temperatures at constant voltage or with different applied currents at constant temperature cross each other at the same point. The parameters m_0 and m_1 in the equation $t_m = m_0 + m_1 n^{0.5}$ were correlated with the column temperature and the operating current. The activation energy of diffusion was also obtained for the plot of log m_1 *versus* $1/T$ at constant voltage. Linear relationships between m_0 and m_1 were found for these peptides which thus result in the intersection point in the plots of t_m *vs.* $n^{0.5}$.

INTRODUCTION

Capillary electrophoresis is a high-resolution technique and free-solution capillary electrophoresis (FSCE) has been demonstrated to be a very useful and operationally simple mode for the analysis of peptides and proteins [1]. Optimization of the electrophoretic behaviour of solutes such as peptides in FSCE requires a deeper understanding of the quantitative relationships between migration parameters and the operating parameters and the intrinsic physical properties of the peptides [2,3].

The column temperature of the capillary plays a significant role in the electrophoretic behaviour of the peptides. Good temperature control is therefore very important for migration reproducibility [4–6].

The separation mechanism in FSCE has been extensively studied [7–13]. The separation mechanism is based on the differential migration of solutes in an electric field due to differences in the charge-to-size ratio of the solutes. Subtle variations in this ratio allow the resolution of peptides with minute differences in structure. Few studies have been published that deal with

* Corresponding author.

0021-9673/93/$06.00

the correlation between migration times and solute physico-chemical properties in FSCE. This originates partly from the lack of a well developed theory for the expected relationships, necessitating simplifying assumptions. It also arises from the difficulty in the estimation of the charge and size of the peptides. Nyberg *et al.* [7] reported a linear correlation between relative migration times *versus* the molecular mass to the 2/3 power divided by the calculated charge (q) for a series of peptides analysed by capillary zone electrophoresis (CZE) in phosphate buffer. Deyl *et al.* [8] also reported a similar correlation for a series of seven cyanogen bromide cleavage fragments from collagens. Grossman and coworkers [9–11] established an empirical correlation for FSCE electrophoretic mobilities in a pH 2.50 buffer with the logarithm of the quantity $q+1$ divided by the number of amino acid residues to the 0.43 power for 40 different peptides. Rickard *et al.* [12] measured electrophoretic mobilities (μ) of several protein digests and correlated the results with different charge-to-size parameters, namely $q/M^{1/3}$, $q/M^{1/2}$ and $q/M^{2/3}$, where M = molecular mass. They found the best fit for μ *vs.* $q/M^{2/3}$.

In this work, the influence of column temperature on the electromigration times of polyglycines was studied. A quantitative relationship between migration times and column temperature is presented, with which the activation energy of diffusion (AED) was derived.

Hydrophilic peptides differing in only glycine amino acids were chosen as model molecules to study the quantitative relationships between migration times and the physico-chemical properties of the peptides. These peptides have been shown to have weak retention in RP-HPLC [14,15]. Systematic correlations of migration times with physico-chemical properties of polyglycine peptides were made using different operation modes.

EXPERIMENTAL

FSCE was conducted on a Spectra-Phoresis-500 system (Spectra-Physics Analytical, Fremont, CA, USA) with a 75 cm × 50 μm I.D. fused-silica capillary column. Prior to use, the column was conditioned with 0.5 M phosphate buffer at 60°C, 50 mM buffer at 20°C and 50 mM buffer at 20°C under a voltage of 25 kV. The column was washed with the separation buffer after each separation. Analytical-reagent grade NaH_2PO_4 was used. Peptides were purchased from Serva (Heidelberg, Germany). Water was doubly distilled. Ultraviolet absorption measurement at 200 nm was used for detection.

RESULTS AND DISCUSSION

Two electrokinetic phenomena, electrophoresis and electroosmosis, occur when an external electric field is applied across an electrolyte-filled capillary column. Electrophoresis is the movement of charged particles in response to the applied field.

