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RESEARCH ARTICLE

Speciation Studies of Phenylalanine complexes of Ca(II), Mg(II) and Zn(II) in acetonitrile-water mixtures

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Abstract: *Equilibrium study on complex formation of Phenylalanine with Ca(II), Mg(II) and Zn(II) has been investigated pH metrically in acetonitrile-water mixtures 0.0-60.0% v/v at 303.0 K and 0.16 M ionic strength. The predominant species detected for Ca(II), Mg(II) and Zn(II) are ML, ML_2 and ML_2H_2.. Models containing different numbers of species were refined by using the computer program MINIQUAD75. The best-fit chemical models were arrived at based on statistical parameters. The trend in variation of complex stability constants with change in the dielectric constant of the medium is explained on the basis of electrostatic and non-electrostatic forces.*

Key words: *Complex equilibria, Chemical speciation, Phenylalanine, Acetonitrile, Metals*

INTRODUCTION:

The speciation study of metal ion complexes is useful to understand the role played by the active site cavities in biological molecules and the bonding behavior of protein residues with the metal ion. The species refined and their relative concentrations under the experimental conditions represent the possible forms of amino acids in bio fluids.
Calcium was among the first materials known to be essential in the diet. Milk and the dairy products are the important food sources of calcium for human populations [1-6]. Serum calcium is maintained within a narrow normal range, chiefly by resorption from the skeleton and alteration of urinary calcium loss and absorption from gut [7]. Calcium is essential for living organisms because it is a structural component of bones, teeth and soft tissues and activates many metabolic processes. Calcium is used to regulate the permeability and biological membrane (such as cell walls) potentials, which in turn control muscle and blood vessel expansion and contraction, nerve functions, secretion of hormones and enzymes and transmitting impulses throughout the nervous system [8]. Less than 1% of total body calcium is needed to support these functions. Calcium is also essential for proper blood clotting. The remaining 99% of the body's calcium supply is stored in the bones and teeth where it is present as phosphate i.e. hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), together with small amounts of $CaCO_3$, providing rigidity to the structure [9]. Bone itself undergoes continuous remodeling, with constant resorption and deposition of calcium into new bone [10].

Magnesium is a silvery white metal that ignites easily in air and burns with a bright light. Magnesium is used in products that benefit from being lightweight, such as car seats, luggage, laptops, cameras and power tools. It is also added to molten iron and steel to remove sulfur [1-6]. It is the main intracellular earth metal cation with a free concentration in the cytosol around 0.5 mmol/ L [11-15]. Magnesium absorption is presumed to occur throughout the small intestines of humans. Kidney is the important organ involved in Magnesium homeostatis. Magnesium is needed for more than 300 biochemical reactions in the body. It helps to maintain normal muscle and nerve function, keeps heart rhythm steady, supports a healthy immune system and keeps bones strong. Magnesium also helps to regulate blood sugar levels, promotes normal blood pressure and is known to be involved in energy metabolism and protein synthesis. Increasing magnesium intake may be a valid strategy to enhance cognitive abilities [16]. The chemistry of Mg^{2+} ion, as applied to enzymes, uses the full range of this ion's unusual reaction chemistry to fulfill a range of functions [17-19]. Mg^{2+} generally interacts with substrates through inner sphere coordination, stabilizing anions or reactive intermediates, also including binding to ATP and activating the molecule to nucleophilic attack. ATP, the main source of energy in cells, must be bound to a magnesium ion in order to be biologically active. What is called ATP is often actually Mg-ATP.

