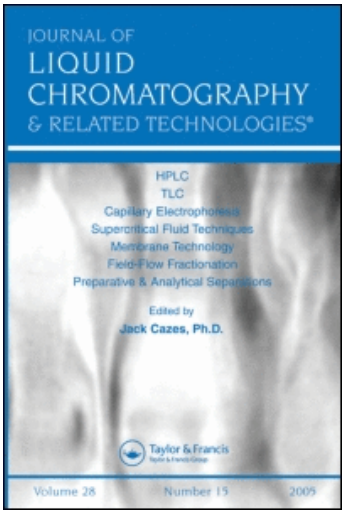

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Study on Migration Behavior of Organic Acids of Low Molecular Weights in Capillary Zone Electrophoresis

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STUDY ON MIGRATION BEHAVIOR OF ORGANIC ACIDS OF LOW MOLECULAR WEIGHTS IN CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

An equation to describe the migration behavior of organic acids of low molecular weight in capillary zone electrophoresis(CZE) is derived based on the knowledge of the electric chemistry and the theory of acid-base equilibrium. The equation can be readily applied to predicted the migration order for organic acids in CZE. The predictions using the equation agree well with the experiment results by us and other workers. Also, the equation may be used for the estimation of electroosmotic mobility and the calculation of the dissociation constants for acids.

INTRODUCTION

As far back as the 1960s, high performance capillary electrophoresis (HPCE) was first introduced by Hjerten.[1] Afterwards, Mikkers research group[2,3] and Jorgenson and Lukacs[4,5] developed this technique. Now it has become an important analytical separation technique,[6,7] because of its efficiency, flexibility and accuracy.

Recently, capillary zone electrophoresis has proven very effective for the separation and determination of weak organic acids or bases of low molecular weights. Lin and co-workers[8] have separated aromatic and thiazole compounds, including benzotriazole, 5-tolyltriazole, dimethylbenzotriazole, and 2-mercaptobenzothiazole by capillary zone electrophoresis. Turnes and co-workers[9] have described the separation of pentachlorophenol and other mono-, di-, tri- and tetrachlorinated phenols from variour compounds. The separation of B vitamins was reported by Huopalahti,[10] while Bruin et al.[11] has separated 7 different amino acids using indirect detection. Capillary zone electrophoresis using phthalate ion as a carrier electrolyte has been employed in the separation of oxalate, tartrate, malate, succinate, lactate, acetate, propionate, butyrate, and caprylate anions.[12] The use of capillary electrophoresis in the separation of ephedrine alkaloids has also been reported.[13]

In addition, it has been demonstrated that capillary electrophoresis is useful in the determination of the dissociation constants of acids and bases. Some authors[14,15] have proposed a formula to determine pK_α values by CZE. However, the formula contained many parameters, such as ionic radii and activity coefficients, which were not readily available for the majority of compounds, making the application in most cases difficult or even impossible. Afterwards, Smith and Khaledi[16] proposed a simple formula to describe the relationship between the mobilities and dissociation constants of analytes. Although it has been successfully used in the determination of the dissociation constants for analytes by CZE,[17,18] as yet that formula has not been proven theoretically.

While much work has been done to determine the influence of various factors on migration times and apparent mobility, our laboratory has developed a model to evaluate the influence of sample injection time, in stacking and non-stacking modes, on the migration time.[19] The effect of pH and ionic strength of the buffer on the migration behavior of peptides was studied by Cifuentes and Poppe.[20] Cordier and co-workers[21] reported the use of computer-aided prediction of the migration times. However, little attention has been devoted to describing the relationship among migration times of organic acids or bases in capillary electrophoresis and their dissociation constants.

The main aim of this paper is to demonstrate a new relationship between the migration times for organic acids and their dissociation constants and molecular weights in CZE. This relationship was successfully used to predict the migration order of organic weak acids in CZE, estimate the electroosmotic flow mobility, and calculate dissociation constants.