The velocity of a particular ion (ν_{ep}) under the influence of an applied electric field strength E is approximately

$$\nu_{ep} = \mu_{ep}E \tag{1}$$

where E ($=V/L$) is the electric field strength, V the applied potential, L the length of the capillary and μ_{ep} the electrophoretic mobility of the ion.

The electrophoretic mobility of a particle is defined as the steady-state velocity per unit field strength. The electrophoretic mobility is of considerable importance in FSCE. The simplest way to describe it is to picture ions moving through the solvent at a steady-state velocity when the electric driving force is exactly balanced by the drag force. Hence, the electrophoretic mobility for spherical particles can be generally expressed by the following equation:

$$\mu_{ep} = q/6\pi\eta a \tag{2}$$

where q is the charge of the particle's ionic cloud, a is the hydrodynamic radius of the species and η is the viscosity of the buffer.

A more accurate mathematical treatment would include a numerical factor [$f(Ka)$]; thus μ_{ep} can be expressed as

$$\mu_{ep} = \frac{q}{6\pi\eta a} f(Ka) \tag{3}$$

where K the reciprocal of the analyte double-layer thickness and $f(Ka)$ depends on Ka of the analyte in the buffer.

In columns where the local electric field strength, E, and the zeta potential are constant and homogeneous throughout the entire column, the bulk electroosmotic velocity ν_{e0} can be expressed as

$$\nu_{e0} = \mu_{eo} E \tag{4}$$

where μ_{eo} is the electroosmotic mobility of buffer, which can be written as

$$\mu_{eo} = \frac{\varepsilon \zeta_c}{\eta} \tag{5}$$

where ζ_c is the zeta potential of the inner wall of the capillary column. The ηs in eqns. 3 and 5 are considered to be identical, hence the net migration equation is given by

$$\nu_m = \frac{E}{\eta}[(q/6\pi a)f(Ka) + \varepsilon\zeta_c] \tag{6}$$

where ν_m is the net migration velocity. Hence the migration time, t_m, can be expressed as

$$t_m = \frac{L\eta}{E\left[\zeta_c \varepsilon + \frac{q}{6\pi a} f(Ka)\right]} \tag{7}$$

The column temperature (T) can potentially be an important factor in FSCE. Precise control of the temperature is essential for obtaining adequate precision of the migration time. Examination of eqn. 7 reveals that no term except η is temperature dependent, the changes in ε, ζ_c and $f(Ka)$ as a function of T being negligibly small. Therefore, the changes in electromigration time with column temperature in FSCE are predominantly due to the temperature-induced viscosity changes of water. Viscosity has been extensively studied in electrochemistry. The viscosity of a liquid usually has a temperature dependence that is fairly well represented by an equation of the form [16]

$$\eta = A_0 \cdot 10^{\frac{\Delta E_a}{RT}} \tag{8}$$

where ΔE_a is the activation energy of diffusion, often of the order of one third of the heat of vaporization, and A_0 is the pre-exponential factor and is a constant often called the frequency factor.

Therefore, under constant electric field strength, the quantitative correlation between migration times (t_m) and column temperature is derived as

$$t_m = A \cdot 10^{\frac{\Delta E_a}{RT}} \tag{9}$$

where A is a constant. Therefore, under a constant field strength, a linear relationship between the logarithm of migration time and the reciprocal column temperature is derived:

$$\log t_m = \log A + B/T \tag{10}$$

where A (see eqn. 9) and B are constants and can be written as

$$A = A_0 L/E\left[\varepsilon\zeta_c + \frac{q}{6\pi a} f(Ka)\right] \tag{11}$$

$$B = \Delta E_a / R \tag{12}$$

Table I gives the values of log A, B, A and ΔE_a for five polyglycines under constant voltage operation. Correlation coefficients (r) are also given.

As can be seen from Table I, excellent correlations were observed for log t_m *versus* $1/T$. The data appear to fit a straight line with nearly the same slope; for $(gly)_3$, with a slope of $0.0716 \cdot 10^4$ K,

$$E_a = R \cdot \text{slope} = (8.314\ \text{J/mol} \cdot \text{K})(0.0716 \cdot 10^4\ \text{K})$$
$$= 5.953\ \text{kJ/mol}$$

The activation energies of diffusion do not change very much with the different peptides, showing the parallel electrophoretic behaviour of migration time *versus* column temperature for these peptides. Therefore, in the temperature range investigated, parallel electrophoretic behaviour in the plots of log t_m *vs.* $1/T$ was found, which indicates that the relative migration times or electrophoretic selectivity factors do not vary with the column temperature [6].

