*Journal of Chromatography*, 585 (1991) 364–368
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 574

# Short Communication

# Electrophoretic mobility and dissociation constants of tripeptides evaluated by isotachophoresis

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(Received April 23rd, 1991)

ABSTRACT

Ionic mobilities and dissociation constants of twenty peptides (seventeen tripeptides and a few oligoglycines) were evaluated on the basis of the observed isotachophoretic qualitative indices $R_E$ in the pH range 8.1–9.5 by the use of a least-squares method. Good correlation was obtained among the mobilities of amino acids, dipeptides and tripeptides when the clay ball model and the conventional formula weight were adopted.

## INTRODUCTION

Ionic mobility and dissociation constants of ionic substances are the essential physico-chemical constants for the theoretical treatment of electrophoresis, including separation optimization. As discussed previously [1], isotachophoresis itself is a useful method for the evaluation of the mobility, dissociation constant and ion-pair formation constant when the pH dependence of the qualitative indices are measured accurately and data reduction software is available. The unique feature of the method in comparison with conventional techniques is that a sample mixture can be treated and the necessary amount for the measurement is very small (less than 1 $\mu$mol).

As reviewed by Pospichal *et al.* [2], the number of samples for which the mobility has been obtained is not more than about 500. We have evaluated a considerable number of absolute mobilities ($m_0$) and p$K_a$ values by the use of isotachophoresis, including those of 25 amino acids [3] and 28 dipeptides [4].

In this work, the $m_0$ and p$K_a$ values of twenty peptides (seventeen tripeptides and a few oligoglycines) were evaluated on the basis of the observed isotachophoretic qualitative indices $R_E$ in the pH range 8.1–9.5. The least-squares method was used, utilizing a simulation technique of the isotachophoretic steady state. The correlation of the mobility among amino acids, dipeptides and tripeptides is briefly discussed on the basis of the clay ball model [4] and the conventional formula weight dependence.

## EXPERIMENTAL

### *Samples*

The twenty peptides used are summarized in Table I together with their abbreviations and formula weights. Oligoglycines were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and the other peptides from Sigma (St. Louis, MO, USA). Sample solutions (5 m$M$) were prepared by dissolving these dipeptides in distilled water. When the

0021-9673/91/$03.50 

TABLE I

PEPTIDE SAMPLES, ABBREVIATIONS AND MOLECULAR WEIGHTS

| Peptide | Abbreviation | Molecular weight |
|---|---|---|
| L-Alanyl-L-alanyl-L-alanine | $(Ala)_3$ | 231.2 |
| DL-Alanylglycylglycine | AlaGlyGly | 203.2 |
| DL-Alanyl-DL-leucylglycine | AlaLeuGly | 259.3 |
| Diglycine | $(Gly)_2$ | 132.1 |
| Triglycine | $(Gly)_3$ | 189.2 |
| Tetraglycine | $(Gly)_4$ | 246.2 |
| Pentaglycine | $(Gly)_5$ | 303.3 |
| Hexaglycine | $(Gly)_6$ | 360.3 |
| Glycylglycyl-L-isoleucine | GlyGlyIle | 245.3 |
| Glycylglycyl-D-leucine | GlyGlyLeu | 245.3 |
| Glycylglycyl-L-phenylalanine | GlyGlyPhe | 279.3 |
| Glycylglycyl-L-valine | GlyGlyVal | 231.3 |
| Glycyl-L-histidylglycine | GlyHisGly | 269.3 |
| Glycyl-DL-leucyl-DL-alanine | GlyLeuAla | 259.3 |
| Glycyl-L-leucyl-L-tyrosine | GlyLeuTyr | 351.4 |
| Glycyl-L-prolyl-L-alanine | GlyProAla | 243.3 |
| Glycyl-D-phenylalanyl-L-phenylalanine | GlyPhePhe | 369.4 |
| L-Leucylglycylglycine | LeuGlyGly | 245.3 |
| DL-Leucylglycyl-DL-phenylalanine | LeuGlyPhe | 335.4 |
| L-Leucyl-L-leucyl-L-leucine | $(Leu)_3$ | 357.5 |
| L-Seryl-L-seryl-L-serine | $(Ser)_3$ | 279.3 |

solubility was low, a small amount of sodium hydroxide solution was added. The internal standards used to correct the asymmetric potential of PGD [5] were butyric acid, glycine and diglycine.

