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Evaluation of protonation constants of glycylglycine in acetonitrile– and ethylene glycol–water mixtures

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ABSTRACT

Knowledge of the protonation constants of dipeptides is important and necessary for complete understanding of their physiochemical behavior. The effect of dielectric constant of medium on protonation equilibria has been studied by determining protonation constants of glycylglycine pH-metrically in various concentrations (0–60%v/v) of acetonitrile–water and ethylene glycol–water mixtures, at an ionic strength of 0.16 mol L^{-1} and at 303.0 ± 0.1 K. The protonation constants were calculated with the computer program MINIQUAD75 and selection of the best fit chemical models is based on the statistical parameters. Linear and non-linear variations of stepwise protonation constants with reciprocal of dielectric constant of the solvent mixtures have been attributed to the dominance of electrostatic and non-electrostatic forces, respectively. Distribution of species, protonation equilibria, and effect of influential parameters on the protonation constants have also been presented.

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KEYWORDS
Glycylglycine; acetonitrile; ethylene glycol; protonation constants; MINIQUAD75; dielectric constant

Introduction

Peptides are an amazing class of compounds constructed from relatively simple building blocks, the amino acids. They are components of tissues [1] exhibiting a remarkable range of biological properties acting as antibiotics, hormones, food additives, poisons, or painkillers. [2] Investigations involving the peptides in acid–base reactions [3,4] particularly, small peptides have attracted great attention in relation to the bioinorganic chemistry because these compounds are usually considered as good model systems to attain a better insight into the characteristics of naturally occurring metalloproteins. [5,6] On the other hand, several metal complexes containing peptide groups have displayed diverse pharmacological activities. For instance, copper complexes with amino acids and peptides as ligands show anti-inflammatory and cytostatic activities.

Vast data are available on the protonation and stability constants of the amino acids and simple peptides in water and organic solvents. However, the protonation constants of amino acids and peptides in organic solvents are often different from those in water, as organic media tend to be lipophilic rather than hydrophilic.[7,8] Little is known about the chemistry of amino acids and simple peptides in mixed solvents, in regard to their protonation constants and experiments shown that one solvent alone is not an ideal model for *in vivo* reactions. It has been suggested that mixture of solvents such as organic solvent–water mixtures provide a better model for *in vivo* reactions. Therefore, the influence which the solvent exerts on the protonation constant (*K*) values depends upon the extent and nature of the solute–solvent interaction, which involves species participating in the acid–base equilibrium.[9] Hence, studies in media made up of organic solvent–water mixtures should provide some understanding of the chemistry of peptides in living systems.

Glycylglycine (GG) is the simplest dipeptide made from the residues of two glycine molecules. Besides an important component of the tissue, glycylglycine is a potentially useful zwitterionic buffer in the physiological pH range (6.0–8.5). Ethylene glycol (EG) is a protophilic dipolar protic solvent and acts as a structure former. EG, having two hydroxyl groups, is distinctly different from monohydric alcohols. EG is more acidic (less basic) than water [10] due to the electron withdrawing effect [11] of the CH_2 group. EG offers several advantages as solvent in titration of weak bases.[12] EG plays an important role in protein conformation studies [13,14] because it is a weak protein denaturant compared to urea or other organic solvents such as ethanol, dioxan, etc. The protonation equilibria of glycylglycine have been studied in the presence of EG to understand the influence of solvent on the chemical speciation.

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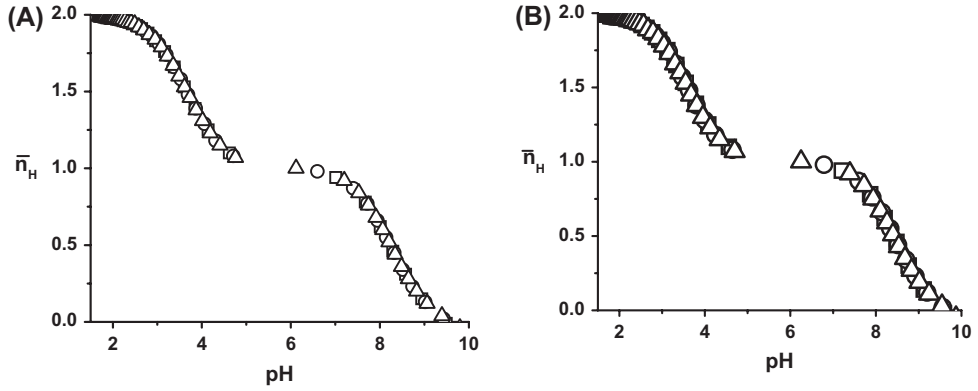

Figure 1. Plots of $\bar{n}_H$ vs. pH of glycylglycine in 30%v/v AN–water mixture (A) and EG–water mixture (B) (□) 0.25 (○) 0.375 (Δ) 0.50 mmol.