The activation energy of diffusion obtained in this paper is of the order of one sixth of the heat of vaporization of water. The heat of vaporization for water is about 40.6 kJ/mol. Therefore, the plots of log t_m *vs.* $1/T$ under constant electric

TABLE I

LOG A, B, ACTIVATION ENERGY OF DIFFUSION (ΔE_a) AND PRE-EXPONENTIAL FACTOR (A) FOR FIVE POLYGLYCINE PEPTIDES

At constant voltage, 25 kV; column, 75 cm × 75 μm I.D., fused silica; buffer, 50 m*M* phosphate buffer (pH 2.5). Migration times used for regression were taken from ref. 17.

Peptide	log A	B	r	A	ΔE_a(kJ/mol)
$(Gly)_2$	−1.478	724.70	0.9989	0.0333	6.03
$(Gly)_3$	−1.397	716.00	0.9987	0.0401	5.95
$(Gly)_4$	−1.398	730.24	0.9988	0.040	6.07
$(Gly)_5$	−1.388	738.00	0.9989	0.0409	6.14
$(Gly)_6$	−1.390	747.30	0.9987	0.0407	6.21

field strength offer a method for the calculation of the activation energy of diffusion in FSCE. The pre-exponential factor has little effect on the temperature dependence of the migration time.

Under constant-current operation, if the voltage does not change significantly with column temperature, an approximately linear relationship between log t_m and $1/T$ can also be found. However, the linear correlations of log t_m *versus* $1/T$ are not better with constant-current operation. The regression results for the five peptides at constant current are as follows:

$(gly)_2$: $\log t_m = 158.94/T + 0.470$ $(r = 0.9739)$

$(gly)_3$: $\log t_m = 157.06/T + 0.531$ $(r = 0.9713)$

$(gly)_4$: $\log t_m = 158.96/T + 0.573$ $(r = 0.9712)$

$(gly)_5$: $\log t_m = 159.07/T + 0.610$ $(r = 0.9693)$

$(gly)_6$: $\log t_m = 157.12/T + 0.647$ $(r = 0.9641)$

There are at least two aspects that should be stated explicitly when examining the influence of temperature on migration times at constant applied current (I). First, at the constant I, an increase in temperature would increase the mobility by decreasing the buffer viscosity, whereas as the temperature is increased the solution resistance decreases and the voltage required to maintain a constant current would decrease, which could counteract the effect of increased mobility with increasing temperature. In contrast, at constant voltage (E = constant), an increase in temperature would increase the mobility by decreasing the viscosity, which results in a decrease in migration times. Therefore, the effect of column temperature on migration times is much more significant under constant-voltage than under constant-current operation in FSCE. Therefore, the temperature effect in FSCE is different from that in RP-HPLC [18].

Figs. 1 and 2 show the electropherograms of these peptides under constant-voltage and constant-current operation.

In order to explore the relationships between the peptides' migration and physico-chemical parameters in FSCE, peptides differing only in glycine amino acids were chosen as model mole-

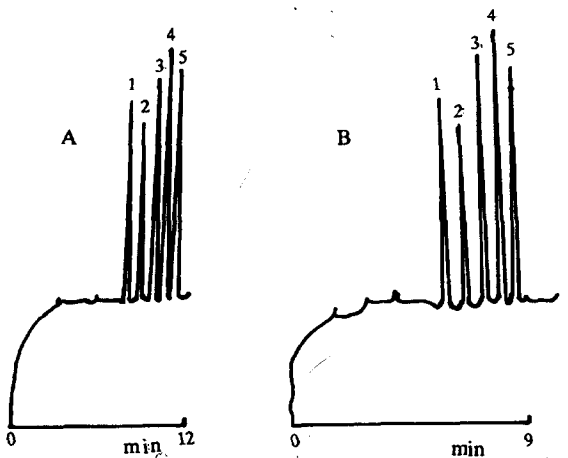

Fig. 1. Electropherograms of five hydrophilic peptides at applied column temperatures of (A) 30°C and (B) 50°C at constant voltage (25 kV). Peaks: 1 = $(gly)_2$; 2 = $(gly)_3$; 3 = $(gly)_4$; 4 = $(gly)_5$; 5 = $(gly)_6$.