*Operational electrolyte system*

The leading electrolyte was 10 m*M* hydrochloric acid and the pH of leading electrolyte ($pH_L$) was adjusted to 8.10 (No. 1) and 8.41 (No. 2) by adding tris(hydroxymethyl)aminomethane, 8.80 (No. 3) and 8.92 (No. 4) by adding 2-amino-2-methyl-1,3-propanediol and to 9.50 (No. 5) by adding ethanolamine. The above $pH_L$ range was determined on the basis that the $pK_a$ values of dipeptides are in the range *ca.* 8–9 [4] and the $pK_a$ values of tripeptides will not be much different from these values. All of the leading electrolytes contained 0.1% of hydroxypropylcellulose (HPC) to suppress electrode reactions and electroendosmosis.

The terminating electrolytes used were 10 m*M* $\beta$-alanine and 10 m*M* glycine. The pH of the terminating electrolyte was adjusted to *ca.* 10 by adding barium hydroxide in order to reduce the dilution effect caused by $HCO_3^-$ [6,7]. pH measurements were carried out using a Horiba Model F7ss expanded pH meter.

Isotachopherograms were obtained using a Shimadzu IP-2A isotachophoretic analyser equipped with a potential gradient detector (PGD). The temperature was thermostated at 25°C. The PTFE separating tube used was 40 cm × 0.5 mm I.D. The driving current applied was 50 $\mu$A. Fig. 1 shows typical isotachopherograms for several peptides observed with the use of electrolyte system No. 3 ($pH_L$ = 8.8).

The computer program SIPS-LSQ developed by us was used for the evaluation of $m_0$ and/or $pK_a$. The method is based on the simulation of an isotachophoretic zone to evaluate the isotachophoretic qualitative indices $R_E$; the difference between the observed and the simulated $R_E$ values was minimized by varying $m_0$ and/or $pK_a$ repeatedly. The program was written in Fortran 77. A typical calculation took 1 min on an NEC PC-9801RA microcomputer (80386–80387, clock 20 MHz).

## RESULTS AND DISCUSSION

*Evaluated mobility and $pK_a$*

The observed $R_E$ values are summarized in Table II together with the $R_E$ values of the internal standards. The asymmetric potential of PGD was corrected using mainly the simulated values for butyric acid [5]. The step heights in the isotachopherogram might be decreased by the dilution effect of carbonate from the terminating zone [6,7]. Therefore, the observed $R_E$ values might be affected to some extent, although not seriously.

Table III summarizes the absolute mobility ($m_0$) and $pK_a$ values for twenty peptides at 25°C evaluated by the least-squares method using the $R_E$ values in Table II. The mean deviation between the observed and the best-fitted $R_E$ values was 0.5–1.5% (maximum 2.4% for GlyPhePhe). Table III also shows the mobility and $pK_a$ values of the constituent amino acids of the peptides and the internal standards. The $pK_a$ values of oligopeptides were considerably different from those of the constituent amino acids [3]. However, the $pK_a$ values of homologous oligopeptides of $(Gly)_2$–$(Gly)_6$ were

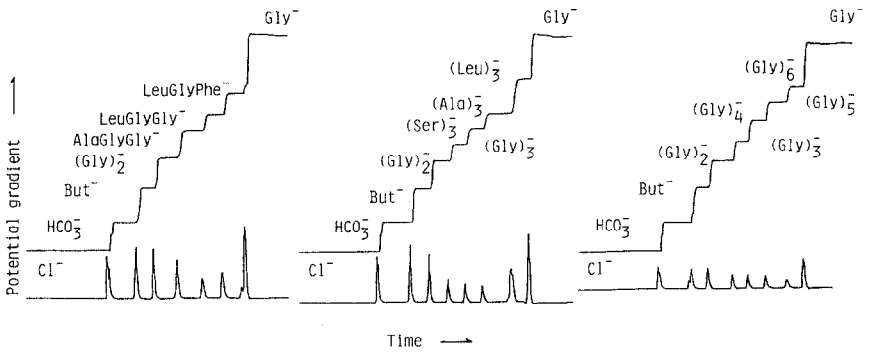


Fig. 1. Observed isotachopherograms of butyric acid, $(Gly)_2$, AlaGlyGly, LeuGlyGly, LeuGlyPhe, $(Gly)_3$, $(Ser)_3$, $(Ala)_3$, $(Leu)_3$, $(Gly)_4$, $(Gly)_5$ and $(Gly)_6$. Butyric acid (But) and $(Gly)_2$ were the internal standards. The leading electrolyte was 10 m*M* hydrochloric acid buffered by ammediol at $pH_L = 8.80$. The terminator was 10 m*M* glycine (pH $\approx$ 10 by adding barium hydroxide).