Acetonitrile (AN) is a weak base [15] and a much weaker acid [16] than water. Hence, cations and anions have lower solvation energies in AN than in water, except in those cases where there is specific interaction with the solvent.[17] It is a protophobic dipolar aprotic solvent, and it does not form any hydrogen bond with solute species. The protophobic character of AN may be due to the formation of dimers which are shown to exist from IR studies.

In this study, the protonation constants of glycylglycine have been determined pH-metrically in aqua-organic mixtures containing AN and EG frequently used as media in biochemistry and biology laboratories.

Materials and methods

Experimental

All the chemicals used in this investigation were of analytical reagent grade purity. Solution (0.05 mol L^{-1}) of glycylglycine (Merk, India) was prepared in triple-distilled water by maintaining 0.05 mol L^{-1} hydrochloric acid concentration to increase the solubility. AN and EG (Merk, India) were used without further purification. Carbonate-free sodium hydroxide (Qualigens, India) pellets were used for the preparation of 0.4 mol L^{-1} solution. Hydrochloric acid (Qualigens, India) of 0.2 mol L^{-1} was prepared. Sodium chloride (Qualigens, India) of 2.0 mol L^{-1} was prepared to maintain 0.16 mol L^{-1} ionic strength in the titrant. Triple-distilled water was used throughout the experiment. The strengths of acid and alkali were determined using Gran plot method.[18] To assess the errors that might have crept into the determination of the concentrations, the data were subjected to analysis of variance of one-way classification (ANOVA).[19]

Alkalimetric titrations

Alkalimetric titrations were carried out with varying composition of AN and EG (0.0–60.0%v/v) maintaining an ionic strength of 0.16 mol L^{-1} with sodium chloride at 303.0 ± 0.1 K. The purpose of this study was to mimic the equilibria taking place in biofluids, where the temperature is 37 °C. But at that temperature, the solvent may be evaporated. Hence, a temperature of 30 °C or 303 K has been chosen in this study. Amount of glycylglycine in the titrant ranged between 0.25 and 0.50 mmol. Elico Li-120 pH meter was used. Potassium hydrogen phthalate (0.05 mol L^{-1}) and borax (0.01 mol L^{-1}) solutions were used to calibrate the pH meter. The glass electrode was equilibrated in a well-stirred AN–water and EG–water mixture containing inert electrolyte for several days. At regular intervals, strong acid was titrated against alkali to check the complete equilibration of the glass electrode. The calomel electrode was refilled with AN (or EG)–water mixture of equivalent composition of the titrant. Experimental procedure and titration assembly have been detailed elsewhere.[20] The approximate protonation constants of glycylglycine were calculated with the computer program SCPHD.[21] The best fit chemical model for each system investigated was arrived at using non-linear least squares computer program, MINIQUAD75.[22]

Results and discussion

Normal biochemical processes occur in aqueous solutions at about neutral pH. Physiological pH is about 7.2–7.4. Amino acids present in biomolecules can gain or lose protons depending on the availability of hydrogen ions in the solution. This situation results in the simultaneous existence of a number of protonation–deprotonation equilibria in solution. Secondary formation functions like average number of protons bound per mole of ligand ($\bar{n}_H$) and number of moles of alkali consumed per mole of ligand (*a*) are useful to detect the number of polymeric species and equilibria. Plots of $\bar{n}_H$ vs. pH (formation curves) for different concentrations of the ligand should overlap if there is no formation of polymeric species. Overlapping formation curves for glycylglycine in AN– and EG–water mixtures shown in Figure 1 ruled out the polymerization of the ligand molecule. The pH values at half integral values of $\bar{n}_H$ correspond to the protonation constants of the ligand. Two half integrals (0.5 and 1.5) of glycylglycine in case of both the media shown in Figure 1