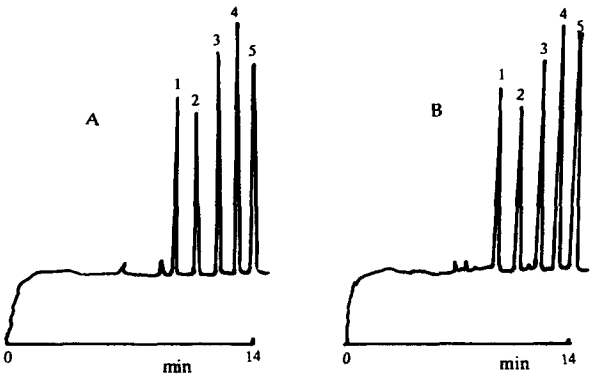

Fig. 2. Electropherograms of five peptides at the applied column temperatures of (A) 30°C and (B) 50°C at constant current (80 μA). Peaks: 1 = $(gly)_2$; 2 = $(gly)_3$; 3 = $(gly)_4$; 4 = $(gly)_5$; 5 = $(gly)_6$.

cules to study the mobility model. As the charges for these species are nearly identical, this series could be a good test for a mobility model to characterize the electrophoretic systems. They are much like homologous series in chromatography.

As the separation was performed at pH 2.5, the electroosmotic flow was negligible. The migration times were therefore not corrected for electroosmotic flow in this present study.

From eqn. 2, the electrophoretic mobility is directly related to the radius of the species. To investigate the dependence of migration times on peptide size, these peptides were treated as a classical polymer in solution. For an unperturbed random coil, the simplest model of a freely joined chain can be used. The hydrodynamic radius is given by

$$a = bn^{0.5} \tag{13}$$

where b is the apparent size of an individual monomer unit and n the number of single units in the polymer, here referring to the number of amino acid residues.

For a series of polyglycines, the net charges are equal according to the Henderson–Hasselbach equation [19]. Therefore, for the equally charged polyglycine peptides, substituting eqn. 13 into eqn. 2, the migration times have the following linear relationship with respect to n:

$$t_m = m_0 + m_1 n^{0.5} \tag{14}$$

where m_0 and m_1 are constants; m_0 is the extrapolated t_m value for $n = 0$ and the slope m_1 is

$$m_1 = 6\pi b\eta L/qE \tag{15}$$

In order to test the validity of eqn. 14, migration times of these peptides in different operating modes were plotted as functions of operating parameters in FSCE. The results are shown in Figs. 3–5 and in Tables II and III.

Table II shows the variation of the migration times of (gly)n peptides with the column temperature under constant-current operation in FSCE. Table III shows the variation of t_m of these peptides with the different applied currents at constant temperature for these peptides. The parameters m_0 and m_1 and the correlation coefficients r are also given.

As can be seen from these tables, the correlation coefficients of the linear fit all approached 1.0. Therefore, for a series of equally charged polyglycines, linear relationships between t_m and $n^{0.5}$ were obtained, and this correlation holds