Zinc is the second most essential trace metal after iron and it plays an important role in biological systems [20-23]. Enzymes with a zinc atom in the reactive centre are widespread in biochemistry, such as the alcohol dehydrogenase in humans. It is found primarily in eye, kidney, liver, muscle and prostate. It is particularly rich in semen and is a key factor in prostate gland function and reproductive organ growth. It is a versatile ion as it can bind to different combinations of ligand types resulting in a broad range of stability, reactivity and functions. Zinc is transported through the blood by albumin and transferrin. Since transferrin also transports iron, excessive iron reduces zinc absorption, and vice-versa [24]. Amino acids and peptides increase the absorption of zinc in the gastrointestinal tract. A mixture of zinc and histidine in the ratio of 1:2 was to be quickly absorbed in comparison to zinc sulphate. This is because histidine keeps zinc in solution, without making it available for precipitation or to other ligands that do not have a direct effect on membrane zinc transport for complexation. An estimated 3,000 proteins in the human body contain zinc as prosthetic group. There are over 200 enzymes that contain zinc as a cofactor and about the same number of transcription factors. Zinc-containing enzymes are used by the body to regulate growth and development, promote fertility and aid digestion and nucleic acid synthesis. Zinc ion is a good Lewis acid, making it a useful catalytic agent in hydroxylation and other enzymatic reactions. Also zinc has flexible coordination geometry, allowing enzymes using zinc to rapidly shift conformations and perform biological reactions [25]. Mild or marginal deficiency of zinc in humans is characterized by neurosensory changes, oligospermia in males, hyperammonemia, decreased serum testosterone in males, decreased serum thymulin activity, decreased natural killer cell activity, alterations in T cell subpopulations, impaired neuropsychological functions [26] and decreased ethanol clearance [27]. Higher concentrations of zinc in human body suppress copper and iron absorption. Free zinc ions react with hydro chloric acid of gastric juice to form corrosive zinc chloride, which can cause damage to the stomach lining [28].

Phenylalanine (Phe) is an essential amino acid classified as nonpolar because of the hydrophobic nature of the benzyl side chain and it forms stable complexes with heavier metals according to Hard and Soft Acid Base theory. It is found naturally in the breast milk of mammals, cheeses, eggs, fish, meat and poultry. It is used in the manufacture of food and drink products and sold as a nutritional supplement for its analgesic and antidepressant effects. L-Phenylalanine is biologically converted into L-tyrosine, one of the DNA-encoded amino acids. L-tyrosine in turn is converted into L-DOPA which is further converted into dopamine, norpinephrine (noradrenaline), and epinephrine (adrenaline). The latter three are known as the catecholamines. The genetic disorder phenylketonuria (PKU) is the inability to metabolize phenylalanine. Individuals with this disorder are known as "phenylketonurics". In plants, Phenylalanine is the starting compound used in the flavonoid biosynthesis. Lignan is derived from phenylalanine and from tyrosine. Phenylalanine is converted to cinnamic acid by the enzyme Phenylalanine ammonia-lyase [29].
Acetonitrile(AN) is a colorless polar aprotic solvent [30]. It behaves as a weaker base [31] and as a much weaker acid [32] than water. It has a dielectric constant of 36 and autoprotolysis constant of 33.6. AN also acts as a strongly differentiating solvent with a modest solvating power for many polar ionic solutes [33].

Hence Phe is selected for speciation studies of its complexes with Ca(II), Mg(II) and Zn(II) in acetonitrile (AN)-water mixtures. The protonation constants of Phe and Mal in EG- and AN- water mixtures were reported earlier [34, 35].

EXPERIMENTAL

Materials

Acetonitrle (Merck, Mumbai) was used as received. Aqueous solutions of Phenylalanine and sodium chloride (E-Merck, Germany) were prepared. Metal solutions of Ca(II), Mg(II), Zn(II) chlorides were prepared. To increase the solubility of Phe and to suppress the hydrolysis of metal salts, the mineral acid concentration in the above solutions was maintained at 0.05M. To assess the errors that might have crept into the determination of the concentrations, the data have been subjected to analysis of variance of one way classification (ANOVA). The strength (concentration) of alkali has been determined using the Gran plot method [36, 37].