THEORY

The electrophoretic mobility of an analyte is readily measured from an electropherogram as[22]

$$\mu_{app} = \mu_{ep} + \mu_{eo} \tag{1}$$

and

$$\mu_{app} = \frac{L \cdot l}{V \cdot t_M} \tag{2}$$

where μ_{app} is the apparent mobility of the analyte, L and l denote the total and effective length of the capillary, respectively, V the applied voltage and t_M the migration time of analyte. Further, μ_{eo} is the mobility of the electroosmotic flow, which is calculated from the migration time of a neutral compound (t_{eo}) according to the following formula:[22]

$$\mu_{eo} = \frac{L \cdot l}{V \cdot t_{eo}} \tag{3}$$

From 2 and 3, Eq.1 can be rearranged as

$$\frac{L \cdot l}{V \cdot t_M} = \mu_{ep} + \frac{L \cdot l}{V \cdot t_{eo}} \tag{4}$$

When a charged particle is placed in an electric field (E), it experiences a force which is proportional to its effective charge *(q)* and the electric field strength. Thus, this particle will get an acceleration. According to Newton's second law, we have:

$$m\frac{dv}{dt} = q \cdot E - f \cdot v \tag{5}$$

where m represents the mass of the particle, dv/dt is acceleration, q is the charge number of the particle, E is the electric field strength and f translational friction coefficient. However, the velocity of this particle cannot increase forever and a stable equilibrium quickly sets up. Thus Equation 5 can be derived as follows:

$$q \cdot e = f \cdot v \tag{6}$$

If the particle is a spheroid, we will substitute Stokes law[22]

$$f = 6\pi\eta R \tag{7}$$

into Eq.6, we get

$$q/(6\pi\eta R) = v/E \tag{8}$$

Based on the fundamental knowledge of electric chemistry, we can get

$$\mu_{ep} = \frac{v}{E} \tag{9}$$

For a spherical particle, the relationship between radius and mass exist

$$m = \rho \cdot (\frac{4}{3}\pi R^3) \tag{10}$$

where ρ is the atomic density of the particle. Thus, from Eq.8, 9 and 10, we get

$$\mu_{ep} = A\frac{q}{\sqrt[3]{m}} \tag{11}$$

and

$$A = \frac{1}{6\pi\eta(\frac{3}{4\rho\pi})^{\frac{1}{3}}} \tag{12}$$

In general , the charge of a simple ion can be expressed as[24]

$$q = z \cdot e \tag{13}$$

where e is the charge of an electron and z is the valence of the ion. In an acid-base equilibrium system, the concentrations of the acid and its conjugate base vary with the concentration of H^+ in solution. The ratio of acid or base concentration to the total concentration is defined as molar fraction, expressed as δ. For example, the molar fractions δ_0 and δ_1 for a monoacid can be given by:[25]

$$\delta_0 = \frac{[HA]}{C} = \frac{[H^+]}{[H^+] + K\alpha} \tag{14}$$

and

$$\delta_1 = \frac{[A^-]}{C} = \frac{K\alpha}{[H^+] + K\alpha} \tag{15}$$

For a binary acid molecule, the molar fractions δ_0,δ_1 and δ_2 can be obtained by:

$$\delta_0 = \frac{[H_2A]}{C} = \frac{[H^+]^2}{[H^+]^2 + K\alpha1 \cdot [H^+] + K\alpha1 \cdot K\alpha2} \tag{16}$$

$$\delta_1 = \frac{[HA^-]}{C} = \frac{K\alpha1 \cdot [H^+]}{[H^+]^2 + K\alpha1 \cdot [H^+] + K\alpha1 \cdot K\alpha2} \tag{17}$$

$$\delta_2 = \frac{[A^{2-}]}{C} = \frac{K\alpha1 \cdot K\alpha2}{[H^+]^2 + K\alpha1 \cdot [H^+] + K\alpha1 \cdot K\alpha2} \tag{18}$$