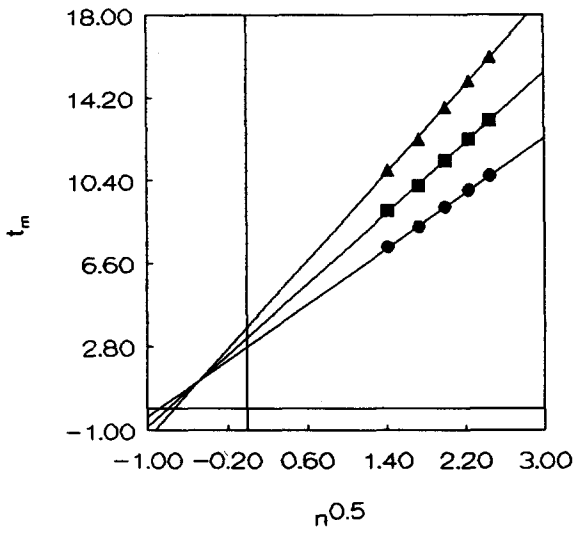

Fig. 3. Linear plots of t_m *versus* $n^{0.5}$ for a series of polyglycines at constant voltage at different column temperatures: ▲ = 15; ■ = 25; ● = 35°C. Electrophoretic conditions as in Table I. Regression results: (▲) $t_m = 3.66 + 5.05n^{0.5}$ (r = 0.9995); (■) $t_m = 3.21 + 4.05n^{0.5}$ (r = 0.9995); (●) $t_m = 2.80 + 3.19n^{0.5}$ (r = 0.9996).

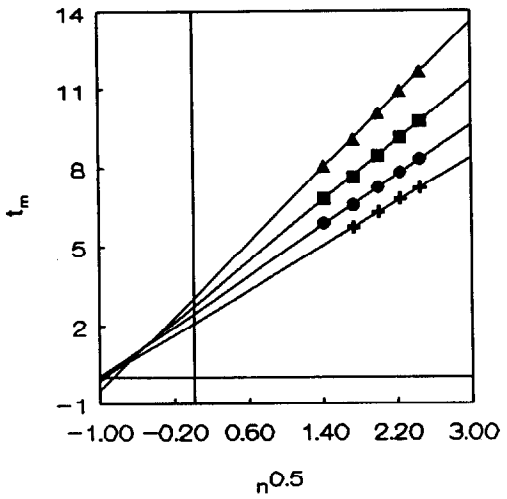

Fig. 4. Linear relationships between t_m and $n^{0.5}$ for hydrophilic peptides at constant voltage at different column temperatures: ▲ = 30; ■ = 40; ● = 50; + = 60°C. Other conditions as in Table I. Regression results: (▲) $t_m = 3.02 + 3.52n^{0.5}$ ($r = 0.9994$); (■) $t_m = 2.71 + 2.87n^{0.5}$ ($r = 0.9996$); (●) $t_m = 2.44 + 2.40n^{0.5}$ ($r = 0.9996$); (+) $t_m = 2.07 + 2.11n^{0.5}$ ($r = 0.9999$).

even with the use of different column temperatures and different applied currents (see Figs. 3–5).

Figs. 3 and 4 demonstrate a set of linear relationships between migration times and $n^{0.5}$

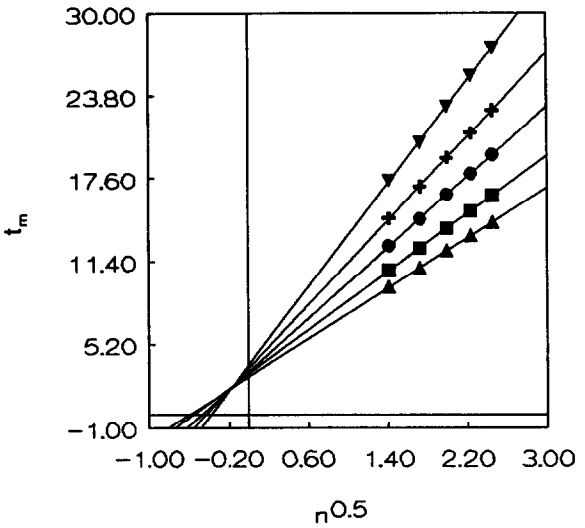

Fig. 5. Linear correlations of t_m *versus* $n^{0.5}$ for five peptides at constant temperature with different applied currents: ▲ = 100; ■ = 90; ● = 80; + = 70; ▼ = 60 μA. For electrophoretic conditions and correlation results, see Table III.

for the five polyglycines with different temperatures at constant voltage. Fig. 5 illustrates a series of straight lines for t_m *versus* $n^{0.5}$ with different applied currents at constant temperature.