Apparatus

The titrimetric data were obtained with a calibrated ELICO (Model L1-120) pH-meter (readability 0.01) which can monitor the changes in H^+ concentration. The pH meter was calibrated with 0.05 M potassium hydrogen phthalate in acidic region and 0.01 M borax solution in basic region. The glass electrode was equilibrated in a well-stirred AN-water mixture containing inert electrolyte. All the titrations were carried out in the medium containing varying concentrations of AN (0-60.0 % v/v) maintaining an ionic strength of 0.16 M with sodium chloride at 303.0 ± 0.1 K. The effect of variations in asymmetry potential, liquid junction potential, activity coefficient, sodium ion error and dissolved carbon dioxide on the response of glass electrode were accounted for in the form of correction factor [38].

Procedure

For the determination of stability constants of metal-ligand binary species, initially titrations of strong acid with alkali were carried out at regular intervals to check whether complete equilibration was achieved. Then the calomel electrode was refilled with AN-water mixture of equivalent composition as that of titrand. In each of the titrations, the titrand consisted of approximately 1 mmol mineral acid in a total volume of 50 mL. Titrations with different ratios (1 : 2.5, 1 : 3.75 and 1 : 5.0 for Ca(II), Mg(II) and Zn(II)) of metal-to-ligand were carried out with 0.4 mol L^{-1} sodium hydroxide. Other experimental details are given elsewhere [39].

Table 1. Exhaustive modeling of Ca(II)-Phenylalanine complexes in 30% v/v AN-water. mixture. pH range = 2.8-9.9; Number of points = 42.

Model no.	log β_{mlh} (SD) 110	120	122	U_{corr}	Ske-Wness	Kur-tosis	χ^2	R-Factor
1.	9.67(22)	---	---	5.01	1.43	3.85	22.18	0.0207
2.	------	10.43(30)	----	3.08	-0.11	4.60	22.20	0.0271
3.	------	------	25.06(21)	3.21	1.55	4.32	23.19	0.0206
4.	-----	10.01(15)	25.07(29)	2.26	1.20	4.77	24.73	0.0232
5.	9.60(13)	------	25.32(50)	1.8	1.55	2.32	36.19	0.0206
6.	9.66(14)	10.85(22)	-------	1.85	1.43	3.32	22.99	0.0207
7.	9.13(6)	10.88(5)	25.02(7)	3.36	0.05	2.57	22.83	0.0143

Table 2: Best fit chemical models of Ca(II), Mg(II) and Zn(II) - Phemylalanine complexes in AN-water mixture.

% v/v AN	log β_{mlh}(SD) 110	120	122	NP	U_{corr}	χ^2	Kur-Tosis	Skew-ness	R-factor	pH-range
				Ca(II)						
0.0	7.94(5)	10.64(7)	23.56(9)	48	9.77	8.77	5.38	1.38	0.0315	2.7-9.9
10.0	7.32(8)	10.04(5)	23.79(11)	45	1.39	43.81	6.06	0.20	0.0220	2.8-9.9
20.0	8.38(3)	10.82(6)	24.56(10)	48	1.40	3.11	3.54	-0.11	0.0282	2.8-9.9
30.0	9.13(6)	10.88(5)	25.02(7)	42	3.36	22.83	2.57	0.05	0.0143	2.8-9.9
40.0	9.52(7)	11.32(6)	25.36(6)	47	0.65	17.50	5.20	0.58	0.0068	2.8-9.9
50.0	10.03(6)	12.92(8)	26.5(9)	51	4.07	10.29	6.10	0.86	0.0323	2.8-9.9
60.0	10.18(8)	13.56(9)	26.78(10)	50	3.59	7.01	2.47	-0.14	0.0294	2.8-9.9
				Mg(II)						
0.0	7.69(6)	11.01(5)	22.02(6)	41	3.34	5.68	3.55	1.43	0.0236	2.6-10.0
10.0	7.82(8)	11.52(12)	22.92(8)	40	4.88	5.47	2.97	0.10	0.0149	2.7-9.9
20.0	8.02(11)	12.04(10)	23.22(5)	49	3.89	5.87	2.41	0.26	0.0443	2.7-9.9
30.0	9.03(9)	12.52(6)	23.39(3)	44	6.04	13.45	5.21	1.12	0.0214	2.7-9.9
40.0	9.29(8)	12.72(8)	24.49(9)	37	4.20	15.34	2.62	0.29	0.0164	2.7-9.9
50.0	9.96(10)	13.06(8)	24.84(7)	49	0.29	18.95	3.99	-0.02	0.0227	2.7-9.9
60.0	10.33(9)	13.39(9)	25.16(5)	46	1.11	4.46	2.61	0.03	0.0091	2.7-9.9
				Zn(II)						
0.0	8.04(7)	12.14(2)	22.52(6)	56	5.11	6.41	4.96	1.05	0.0340	2.8-10.0
10.0	8.22(6)	12.76(8)	23.23(8)	59	3.14	20.90	5.57	1.20	0.0351	2.7-10.1
20.0	8.66(5)	13.12(7)	24.04(3)	59	2.18	27.07	5.52	0.77	0.0254	2.7-10.1
30.0	8.75(11)	13.53(7)	24.22(4)	54	9.44	23.38	5.38	1.35	0.0381	2.7-10.1
40.0	8.92(9)	13.85(8)	24.64(3)	52	3.76	23.38	4.62	0.19	0.0147	2.7-10.1
50.0	9.02(10)	14.04(9)	25.21(5)	59	0.55	21.37	4.12	0.64	0.0251	2.7-10.1
60.0	9.13(5)	14.55(10)	25.55(6)	56	5.06	14.19	3.45	0.57	0.0176	2.7-10.1