Therefore, the average charges of the monoacid or binary acid separated by capillary electrophoresis can be calculated according to the following equations:

$$q_{mono} = (\delta_0 \times 0 + \delta_1 \times 1) \cdot e = \delta_1 \cdot e \tag{19}$$

and

$$q_{binary} = (\delta_0 \times 0 + \delta_1 \times 1 + \delta_2 \times 2) \cdot e = \delta_1 \cdot e + 2\delta_2 \cdot e \tag{20}$$

It should be noted that the negative sign of the charge is omitted in calculation. In general, if an organic molecule contains n dissociable hydrogens, its charge can be expressed as:

$$q = e \cdot \frac{\sum_{i=0}^{n} \left(i \cdot [H^+]^{n-i} \cdot \prod_{j=0}^{i} K_{\alpha j} \right)}{\sum_{i=0}^{n} \left([H^+]^{n-i} \cdot \prod_{j=0}^{i} K_{\alpha j} \right)} \qquad (21)$$

with $K_{\alpha 0} \equiv 1$

where $K_{\alpha j}$ represents the dissociation constant of the jth grade of the organic acid. Substituting Eq.11 and Eq.21 into Eq. 4, we find

$$\frac{L \cdot l}{V \cdot t_M} = A \cdot \frac{e}{\sqrt[3]{m}} \cdot \lambda + \frac{L \cdot l}{V \cdot t_{eo}} \qquad (22)$$

where

$$\lambda = \frac{\sum_{i=0}^{n} (i \cdot [H^+]^{n-i} \cdot \prod_{j=0}^{i} K_{\alpha j})}{\sum_{i=0}^{n} ([H^+]^{n-i} \cdot \prod_{j=0}^{i} K_{\alpha j})} \qquad (23)$$

This relationship suggests that the reciprocal of the migration time is approximately proportional to $\lambda/(MW)^{1/3}$. However, the actual situation is more complicated when the medium is a conducting solvent and the moving particle is surrounded by an ionic atmosphere of the opposite charge. From a purely inorganic chemistry viewpoint, an anion is more likely to deform than an ion under the influence of an electric field.[26] On the assumption that the shape of the molecule changes into cylinder from spheroid under the influence of an electric field and its volume is constant, we have

$$h \cdot \pi \cdot r^2 = \frac{4}{3} \cdot \pi \cdot R^3 \qquad (24)$$

where r and *h* are the radius and the height of the cylinder, respectively. If the friction on the deformed molecule is equal to that on a spherical molecule with radium r, by combination Eqs. 24, 7, and 10, we get

$$\mu_{ep} = B \cdot \frac{\lambda}{m^{0.5}} \qquad (25)$$

Table 1

Dissociation Constants, Molecular Weights and Migration Times for 8 Organic Acids

Organic Acid	Molecular Weight	pK^a_{a1}	pK^a_{a2}	Migration Time[b]/Min
Oxalic	90.04	1.23	4.19	2.633
Tartaric	150.09	2.98	4.34	3.000
Malic	134.09	3.40	5.11	3.267
Succinic	118.09	4.10	5.61	3.633
Lactic	90.08	3.08		3.780
Acetic	60.06	4.75		3.900
Butyric	88.11	4.81		4.253
Propionic	74.08	4.87		4.300

[a] Data from Reference 27.
[b] Data from Reference 12.

with B defined by Eq.26:

$$B = \frac{\rho}{6 \cdot \eta} \cdot \sqrt{\frac{h}{\pi}} \tag{26}$$

The mass of the individual molecule (m) is equal to the ratio of its molecular weight to Avogadro constant(N_0).Consequently, Eq. 23 becomes

$$\frac{L \cdot l}{V \cdot t_M} = B \cdot \frac{e}{(MW)^{0.5}} \cdot \frac{\sum_{i=0}^{n} (i \cdot [H^+]^{n-i} \cdot \prod_{j=0}^{i} K\alpha j)}{\sum_{i=0}^{n} ([H^+]^{n-i} \cdot \prod_{j=0}^{i} K\alpha j)} + \frac{L \cdot l}{V \cdot t_{eo}} \tag{27}$$