As can be clearly seen, the best linear correlations were obtained when the migration times were plotted against $n^{0.5}$. The plots show good linear correlations.

The slope (m_1) at constant voltage, which mainly characterizes the viscosity of the running buffer, decreases with increasing column temperature for the peptides. Hence m_1 can also be written as

$$m_1 = A_1 \cdot 10^{\frac{\Delta E_a}{RT}} \quad (16)$$

and a linear correlation between log m_1 and $1/T$ is obtained under a constant electric field strength:

$$\log m_1 = \log A_1 + B_1/T \quad (17)$$

where $A_1 = 6\pi bLA_0/qE$ and $B_1 = \Delta E_a/R$. Therefore, the slope m_1 in the plot of t_m *vs.* $n^{0.5}$ can also be directly related to the activation energy of diffusion. A linear log m_1 *vs.* $1/T$ plot has been obtained with a slope of 826.15 K (see Fig. 6). Therefore, ΔE_a calculated from the plot of log m_1 *versus* $1/T$ is 6.87 kJ/mol, which is higher than that obtained from log t_m *vs.* $1/T$ plots.

It can be seen from Tables II and III that a non-zero intercept was found in plots of t_m *versus* $n^{0.5}$. The intercept represents extrapolated values of t_m for $n = 0$ species, and its non-zero value probably reflects the migration properties of this series of peptides at $n = 0$. Under the given operating conditions, this value is constant. From the regression results in Figs. 3 and 4, the log m_0 values are quantitatively correlated with the reciprocal of column temperature, which implies that there is a linear correlation between m_1 and m_0 under constant-voltage operation. The regression equations for log m_1 and log m_0 *versus* $1/T$ are

$$\log m_0 = 510.10/T - 1.202 \ (r = 0.9951)$$

$$\log m_1 = 826.15/T - 2.168 \ (r = 0.9973)$$

With different applied currents at constant temperature, the reciprocals of m_0 and m_1 are

TABLE II

EFFECT OF COLUMN TEMPERATURE ON MIGRATION TIMES AT CONSTANT APPLIED CURRENT AND THE PARAMETERS m_0 AND m_1 FOR FIVE PEPTIDES

Constant current, 80 μA; other conditions as in Table I.

Temperature (°C)	Migration time (min)					m_0	m_1	r
	$(Gly)_2$	$(Gly)_3$	$(Gly)_4$	$(Gly)_5$	$(Gly)_6$			
20	10.18	11.56	12.93	14.10	15.13	3.29	4.83	0.9996
25	9.92	11.23	12.54	13.65	14.63	3.37	4.59	0.9996
30	10.00	11.34	12.66	13.80	14.80	3.33	4.67	0.9996
35	9.66	10.97	12.26	13.36	14.33	3.18	4.55	0.9996
40	9.63	10.94	12.22	13.33	14.35	3.08	4.58	0.9996
50	9.17	10.42	11.64	12.68	13.63	2.99	4.33	0.9997
60	8.72	9.9	11.04	12.03	12.91	2.91	4.07	0.9997

linearly related to the applied current, which indicates that there is a linear m_0 *vs.* m_1 plot (see Fig. 7). The regression equations for $1/m_0$ and $1/m_1$ and the current are

$$1/m_1 = 0.00276I - 0.0652 \quad (r = 0.9977)$$

$$1/m_0 = 00205I + 0.150 \quad (r = 0.9985)$$

where I is the applied current.

The linear correlation for m_0 *vs.* m_1 indicates that the extrapolation of the t_m *vs.* $n^{0.5}$ plots for polyglycines at different temperatures at constant voltage or with different currents at constant temperature leads to a common intersection point, characterizing another feature of these peptides (see Figs. 3–5). Further elucidation to understanding this convergence point is required.