U_{corr} = U/ (NP-m) X 10^8; where m = number of species; NP = Number of experimental points

Table 3: Effect of errors in influential parameters on the Ca(II) Phenylalanine complex stability constants in 20% v/v AN-water mixture.

Ingredient	% Error	log β (SD)		
		110	120	122
	0	8.38(3)	10.82(6)	24.56(10)
Alkali	-5	10.12(82)	Rejected	Rejected
	-2	9.09(62)	Rejected	25.13(29)
	+2	9.41(42)	10.19(35)	25.66(31)
	+5	Rejected	11.21(48)	Rejected
Acid	-5	Rejected	Rejected	Rejected
	-2	9.34(12)	10.92(67)	Rejected
	+2	8.98(65)	10.87(63)	25,01(45)
	+5	10.51(9)	Rejected	Rejected
Ligand	-5	8.51(19)	10.82(65)	24.72(65)
	-2	8.54(16)	10.84(32)	24.32(16)
	+2	8.51(32)	10.85(43)	24.28(28)
	+5	8.35(12)	10.89(65)	24.81(59)
Metal	-5	8.75(62)	10.85(68)	24.72(38)
	-2	8.34(12)	10.14(11)	24.14(11)
	+2	8.24(21)	10.21(13)	24.15(12)
	+5	8.73(54)	10.47(52)	24.86(21)