If h in Eq.26 is only dependent on the applied voltage, the reciprocal of the migration time is linearly correlated with $\lambda/m^{0.5}$. It is found that the major difference between Eq. 22 and Eq. 27 lies in the exponent term of the m^x term. The exponent, x, is concerned with the degree of deformation experienced by the analyte molecule. In order to determine the proper value for x, we have correlated the reciprocal of migration time listed in Table 1 with λ/m^x for

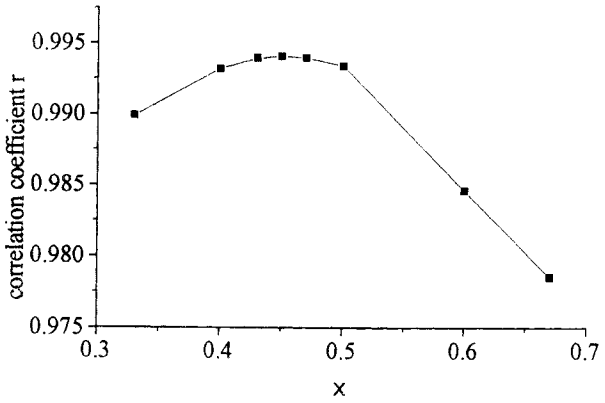

Figure 1. The plot of the correlation coefficient r as a function of exponent x in $(MW)^x$ term. Data of the migration times for 8 organic acids was from Ref. 12. Capillary: untreated fused silica, 70cm ×75 μm. Injection:1 to 5 sec electrokinetic,-10kV. Buffer: phthalate/tetradecyltrimethyl ammonium bromide/2-(N-morpholino)-ethanesul-fonic acid. Voltage:-30 kV. Temperature:20 °C.

different x values. It is found from Figure 1 that the best fit, as determined by the largest value of the correlation coefficient, is with $\lambda/m^{0.45}$. Figure 2 gives a plot of the reciprocal of migration time vs. $\lambda/m^{0.45}$. The plot was linear with a correlation coefficient of > 0.994.

Therefore, the following relationship can be obtained

$$\frac{L \cdot l}{V \cdot t_M} = F \cdot \frac{e}{(MW)^{0.45}} \cdot \frac{\sum_{i=0}^{n} (i \cdot [H^+]^{n-i} \cdot \prod_{j=0}^{i} K\alpha j)}{\sum_{i=0}^{n} ([H^+]^{n-i} \cdot \prod_{j=0}^{i} K\alpha j)} + \frac{L \cdot l}{V \cdot t_{EO}} \qquad (28)$$

where F is mainly dependent on the viscosity of the electrolyte solute, the applied voltage and the molecular structure of the analyte.

Equation 28 is the final equation describing the relationship among migration time, dissociation constant and molecular weight.

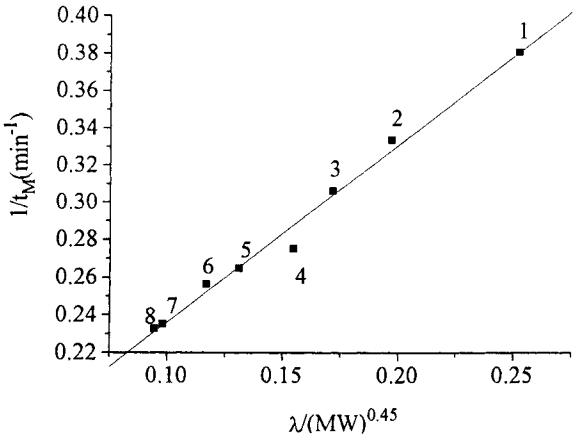

Figure 2. Migration time for 8 organic acids versus $\lambda/(MW)^{0.45}$. Solution: 1=Oxalic acid, 2=tartaric acid, 3=malic acid, 4=succinic acid, 5=lactic acid, 6=acetic acid, 7=butyric acid, 8=propionic acid. Experimental conditions see Fig.1.