TABLE II

OBSERVED $R_E$ VALUES OF PEPTIDES AT DIFFERENT $pH_L$ VALUES

| Sample | Electrolyte system and $pH_L$ | | | | |
|---|---|---|---|---|---|
| | No. 1, 8.10 | No. 2, 8.41 | No. 3, 8.80 | No. 4, 8.92 | No. 5, 9.50 |
| β-Ala[a] | 19.69 | 15.83 | 9.67 | 8.98 | 5.15 |
| $(Ala)_3$ | 5.49 | 4.67 | 4.22 | 4.09 | 3.95 |
| AlaGlyGly | 4.86 | 4.21 | 3.73 | 3.71 | 3.40 |
| AlaLeuGly | 5.72 | 5.01 | 4.40 | 4.30 | 4.10 |
| Butyric acid[a] | 2.43 | 2.43 | 2.43 | 2.43 | 2.42 |
| Gly[a] | 10.77 | 8.75 | 5.64 | 5.27 | 3.37 |
| $(Gly)_2$[a] | 4.19 | 3.64 | 3.09 | 3.00 | 2.70 |
| $(Gly)_3$ | 4.29 | 3.91 | 3.49 | 3.42 | 3.22 |
| $(Gly)_4$ | 4.91 | 4.35 | 3.97 | 3.89 | 3.61 |
| $(Gly)_5$ | 5.50 | 4.74 | 4.38 | 4.31 | 4.03 |
| $(Gly)_6$ | 5.88 | 5.07 | 4.72 | 4.65 | 4.51 |
| GlyGlyIle | 5.12 | 4.58 | 4.19 | 4.12 | 3.88 |
| GlyGlyLeu | 5.10 | 4.66 | 4.21 | 4.11 | 3.83 |
| GlyGlyPhe | 4.95 | 4.59 | 4.17 | 4.08 | 3.83 |
| GlyGlyVal | 4.95 | 4.59 | 4.05 | 3.97 | 3.73 |
| GlyHisGly | 5.47 | 4.74 | 4.18 | 4.07 | 3.87 |
| GlyLeuAla | 5.72 | 4.97 | 4.48 | 4.36 | 4.06 |
| GlyLeuTyr | 6.15 | 5.34 | 4.67 | 4.59 | 4.03 |
| GlyProAla | 6.13 | 5.21 | 4.39 | 4.24 | 3.95 |
| GlyPhePhe | 6.13 | 5.07 | 4.39 | 4.61 | 4.45 |
| LeuGlyGly | 5.04 | 4.48 | 4.11 | 4.09 | 4.04 |
| LeuGlyPhe | 5.54 | 4.88 | 4.60 | 4.58 | 4.54 |
| $(Leu)_3$ | 5.64 | 5.21 | 5.05 | 4.94 | 4.90 |
| $(Ser)_3$ | 4.22 | 3.93 | 3.89 | 3.75 | 3.95 |

[a] Standards or terminators for $R_E$ evaluation: the simulated $R_E$ values are listed.

almost identical with each other. It should be noted that the possible divalent ion of GlyLeuTyr was not taken into account in the present evaluation and therefore the evaluated $m_0$ and $pK_a$ values of GleuLeuTyr might contain considerable errors.

*Determination of mobility of oligopeptides by the clay ball model*

We have reported previously a simple equation to calculate the mobilities of dipeptides from those of the constituent amino acids [4], based on the assumption that the ions are spherical and the ionic volume of the dipeptides is equal to the sum of the volumes of the constituent amino acids, as if two clay balls merged into a larger ball. This model was applied to calculate the absolute mobilities of oligopeptides (ABC...), where A, B, C,... denote the constituent amino acids. The Stokes radii of the oligopeptides ($r_{ABC...}$) and the mobilities ($m_{ABC...}$) can be described on the basis of the present assumption as

$$r_{ABC...} = [3(V_A + V_B + V_C + ...)/4\pi]^{1/3} = (r_A^3 + r_B^3 + r_C^3 + ...)^{1/3} \quad (1)$$

and

$$m_{ABC...} = (m_A^{-3} + m_B^{-3} + m_C^{-3} + ...)^{1/3} \quad (2)$$

For oligoglycines $(Gly)_n$ ($n$ = 2–6), the absolute mobilities calculated by the use of eqn. 2 were $29.7 \cdot 10^{-5}$, $25.9 \cdot 10^{-5}$, $23.6 \cdot 10^{-5}$, $21.9 \cdot 10^{-5}$ and