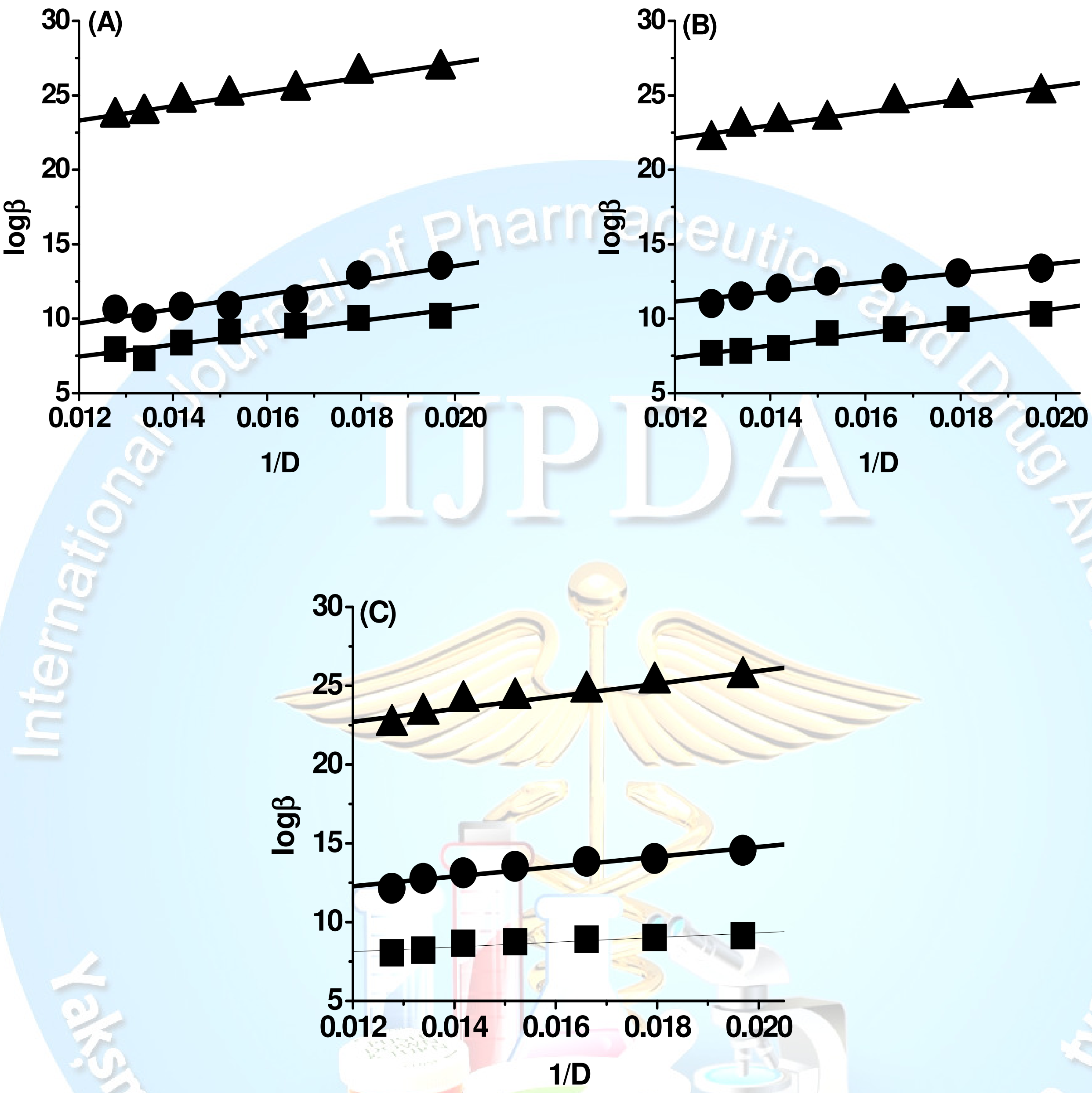


Figure 1. **Variation of stability constant values of metal-phenylalanine complexes with reciprocal of dielectric constants (1/D) in AN-water mixtures at temperature = 303 K and ionic strength = 0.16 M. (A) Ca(II), (B) Mg(II) and (C) Zn(II); (■) log 110, (●) log 120, (▲) log 122.**