EXPERIMENTAL

Instrumentation

The CE system employed was the Quanta 4000E (Waters Chromatography Division of Millipore, Milford, MA, USA) with a negative power supply. Phthalic, p-hydroxybenoic, nicotimic, benzoic, and cinnamic acid were separated with a fused-silica capillary (47.6 cm total length, 75μm internal diameter) obtained from Yongnian Photoconductive Fibre Factory. A window for on-column detection was created 8.6 cm from the end of the capillary. Direct UV detection was accomplished with a zinc lamp and a 214 nm optical filter. Hydrostatic sample time was set 1 s. The separation voltage applied was -20 kV.

Ortho-(o-), meta-(m-), and para-(p-) hydroxybenzoic acids were separated on Waters Accusep™, 60 cm ×75μm I.D. fused-silica capillary. A window for on-column detection was created 7.6 cm from the end of the capillary. Direct UV detection was accomplished with a Hg lamp and a 254 nm optical filter. Hydrostatic sample mode was selected for injection and sample time was set at 20 s. The separation voltage applied was -10kV. The electroosmotic mobility (μ_{eo}) was determined with formamide($HCONH_2$) as neutral marker.

Table 2

Dissociation Constant and Calculated $\lambda/(MW)^{0.45}$ for 5 Organic Acids

Organic Acid	pK^a_{a1}	pK^a_{a2}	$\lambda(MW)^{0.45}$
Phthalic	2.95	5.41	0.1436
p-Hydroxybenoic	4.585	9.23	0.1997
Nicotinic	4.82	11.0	0.1156
Benzoic	4.21		0.1151
Cinnamic	4.44		0.1054

[a] Data from Reference 28.

Chemicals

O-,m-, and p- hydroxybenzoic acids, nicotinic acid (chemical grade), and phthalic acid, benzoic acid(analytical grade) were obtained from Beijing Chemical Plant, Beijing, China. Cinnamic acid was purchased from National Institute for the Control of Pharmaceutical and Biological Product, Beijing, China.

The background electrolyte for the separation of o-,m-,and p-hydroxybenzoic acid was 25.0 mmol/L phosphate containing 10(v/v) of 1-propanol and 0.20 mmol/L of cetyltrimethylammonium bromide(CTAB). The pH of the electrolytic buffer was adjusted to 10.3 by addition of appropriate volume of concentration KOH solution. The separation of phthalic, p-hydroxybenoic, nicotimic, benzoic, and cinnamic acid was performed using 8 mmol/L $Na_2B_4O_7$, 2mmol/L KH_2PO_4, 0.2mmol/L CTAB, and 25%(v/v) acetonitrile (pH 8.90) as running buffer.

RESULTS AND DISCUSSION

Prediction of Migration Order

In order to use Eq. 28 to predict the migration order, the initial step is to look up the values of dissociation constants and molecular weights for the analytes in handbooks. On the assumption that F in Eq. 28 is a constant for different species separated in the same run, the migration order is determined by the value of $\lambda/(MW)^{0.45}$. Table 2 gives the dissociation constants, molecular