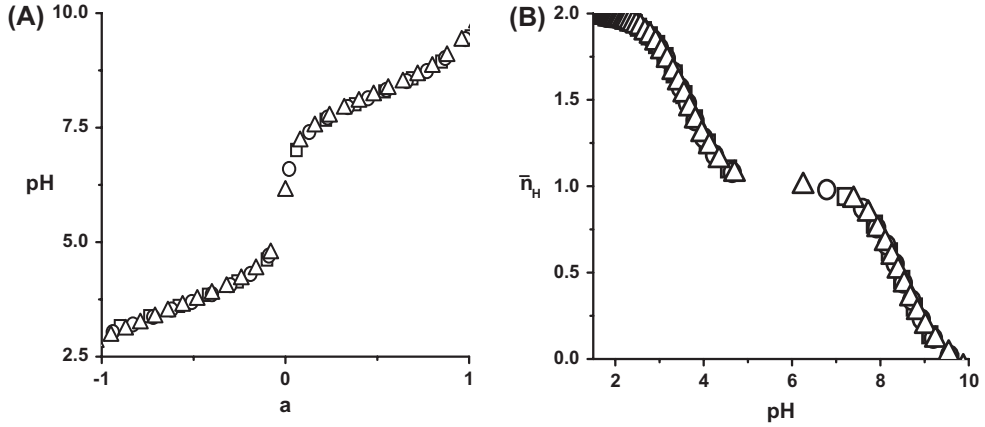

Figure 2. Variation of *a* with pH of glycylglycine in 30%v/v AN–water mixture (A) and EG–water mixture (B) (□) 0.25 (○) 0.375 (Δ) 0.50 mmol.

Table 1. Best fit chemical models of acido-basic equilibria of glycylglycine in AN–water and EG–water mixtures.

	log β_{mlh} (SD)							
Solvent %v/v	011	012	NP	U_{corr}	Skewness	Kurtosis	χ^2	*R*-factor
Acetonitrile (pH range: 2.0–10.0)								
0.0	8.17(11)	11.42(18)	100	2.92	0.11	3.90	11.36	0.0107
10.0	8.23(11)	11.54(11)	92	3.73	−3.13	18.71	51.48	0.0119
20.0	8.32(10)	11.95(11)	92	6.93	−2.36	17.50	30.09	0.0156
30.0	8.24(13)	11.90(12)	91	3.38	−1.81	7.80	28.29	0.0112
40.0	8.18(14)	12.10(12)	96	4.56	−0.77	6.38	33.25	0.0129
50.0	8.14(11)	12.20(13)	95	2.62	−1.68	13.35	44.27	0.0104
60.0	8.16(10)	12.26(10)	69	4.26	−1.84	6.51	23.91	0.0017
Ethylene glycol (pH range: 2.0–10.0)								
0.0	8.17(11)	11.42(18)	100	2.92	0.11	3.90	11.36	0.0107
10.0	8.97(13)	12.90(13)	62	2.62	−0.11	4.96	10.57	0.0090
20.0	9.00(12)	13.07(10)	60	3.83	−1.60	10.18	9.79	0.0098
30.0	8.43(12)	12.03(12)	88	1.76	−1.29	5.16	22.09	0.0140
40.0	8.38(10)	12.17(12)	72	7.16	−2.66	17.47	24.22	0.0195
50.0	8.08(10)	11.48(10)	90	6.88	1.64	5.60	7.08	0.0041
60.0	8.06(10)	11.45(12)	88	7.59	1.56	5.05	5.85	0.0056

Note: $U_{corr} = U/(NP - m) \times 10^8$; NP = number of points; *m* = number of protonation constants; SD = standard deviation.

emphasize the presence of two protonation–deprotonation equilibria in the pH range of this study.

The plots of *a* vs. pH for glyclyglycine in both media were given in Figure 2. The negative values of *a* correspond to the number of moles of free acid present in the titrant and the number of associable protons. The positive values of *a* indicate the number of dissociable protons in the ligand molecules. The maximum value of *a* in Figure 2 is +1, which indicates that glyclyglycine has one dissociable proton.

The best fit models containing the type of species and log values of overall formation constants (log β) along with some of the important statistical parameters of this study are given in Table 1. A very low standard deviation (SD) in log β values and U_{corr} (sum of the squares of deviations in concentrations of ligand and hydrogen ion at all experimental points corrected for degrees of freedom) indicates that the experimental data can be represented by the model. Small values of mean, SD, and mean deviation for the systems corroborate that the residuals are around a zero mean with little dispersion.