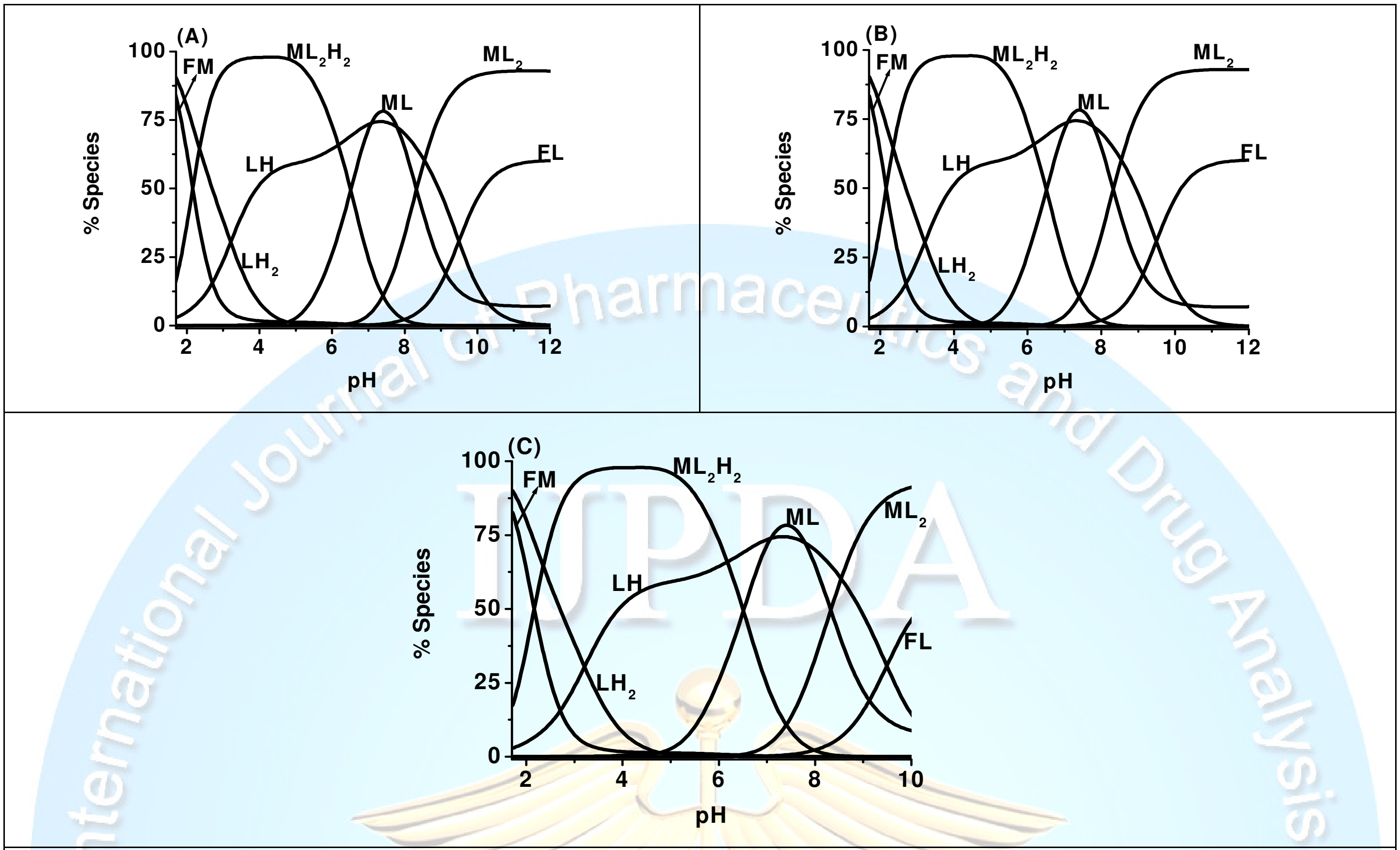
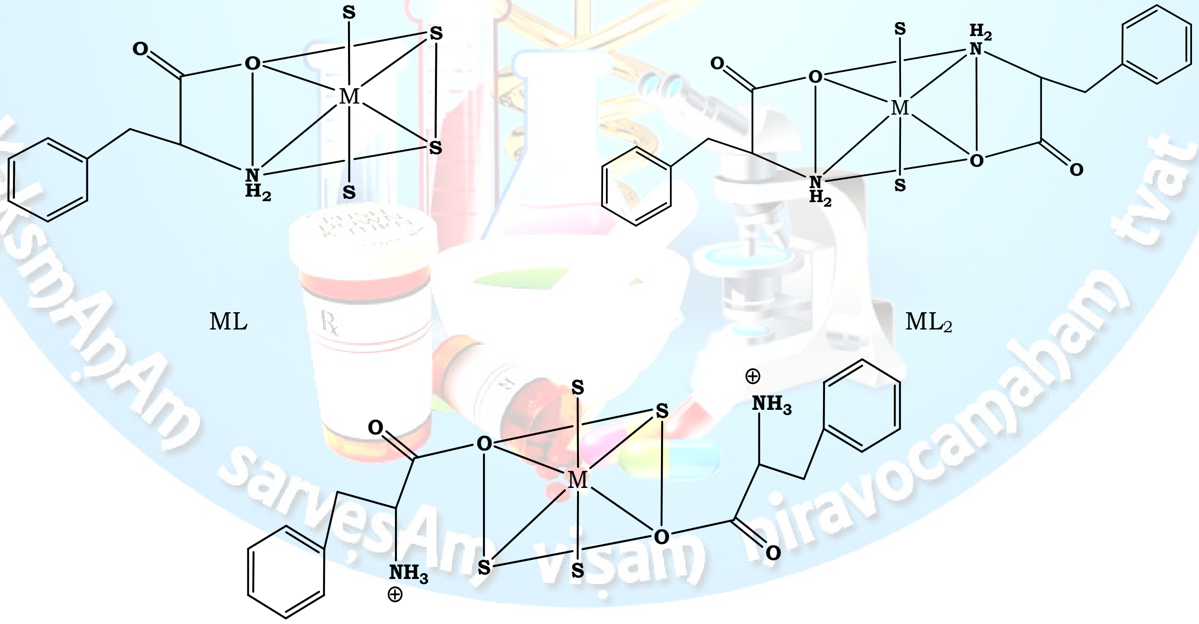