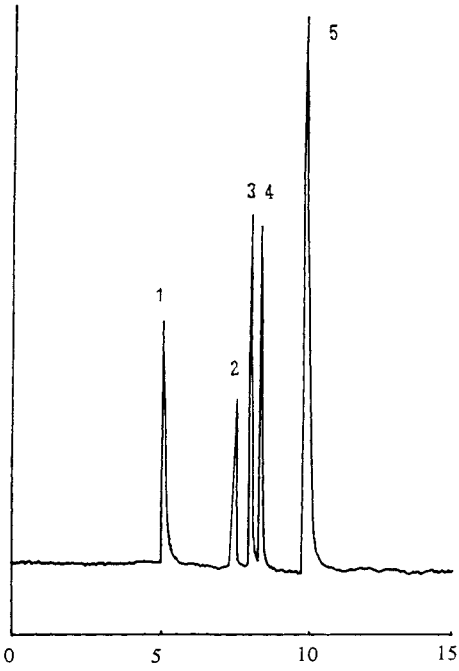

Figure 3. Capillary electropherogram of five aromatic acids separated by reversed electroosmotic flow capillary zone electrophoresis. Peak assignment: 1=phthalic acid, 2=p-hydroxybenzoic acid, 3=nicotimic acid, 4=benzoic acid, 5=cinnamic acid. Electrolyte solution: 8 mmol/L $Na_2B_4O_7$ + 2mmol/L KH_2PO_4 + 0.2mmol/L CTAB+25%(v/v) acetonitrile (pH 8.90).Detection:214 nm. Capillary: untreated fused silica: 47.6 cm total length, 39.0 cm effective length, and 75μm internal diameter. Voltage: -20 kV.Injection: 1s hydrostatic.

weights, the calculated $\lambda/(MW)^{0.45}$ for 5 organic acids. Because CTAB was added to the electrolyte solution to reverse the electroosmotic flow, the greater the value of $\lambda/(MW)^{0.45}$ is, the faster the analyte elutes. The migration order predicted by using Eq.28 flows phthalic acid < p-hydroxybenoic acid < nicotimic acid < benzoic acid < cinnamic acid. This is consistent with our experimental result in Fig. 3.

Equation 28 can also give a satisfactory explanation to Lin's experimental results.[8] They investigated the separation of benzotriazole(BTA), 5-methyl-1H-benzotriazole or tolytriazole (5-TTA), dimethylbenzotriazole (DBTA), and 2-mercaptobenzothiazole (MBT) at different pH. Table 3 gives the pK_α and

Table 3

Dissociation Constant and Molecular Weight for DBTA, 5TTA, BTA and MBT

Analyte	pK_a	MW
MBT	6.74	167.24
BTA	8.18	119.13
5-TTA	8.42	133.15
DBTA	8.63	147.18

molecular weight for these compounds. The $\lambda/(MW)^{0.45}$ for the three triazole compounds follows the order DBTA > 5-TTA > BTA in the pH range from 7.36 to 11.06. Therefore, the migration order will be DBTA < 5-TTA < BTA. This is the same as the result obtained by Lin. At pH = 8.90; the values of $\lambda/(MW)^{0.45}$ for BTA and MBT are 0.0977 and 0.0992, respectively. Therefore, at pH < 8.90 MBT will migrate after BTA, while at pH > 9.24 the migration order will be reversed. This predicted result is close to the result obtained by Lin.

It should be pointed out that when each analyte becomes the fully deprotonated anionic form, the term F in Eq.28 will not be considered as a constant for different species. In the case, it is inadequate to predict the migration order only by the parameter, $\lambda/(MW)^{0.45}$, and the dipole moment also must be considered. For fully deprotonated anionic species, if the direction of electroosmotic flow is from anode to cathode and the molecular weights for analyte are identical, the analyte with the largest dipole moment elutes firstly. This conclusion was supported by the result in literature.[29]

Estimation of μ_{eo}

Usually, the electroosmotic flow velocity is measured using one of compounds assumed to be neutral. There are two drawbacks to this method. Firstly, this method needs to mix the solvent marking electroosmotic velocity with the analytes prior to run. Secondly, when the migration time for the neutral solute is excessively long, the pH of the used background electrolyte possibly will change in the running procedure, resulting in an inaccurate t_{eo}. The method established according to Eq.28 can overcome the above shortcoming. Equation 28 indicates that the reciprocal of migration time is linearly correlated with the parameter, $\lambda/(MW)^{0.45}$. The intercept of the line is

Table 4

Dissociation Constants, Migration Times and Calculated λ for Hydroxybenzoic Acid (HA) Isomers

Analyte	pK^a_{a1}	pK^a_{a2}	t/Min	λ
o-HA	2.98	12.38	13.98	1.0083
m-HA	4.076	9.85	12.17	1.7381
p-HA	4.582	9.23	11.28	1.9216

[a] Data from Reference 30.

