Medium Effects on the Protonation Equilibria of L-Norvaline†

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The protonation constant pKa_2 of L-norvaline has been determined in aqueous solution at different ionic strengths and different temperatures, using the pH-metric technique. The thermodynamic quantities ($\Delta G°$, $\Delta H°$, and $\Delta S°$) have been studied and discussed. Evaluation of the effect of organic solvent of the medium on the protonation processes was also reported and discussed. The organic solvents used were methanol, dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetone, and dioxane. The pKa_2 values for the ionization in a water (1) + dioxane (2) mixture [100 w_2 = 10 %, 20 %, 30 %, 40 %, and 50 %] have been determined at five different temperatures from T = (288.15 to 308.15) K. The thermodynamic quantities were calculated.

Introduction

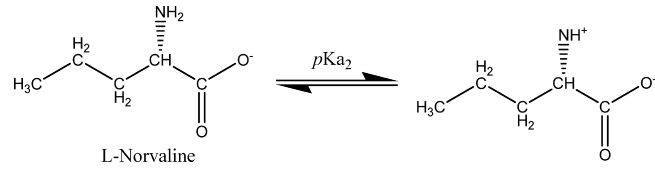
L-Norvaline, the analogue of the branched chain amino acid valine, is a member of a group of natural products known as nonprotein or unusual amino acids. These amino acids, as a group, possess a wide array of chemical structures and often contain functional groups not commonly present in natural products.[1] It was discovered for the first time during a search for the production of antitubercular antibiotics.[2−6]

It has been proposed that L-norvaline has antifungal activity as it inhibits the arginase enzyme thus increasing arginine concentrations.[7] More specifically, L-norvaline has been shown to inhibit homoserine dehydrogenase,[8] an enzyme needed for the conversion of aspartate to isoleucine, methionine, and threonine, and had no effect on the biosynthesis of aspartic acid and asparagine in tumor cells.[9] Human studies with norvaline supplements are not available, and hence no claims can be made regarding the benefit or side effect of norvaline supplementation.

The study of protonation and solvation processes in solutions of L-norvaline is important to elucidate the connection between chemical ability and biological activity. As polarity and activity of water are expected to be lower in an active site cavity of an enzyme than in bulk water, the protonation processes of L-norvaline studied in this investigation were examined in water containing different organic media, from which the thermodynamic data obtained would be useful to researchers in biomedicine. Thus, it is worthwhile to study systematically amino acids, peptides, and proteins in solvents having a different number of hydroxyl groups. These studies may shed some light on the mechanism about how organic solvents affect the stability of proteins.

Recently, the acid base behavior of the essential 20 amino acids, 10 hydroxamic acids, and 10 phenolic compounds in aqueous solution and in different solvent mixtures has been studied.[10−12] These studies provide precise knowledge about the thermodynamic quantities associated with the protonation process of L-norvaline. There have been no reports available on the determination of protonation constants of L-norvaline in aqueous−organic solvent mixtures. By virtue of the presence of the hydroxyl group, L-norvaline has high hydrogen-bonding capability. Thus, solvent effects on the ionization of L-norvaline in various (water + organic solvent) systems are of particular interest, especially with reference to specific (solute + solvent) interactions. The present work reports thermodynamics of the protonation equilibria of L-norvaline in water at different ionic strengths and different solvent mixtures.

Scheme 1. Chemical Structure of L-Norvaline and Its Protonation Equilibrium



L-Norvaline

Experimental Section

Materials and Solutions. Analytical grade L-norvaline (Sigma-Aldrish, USA) was used without further purification. L-Norvaline was assayed in triplicate by titration with a carbonate-free solution of standard NaOH. The assay result showed that the purity of the L-norvaline is 0.9994 ± 0.0005. The organic solvents used (methanol, DMF, DMSO, acetone, and dioxane) were of high purity (A.R. or spectro grade products). Carbonate-free sodium hydroxide solution was prepared by dissolving the Analar pellets in bidistilled water, and the solution was standardized potentiometrically with potassium hydrogen phthalate (Merck. AG). Nitric acid, sodium hydroxide, and sodium nitrate were obtained from Merck p.a.

Apparatus. pH-potentiometric titrations were performed using a Metrohm 796 titroprocessor with a 685 dosimate, a 728 magnetic stirrer, coupled with a dosino buret model 700. The precision of the instrument was ± 0.001 pH unit. pH titrations were carried out in an 80 cm^3 commercial double-walled glass

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Table 1. Protonation Constants (pKa_2) of L-Norvaline at Different Ionic Strengths (I/mol·dm^{-3}) and at Different Temperatures (T/K)

	pKa_2			
I/mol·dm^{-3}	T/K = 298.15	T/K = 308.15	T/K = 318.15	T/K = 328.15
000	10.210 ± 0.002	10.123 ± 0.007	10.034 ± 0.007	9.937 ± 0.008
0.02	9.964 ± 0.004	9.874 ± 0.007	9.785 ± 0.003	9.697 ± 0.006
0.06	9.902 ± 0.003	8.813 ± 0.007	9.742 ± 0.005	9.637 ± 0.006
0.10	9.882 ± 0.006	8.793 ± 0.003	9.702 ± 0.007	9.618 ± 0.005
0.15	9.730 ± 0.007	8.643 ± 0.003	9.554 ± 0.008	9.471 ± 0.007
0.20	9.589 ± 0.003	8.503 ± 0.004	9.417 ± 0.003	9.332 ± 0.007
0.25	9.432 ± 0.004	8.347 ± 0.003	9.263 ± 0.007	9.183 ± 0.003