Figure 2. Distribution diagrams of phenylalanine complexes in 30% v/v AN-water mixture. Temperature = 303 K , ionic strength = 0.16 M. (A) Ca(II), (B) Mg(II) and (C) Zn(II).



ML

ML$_2$

ML$_2$H$_2$

Figure 3. Structures of binary complexes of Ca(II), Mg(II) and Zn(II) with Phe.

Modeling strategy

The computer program SCPHD [40] was used to calculate the correction factor. By using the pH-metric titration data, the binary stability constants were calculated with the computer program MINIQUAD75 [41], which exploits the advantage of the constrained least-squares method in the initial refinement and reliable convergence of Marquardt algorithm. During the refinement of binary systems, the correction factor and the protonation constants of phenylalanine are fixed. The variation of stability constants with the dielectric constant of the medium was analyzed on electrostatic grounds on the basis of solute-solute and solute-solvent interactions.

RESULTS AND DISCUSSION

Alkalimetric titration curves in AN-water mixtures revealed that the acido-basic equilibria of Phenylalanine (LH_2^+, LH and L^-) were active in the pH range 2.0-12.0. Based on the active forms of the ligands in this pH range, models containing various numbers and combination of complex species were fed to MINIQUAD75 along with the alkalimetric titration data. Exhaustive modeling was performed for Ca(II)-Phe in 30% v/v AN-water mixture and the results are given in Table 1. The models indicated better statistics as the number of species was increased, confirming better fit. There was no further improvement in the fit on inclusion of some more species in the model containing CaL, CaL_2 and CaL_2H_2. This indicates that the final model appropriately fits the experimental data. Such exhaustive modeling was performed for all the systems. The best-fit model was selected using the statistical parameters [42] of the least squares residuals.

The final models along with the statistical parameters are given in Table 2. The results of the best-fit models that contain the type of species and overall formation constants along with some of the important statistical parameters are given in Table 2. A very low standard deviation in log β values indicates the precision of these parameters. The small values of U_{corr} (sum of squares of deviations in the concentrations of ingredients at all experimental points corrected for degrees of freedom) indicate that the experimental data can be represented by the model. Small values of mean, standard deviation and mean deviation for the systems corroborate that the residuals are around a zero mean with little dispersion.

For an ideal normal distribution, the values of kurtosis and skewness should be three and zero, respectively. Kurtosis is a measure of the peakedness of the error distribution near a modal 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 the present study indicate that the residuals form leptokurtic as well as platykurtic patterns. The values of skewness recorded in the tables are between -0.11 and 1.43. These data evince that the residuals form part of a normal distribution. Hence, the least-squares method can be applied to the present data. The sufficiency of the model is further evident from the low crystallographic R-value recorded. These statistical parameters thus show that the best-fit models portray the metal-ligand species in AN-water mixture.