Residual analysis [23]

In data analysis with least squares methods, the residuals (the differences between the experimental data and the data simulated based on the model parameters) are assumed to follow Gaussian distribution. When the data are fit into the models, the residuals should be ideally equal to zero. Further, a model is considered adequate only if the residuals do not show any trend. Respecting the hypothesis of the least squares analysis, the residuals are tested for normal distribution. Such tests are χ^2, skewness, kurtosis, and *R*-factor. These statistical parameters of the present data show that the best fit models portray the acido-basic equilibria of glycylglycine in AN–water and EG–water mixtures, as discussed below.

χ^2 test

χ^2 is a special case of gamma distribution, whose probability density function is an asymmetrical function. This distribution measures the probability of residuals forming a part of standard normal distribution with zero

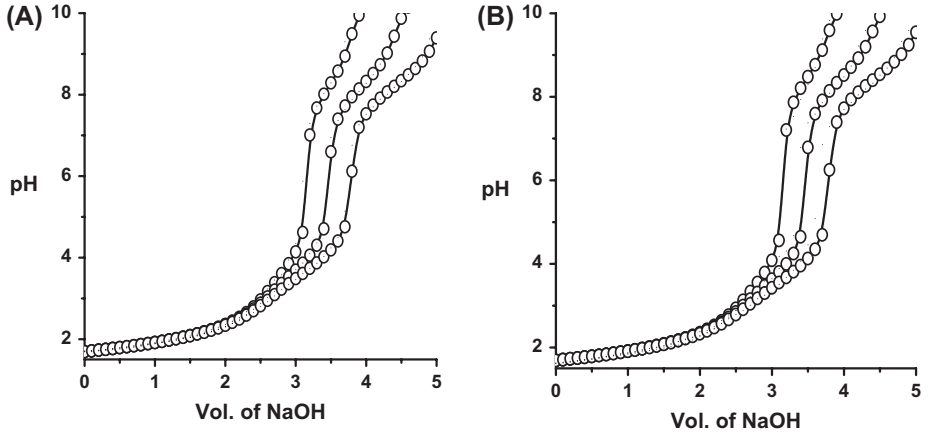

Figure 3. Simulated (○) and experimental (solid line) alkalimetric titration curves in 30%v/v organic solvent; (A) AN and (B) EG: (a) 0.25, (b) 0.375, and (c) 0.50 mmol of GG.

mean and unit SD. If χ^2 calculated is less than the table value, the model is accepted.

Crystallographic R-*test*

Hamilton's *R*-factor ratio test [24] is applied in complex equilibria to decide whether inclusion of more species in the model is necessary or not. In pH-metric method, the readability of pH meter is taken as the R_{limit}, which represents the upper boundary of *R* beyond which the model bears no significance. When different values are obtained for models containing different number of species, models whose values are greater than *R*-table are rejected. The low crystallographic *R*-values given in Table 1 indicate the sufficiency of the model.

Skewness

It is a dimensionless quantity indicating the shape of the error distribution profile. A value of zero for skewness indicates that the underlying distribution is symmetrical. If the skewness is greater than zero, the peak of the error distribution curve is to the left of the mean, and the peak is to the right of the mean if the skewness is less than zero. The values of skewness recorded in Table 1 are between −3.13 and 1.64. These data evince that the residuals form a part of normal distribution; hence, least squares method can be applied to the present data.

Kurtosis

It is a measure of the peakedness of the error distribution near a model value. For an ideal normal distribution, kurtosis value should be three (mesokurtic). If the calculated kurtosis is less than three, the peak of the error distribution curve is flat (platykurtic), and if the kurtosis is greater than three, the distribution shall have sharp peak (leptokurtic). The kurtosis values in this study indicate that the residuals form leptokurtic pattern. Alkalimetric titration data are simulated using the model parameters given in Table 1. These data are compared with the experimental alkalimetric titration data, to verify the sufficiency of the models as shown in Figure 3. The overlap of the typical experimental and simulated titration data indicates that the proposed models represent the experimental data.

Effect of systematic errors in concentrations on best fit model

MINIQUAD75 does not have an inbuilt provision to study the effect of systematic errors in the concentration of ingredients like mineral acid, alkali, and ligand. In order to rely upon the best fit chemical model for critical evaluation and application, a brief investigation was made by introducing pessimistic errors in the ingredients. This type of investigation is useful because the data acquisition was done under varied experimental conditions with different accuracies. The results of a typical system given in Table 2 emphasize that the errors in the concentrations of alkali and mineral acid affect the protonation constants more than that of the ligand.