CONCLUSIONS

Logarithms of migration times in FSCE are quantitatively correlated with the reciprocal of column temperature under a constant electric field strength. The slope of the log t_m *vs.* $1/T$ plots has been directly correlated with the activation energy of diffusion. The electromigration

TABLE III

VARIATION OF THE MIGRATION TIMES WITH APPLIED CURRENT AT CONSTANT COLUMN TEMPERATURE AND COEFFICIENTS m_0 AND m_1 FOR FIVE PEPTIDES

Constant column temperature, 20°C; other conditions as in Table I.

I(μA)	Migration time (min)					m_0	m_1	r
	$(Gly)_2$	$(Gly)_3$	$(Gly)_4$	$(Gly)_5$	$(Gly)_6$			
100	9.48	10.91	12.21	13.33	14.32	2.82	4.70	0.9999
90	10.71	12.35	13.86	15.15	16.30	3.01	5.42	0.9999
80	12.56	14.56	16.39	17.96	19.39	3.16	6.62	0.9999
70	14.60	16.97	19.15	21.03	22.72	3.41	7.87	0.9999
60	17.43	20.32	22.99	25.31	27.40	3.68	9.67	0.9999

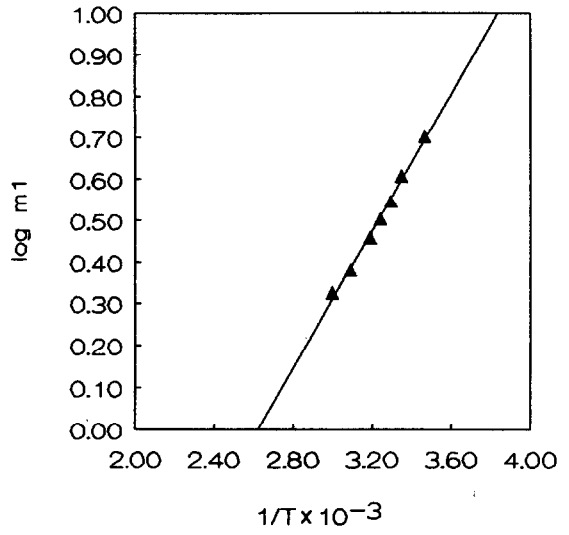

Fig. 6. Linear plot of log m_1 in the equation $t_m = m_0 + m_1 n^{0.5}$ *versus* the reciprocal of column temperature ($1/T$). For other conditions, see Table I. Parameters m_0 and m_1 were taken from Fig. 3 and 4. The regression equation is log m_1 = $(867.15/T) - 2.168$ ($r = 0.9973$).

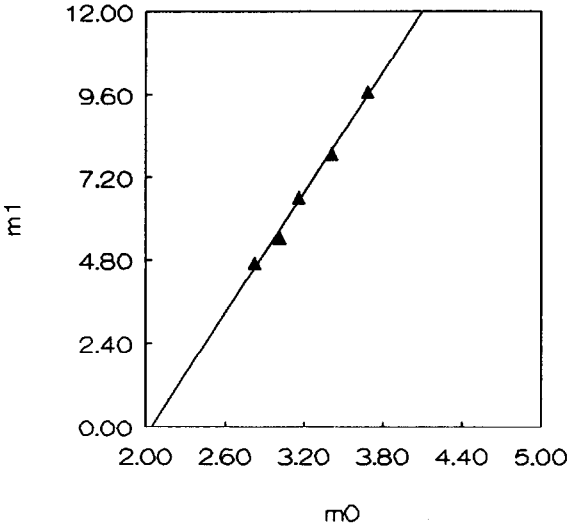

Fig. 7. Linear relationship between the parameters m_1 and m_0 in the equation $t_m = m_0 + m_1 n^{0.5}$ at constant column temperature with different applied currents. For details, see Table III. The regression equation is $m_1 = 5.85 m_0 - 11.97$ ($r = 0.9964$).

times can be correlated from the physico-chemical properties of these peptides. The proposed linear relationships between t_m and $n^{0.5}$ for five polyglycines are very useful for elucidating the migration mechanism in FSCE. The application of this knowledge can then be combined with other basic relationships to develop optimized separation methods using FSCE.

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