Table 5

Dissociation Constants, Molecular Weights, Measured Migration Times and Calculated $\lambda(MW)^{0.45}$ for 7 Amino Acids

Analyte	MW	pK_{a1}	pK_{a2}	t/Min	$\lambda(MW)^{0.45}$
Proline	115.08	10.64		4.31	8.23×10^{-2}
Leucine	131.11	9.74		4.91	10.56×10^{-2}
Valine	117.09	9.74		5.09	11.12×10^{-2}
Alanine	89.06	10.23		5.46	12.12×10^{-2}
Serine	105.06	9.21		5.66	13.51×10^{-2}
Glycine	75.05	9.78		6.09	13.51×10^{-2}
Glutamic acid	147.08	4.28	9.67	8.39	20.22×10^{-2}

readily obtained, and t_{eo} is equal to the reciprocal of the intercept. The following two examples will show that the estimation of the electroosmotic flow by means of Eq.28 is accurate. The dissociation constants, molecular weights, and $q/(MW)^{0.45}$ for hydroxybenzoic acid(HA) isomers are summarized in Table 4. A correlation regression analysis has been done on the data in Table 4. The regression equation of the line obtained by the least square method is $1/t_M = 0.0176\lambda + 0.0535$. μ_{eo} is 2.80×10^{-4} $V^{-1}s^{-1}cm^{-1}$. This is close to the value by our experiment, 2.71×10^{-4} $V^{-1}s^{-1}cm^{-1}$. The signs of the slope and reciprocal in regression equation are consistent. This indicates that the directions of electroosmotic mobility and the electrophoretic mobility are the same. This conclusion is supported by the fact that CTAB was added to the background electrolyte to reverse the electroosmotic flow in our experiment.

As another application case, Table 5 gives the dissociation constants and migration times for 7 different amino acids. The regression equation of the line for $1/t_m$ vs. $\lambda/(MW)^{0.45}$ is $1/t_M = -0.9044 \cdot \frac{\lambda}{(MW)^{0.45}} + 0.2947$.

The calculated t_{eo} is 3.39 min. The t_{eo} estimated in this work agrees very well with that obtained by Bruin,[10] 3.30 min.

Determination of pK_α

For a monoacid, Eq.28 can be rewritten as

$$\mu_{ep} = F \cdot \frac{e}{m^{0.45}} \left(\frac{0 \cdot [H^+]}{[H^+] + K_\alpha} + \frac{1 \cdot K_\alpha}{[H^+] + K_\alpha} \right) = F \cdot \frac{e}{m^{0.45}} \cdot \frac{K_\alpha}{[H^+] + K_\alpha} \tag{29}$$

If the limiting mobility, μ_{A^-}, is defined as

$$\mu_{A^-} = F \cdot \frac{e}{m^{0.45}} \tag{30}$$

then Eq.29 can be simplified as

$$\mu_{ep} = \mu_{A^-} \cdot \frac{K_\alpha}{[H^+] + K_\alpha} \tag{31}$$

Equation 31 can be used to determine the pK_α of the analyte. With the aid of the plot of the electrophoretic mobility of each analyte as a function of buffers' pH, the pK_α of each analyte can be easily determined by estimating the limiting values of μ_{A^-} and measuring the pH corresponding to $\mu_{ep} = \frac{1}{2} \cdot \mu_{A^-}$ from the plot.

Equation 31 can give a theoretical base to the method that has been successfully used[8,16-18] in the determination of the pK_α of the analyte using CZE.

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