Effect of dielectric constant of medium

When the ionization of an acid gives a net increase of ions, a decrease in the dielectric constant of the solvent should be accompanied by an increase in the protonation constant of a weak acid dissolved in it. The variation of protonation constant or change in free energy with co-solvent content depends upon two factors, viz, electrostatic and non-electrostatic forces. Born's classical treatment holds good in accounting for the electrostatic contribution to the free energy change. According to this treatment, the energy of electrostatic interaction or the logarithm of stepwise protonation constant (log *K*) should vary linearly as a function of the reciprocal of the dielectric constant (1/*D*) of the medium.

The linear variation of log *K* values of glycylglycine in AN–water mixture shown in Figure 4 indicates the dominance of electrostatic forces over non-electrostatic

Table 2. Effect of errors in influential parameters on the protonation constants in 30%v/v AN–water and EG–water mixture.

		log β_{mlh} (SD)			
		011	012	011	012
Ingredient	% Error	Acetonitrile		Ethylene glycol	
Alkali	0	8.24(13)	11.90(23)	8.43(27)	12.03(47)
	−5	8.72(33)	12.75(56)	9.03(49)	13.05(84)
	−2	8.42(19)	12.22(33)	8.64(35)	12.39(60)
	+2	8.06(12)	11.58(20)	8.24(24)	11.69(41)
	+5	7.81(22)	11.12(37)	7.98(29)	11.23(47)
Acid	−5	7.85(29)	11.06(46)	8.02(34)	11.16(55)
	−2	8.08(16)	11.57(27)	8.26(27)	11.67(45)
	+2	8.39(18)	12.23(31)	8.61(33)	12.40(57)
	+5	8.64(35)	12.74(63)	8.92(48)	13.03(87)
Ligand	−5	8.18(08)	11.91(15)	8.37(24)	12.05(43)
	−2	8.21(11)	11.90(18)	8.40(26)	12.03(45)
	+2	8.26(15)	11.89(25)	8.45(29)	12.02(49)
	+5	8.29(18)	11.88(31)	8.49(32)	12.01(51)
Log *F*	−5	8.23(13)	11.90(22)	8.42(28)	12.01(48)
	−2	8.24(13)	11.89(22)	8.43(28)	12.02(47)
	+2	8.24(12)	11.90(21)	8.43(27)	12.03(47)
	+5	8.24(12)	11.90(21)	8.43(27)	12.04(46)

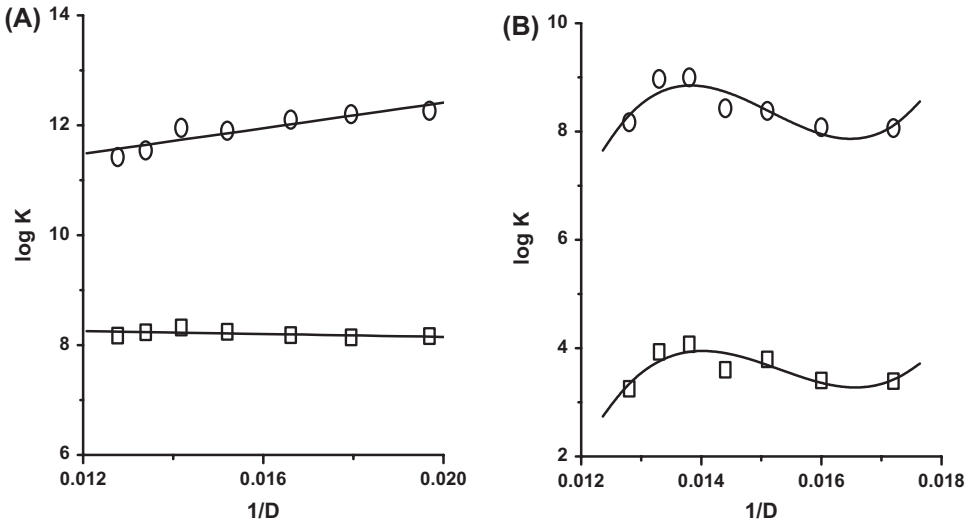

Figure 4. Variation of stepwise protonation constants (log *K*) of glycylglycine with reciprocal of dielectric constant (1/*D*) of AN–water (A) and EG–water (B) mixtures. (□) log K_1, (○) log K_2.

forces. But the non-linear trend in EG–water mixture shows the dominance of non-electrostatic forces. These opposite trends are due to the opposite nature of AN and EG. The cation stabilizing nature of co-solvents, specific solvent–water interactions, charge dispersion, and specific interactions of co-solvent with solute (indicated by the changes in the solubility of different species in the aquo-organic mixtures) account for the deviation of classical linear relationship of log *K* with 1/*D*.