TABLE III

ABSOLUTE MOBILITIES ($m_0$) AND p$K_a$ VALUES OF PEPTIDES, THE CONSTITUENT AMINO ACIDS AND THE INTERNAL STANDARDS

| Sample | $m_0$ | | p$K_a$ Observed |
|---|---|---|---|
| | Observed | Estimated | |
| $(Ala)_3$ | 22.2 | 22.3 | 8.245 |
| AlaGlyGly | 25.0 | 24.5 | 8.254 |
| AlaLeuGly | 21.3 | 21.3 | 8.272 |
| $(Gly)_3$ | 26.1 | 25.9 | 8.102 |
| $(Gly)_4$ | 23.3 | 23.6 | 8.142 |
| $(Gly)_5$ | 21.2 | 21.9 | 8.167 |
| $(Gly)_6$ | 19.3 | 20.6 | 8.107 |
| GlyGlyIle | 21.9 | 22.3 | 8.096 |
| GlyGlyLeu | 21.9 | 22.1 | 8.116 |
| GlyGlyPhe | 21.9 | 22.3 | 8.041 |
| GlyGlyVal | 22.6 | 23.0 | 8.125 |
| GlyHisGly | 22.5 | 23.0 | 8.279 |
| GlyLeuAla | 21.1 | 21.3 | 8.259 |
| GlyLeuTyr[a] | 21.0 | 17.1 | 8.405 |
| GlyProAla | 22.5 | 20.9 | 8.492 |
| GlyPhePhe | 19.7 | 20.2 | 8.216 |
| LeuGlyGly | 21.5 | 22.1 | 7.992 |
| LeuGlyPhe | 19.3 | 20.0 | 7.938 |
| $(Leu)_3$ | 17.6 | 18.3 | 7.730 |
| $(Ser)_3$ | 22.0 | 23.3 | 7.385 |
| | $m_0$ | | p$K_a$ |
| Butyric acid | 33.8 | | 4.820 |
| Glycine | 37.4 | | 9.780 |
| $(Gly)_2$ | 31.5 | | 8.400 |
| β-Alanine | 30.8 | | 10.241 |
| Ala | 32.2 | | 9.857 |
| Gly | 37.4 | | 9.780 |
| Ileu | 26.7 | | 9.765 |
| Leu | 26.4 | | 9.728 |
| Phe | 26.9 | | 9.262 |
| Val | 28.4 | | 9.710 |
| Tyr | 20.0 | | 8.405 |
| | 40.0 | | 10.189 |
| Ser | 33.6 | | 9.302 |

[a] Analysed as a monovalent anion.

$20.6 \cdot 10^{-5}$ $cm^2$ $V^{-1}$ $s^{-1}$. The mean deviation was 3.6%. Although the maximum deviation for $(Gly)_6$ of 6.3% may suggest that the estimate for the higher oligopeptides will be poor, the clay ball model gives a simple rule for calculating rough mobilities of such peptides. The good feature of this model is the applicability to heterogeneous oligopeptides as described previously [4]. The mean deviation for the twenty oligopeptides except for GlyLeuTyr was 1.2%. The worst deviation was found for GlyProAla and $(Ser)_3$ as −7.3 and 5.9%, respectively. By the least-squares method for the nineteen peptides, the following equation between the observed $m_0$ and the estimated $m_{ABC...}$ was obtained:

$$m_0 = 1.076 m_{ABC...} - 2 \cdot 10^{-5} \quad (3)$$

The coefficient in eqn. 3 suggests that the estimated $m_{ABC...}$ values were slightly underestimated in comparison with the observed values and the situation was the same as for dipeptides. The correlation coefficient between the observed $m_0$ and the estimated $m_{ABC...}$ values was 0.95 and the mean deviation between the estimated and the observed $m_0$ values was 1.8%.

On the other hand, it has been well established that the mobility can be correlated with formula weight. For all of the oligopeptides treated, the following equation was obtained by the least-squares method:

$$m_0 = (293.2/\sqrt{FW} + 3.78) \cdot 10^{-5}\ cm^2\ V^{-1}\ s^{-1} \quad (4)$$

where $m_0$ is the absolute mobility of the monovalent ion and $FW$ the formula weight. The mean deviation between the estimated and the observed mobilities was 3.5% for the twenty peptides. The deviation for $(Leu)_3$ and GlyLeuTyr was as high as −9.6% and 7.5%, respectively. If these were omitted, the mean deviation decreased to 2.9%. By the least-squares method for the nineteen peptides, the following equation between the observed $m_0$ and the estimated $m_{ABC...}$ values was obtained when eqn. 4 was used:

$$m_0 = 0.989 m_{ABC...} - 2.3 \cdot 10^{-6} \quad (5)$$

The correlation coefficient was 0.89.

For oligoglycines, a better mobility was obtained by the use of the conventional formula weight. The following equation expresses the mobility of oligoglycines, $Gly_n$ ($n$ = 1–6):

$$m_0 = (291.39/\sqrt{FW} + 4.66) \cdot 10^{-5}\ cm^2\ V^{-1}\ s^{-1} \quad (6)$$

The mean deviation for $(Gly)_n$ ($n$ = 1–6) was 2.2% and the correlation coefficient was 0.992.

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