Effect of systematic errors on best-fit model

In order to rely upon the best chemical model for critical evaluation and application under varied experimental conditions with different accuracies of data acquisition, an investigation was made by introducing pessimistic errors in the influential parameters [43] like concentrations of alkali, mineral acid, ligand and metal (Table 3). The order of the ingredients that influence the magnitudes of stability constants due to incorporation of errors is alkali > acid > ligand > metal. Some species are even rejected when errors are introduced in the concentrations. This study confirms the appropriateness of the chosen best-fit models. This study also indicates the relative sensitivities of model parameters.

Effect of solvent

Variation of logarithmic values of stability constants (log β) with reciprocal of dielectric constant (1/D) are shown in Figure 1. AN is a protophilic, dipolar aprotic and coordinating solvent. It is a structure breaker of water and disrupts the water structure to form AN-water complex [44] of the formula $AN.H_2O$. When small amount of AN is added to water, the water structure breaks down resulting in more basic monomeric water molecules. Hence water molecules compete with the ligands for coordination with metal ions, decreasing the stability of the complexes. But the formation of solvent-water complex decreases the coordinating power of water. Variation of logarithmic values of stability constants (log β) or change in free energy with co-solvent content depends upon two factors, viz., electrostatic and non-electrostatic. Born's classical treatment [45] holds good in accounting for the electrostatic contribution to the free energy change. According to this treatment, the energy of electrostatic interaction is related to dielectric constant. Hence, the log β values should vary linearly as a function of 1/D of the medium. The linear trend observed in the present study (Figure 1) indicates that electrostatic forces are dominating the equilibrium process under the present experimental conditions.

The linear increase indicates the dominance of the structure-forming nature of AN over complexing ability. The cation stabilizing nature of co-solvent, 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 little deviation from the linear relationship.

Distribution diagrams

Phe has one dissociable carboxyl proton and an amino group that can associate with a proton. The different forms of Phe are LH_2^+, LH and L^- in the pH-regions 2.0-3.0, 3.0-9.0 and 9.0-12.0, respectively. Hence, the plausible species in different systems can be predicted from these data. The species refined and determined are ML, ML_2 and ML_2H_2 for Ca(II), Mg(II) and Zn(II).

The species distribution diagrams of various systems are shown in Figure 2. The formation of various binary complex species is shown in the following equilibria.

(a) $M(II) + LH \rightleftharpoons ML + H^+$
(b) $M(II) + LH_2 \rightleftharpoons ML + 2H^+$
(c) $ML + LH \rightleftharpoons ML_2 + H^+$
(d) $ML_2H_2 \rightleftharpoons ML_2 + 2H^+$
(e) $M(II) + 2LH_2 \rightleftharpoons ML_2H_2 + 2H^+$

Equilibria (a) and (b) is proposed for ML species. Equilibria (c) and (d) are proposed for the formation of ML_2 species. The (a) and (b) appears to be more appropriate between them because ML is formed with decreasing concentrations of LH and LH_2. Equilibrium (c) is possible for ML_2 species with decreasing the concentration of LH and (d) appears to be more appropriate because the concentration of MH_2 H_2 is decreasing with increasing concentration of ML_2. Equilibrium (e) is proposed the formation of ML_2H_2 with free metal and decreasing the concentration of LH_2. Depending on the coordinating atoms in the ligand and the nature of the metal ions, structures of the complexes are proposed for the species detected as shown in Figure 3.

CONCLUSIONS

1. The biomimetic studies of metal ion complexes with Phenylalanine in AN-water mixtures indicate the formation of ML, ML_2 and ML_2H_2.
2. The linear increase of log β values with 1/D of the medium indicate the dominance of electrostatic forces over non-electrostatic forces.
3. The order of ingredients influencing the magnitudes of stability constants due to incorporation of errors in their concentrations is alkali > acid > ligand > metal .
4. The stability constants of binary complexes are found to follow the trend Mg(II) < Zn(II) < Ca(II).

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