Distribution diagrams

Glycylglycine has three functional groups (carboxyl, amino, and amido); however, only the carboxyl and amino groups involve in the acid–base equilibria. The protonation–deprotonation equilibria of glycylglycine are shown in Figure 5. As the alkali is added to the titrant containing the ligands, the protonated forms of the ligand lose their protons. In the pH range of this study, glycylglycine loses carboxylic and amino protons successively.

The typical distribution plots shown in Figure 6 produced using the protonation constants from the best fit models Table 1 show the existence of LH_2^+, LH and L^- species. The LH_2^+ species is predominant at low pH, and its concentration decreases exponentially and becomes almost zero around pH 6.5. The most predominant species is LH (zwitterionic) form of glycylglycine present to an extent of 90% in the pH range 4.0–9.0. Around pH 5.0, the formation of the free ligand L^- is observed while its concentration progressively increases and attains its maximum at higher pH in both media.

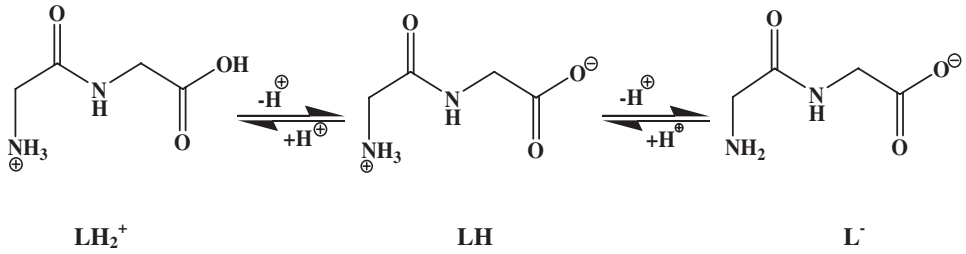

Figure 5. Protonation–deprotonation equilibria of glycylglycine.

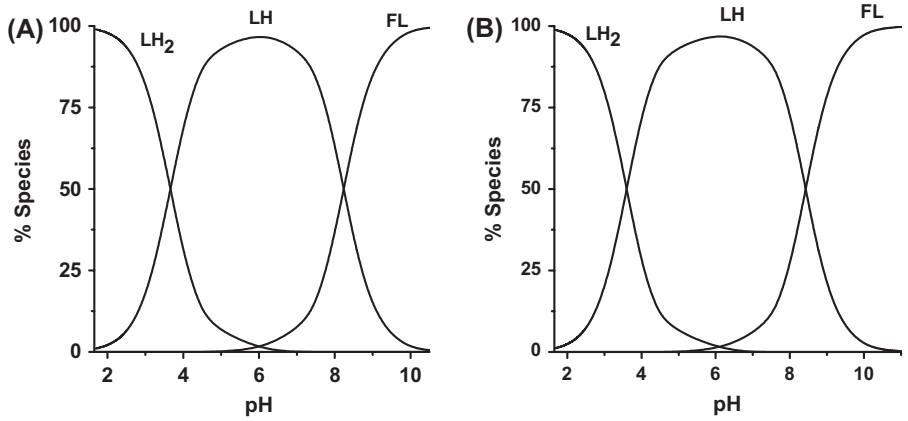

Figure 6. Species distribution diagrams of glycylglycine in (A) 30%v/v AN–water and (B) 30%v/v EG–water mixtures.

Conclusions

Secondary formation functions indicated the existence of two protonation equilibria. The highest form of ligand is LH_2^+ which successively deprotonates to LH and L^- with increasing pH.

Increased standard deviations in the protonation constants with the introduction of errors in the concentrations of ingredients support the appropriateness of the experimental conditions. The change in the magnitude of protonation constant with co-solvent content indicates the influence of dielectric constant of the medium. The linear and non-linear variation of protonation constants in AN–water and EG–water mixtures, respectively, infers the dominance of electrostatic and non-electrostatic forces in the equilibria.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Bogi Srinu, the co-author, holds a MSc degree in Chemistry at the Andhra University, India. He is a research scholar at Andhra University, India. He has published over three scientific and proceedings publications. His research interests are in chemical speciation and water analysis.

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