Table 2. Thermodynamic Quantities (Gibbs Free Energy, $\Delta G°$, Enthalpy, $\Delta H°$, and Entropy, $\Delta S°$, Changes) for the Protonation Equilibria of L-Norvaline at Different Ionic Strengths (I/mol·dm^{-3})

I	$\Delta G°$	$\Delta H°$	$-\Delta S°$
mol·dm^{-3}	kJ·mol^{-1}	kJ·mol^{-1}	J·K^{-1}·mol^{-1}
000	52.952 ± 0.013	22.97 ± 0.013	101.349 ± 0.011
0.02	52.352 ± 0.025	22.92 ± 0.047	99.724 ± 0.046
0.06	52.124 ± 0.043	22.88 ± 0.057	98.259 ± 0.025
0.10	51.492 ± 0.027	22.78 ± 0.045	97.832 ± 0.018
0.15	51.075 ± 0.045	22.56 ± 0.068	97.104 ± 0.038
0.20	50.733 ± 0.035	22.40 ± 0.028	96.135 ± 0.017
0.25	50.150 ± 0.012	22.40 ± 0.015	94.496 ± 0.026

vessel. The ionic strength of the solution is maintained at constant level by using a desired concentration of $NaNO_3$ solution as the supporting electrolyte, and the temperature inside the cell was adjusted to the desired value by circulating thermostatted water using an oil-thermostatted setup. During the course of titrations, a stream of oxygen-free nitrogen was passed through the reaction cell to eliminate the adverse effect of atmospheric carbon dioxide.

Calibration of Glass Electrode Cell. A computer program (GLEE, glass electrode evaluation)[13,14] has been used for the calibration of a glass electrode by means of strong acid−strong base titration. This program provides an estimate of the carbonate contamination of the base, the pseudo-Nernstian standard potential and slope of the electrode, and optionally, the concentration of the base and pK_W. It uses a (nonlinear) least-squares refinement to fit a modified Nernst equation, eq 1 where E is the measured electrode potential; E^0 and s are

$$E = E^0 + s \log[H^+] \tag{1}$$

parameters of the refinement which represent the standard electrode potential and the slope of the plot of E versus log[H$^+$], respectively; and [H$^+$] represents the hydrogen ion concentration.

In acid solutions, the hydrogen ion concentration is obtained from the mineral acid concentration, T_H, as calculated from eq 2; that is, log[H$^+$] = log(T_H).

$$T_H = \frac{a_H v_0 + \gamma b_H v}{v_0 + v_1 + v} \tag{2}$$

a_H is the concentration (mol·dm^{-3}) of acid of which v_0 (cm^3) were added to the titration vessel; b_H is the concentration (mol·dm^{-3}) of base in the buret (by convention given a negative sign); v_1 is the volume (cm^3) of background electrolyte solution added to the titration vessel; and v (cm^3) is the volume of base added from the buret. γ is a correction factor for the base concentration: if γ is refined, the calculated base concentration is γb_H.

In alkaline solutions, the effective concentration of base is usually reduced by the presence of a small amount (preferably < 1 %) of carbonate contamination. The extent of this contamination can be estimated by means of a Gran plot.[13,14] Initially, E^0 is estimated from the acid region[13,14] and s is taken as the ideal Nernstian slope (T/5.0399 mV). Then, eqs 3 and 4 are fitted by linear least-squares.

Acid region:

$$(v_0 + v_1 + v)10^{\frac{E-E^0}{s}} = m^a v + c^a \tag{3}$$

Alkaline region:

$$(v_0 + v_1 + v)10^{\frac{E^0-E}{s}-pK_a} = m^b v + c^b \tag{4}$$

A typical Gran plot is designed. From the slopes and intercepts of the fitted lines, two estimates are obtained of the volume of base consumed at the equivalence point: $v_e{}^a = -c^a/m^a$ from the acid region and $v_e{}^b = -c^b/m^b$ from the alkaline region. Assuming that the difference is due to carbonate, the effective base concentration is reduced by the factor $v_e{}^a/v_e{}^b$ in the alkaline region. The mineral acid concentration in the alkaline region is then given by eq 5.

$$T_H = \frac{a_H v_0 + \gamma \frac{v_e{}^a}{v_e{}^a} b_H v}{v_0 + v_1 + v} \tag{5}$$

T_H is negative and log[H$^+$] = −pK_W − log(−T_H).

With these estimates of log[H$^+$], the standard potential and slope can be obtained by least-squares fitting of eq 1. The whole process, including the Gran plot, is repeated with the refined values of E^0 and s.

Briefly, the standardization of the electrode system was carried out, each time in organic solvent−water mixtures studied by Gran's method.[13,14] An estimated amount of solution, at the same conditions as temperature, ionic strength, and solvent composition, was placed in a double-walled, thermostatted vessel. The potential was allowed to stabilize after each addition of acid or base, and then the value was used to obtain the standard potential of the cell, E^0. The electrode was immersed in background solution, and it was titrated with a strong base in the same experimental conditions of ionic strength and solvent composition. Usually, starting from pH∼2 of the background solution, about 10 or 12 additions are enough to verify that E^0 is accurately determined. In the second step, a suitable amount of L-norvaline was added to the pretitrated background solution. From the potential values of the pretitrated background and that of the L-norvaline, and the volume of NaOH added, pK_a values were calculated using ESAB[15,16] and PKPOT[17] programs. These programs allow the thermodynamic acid−base constants in

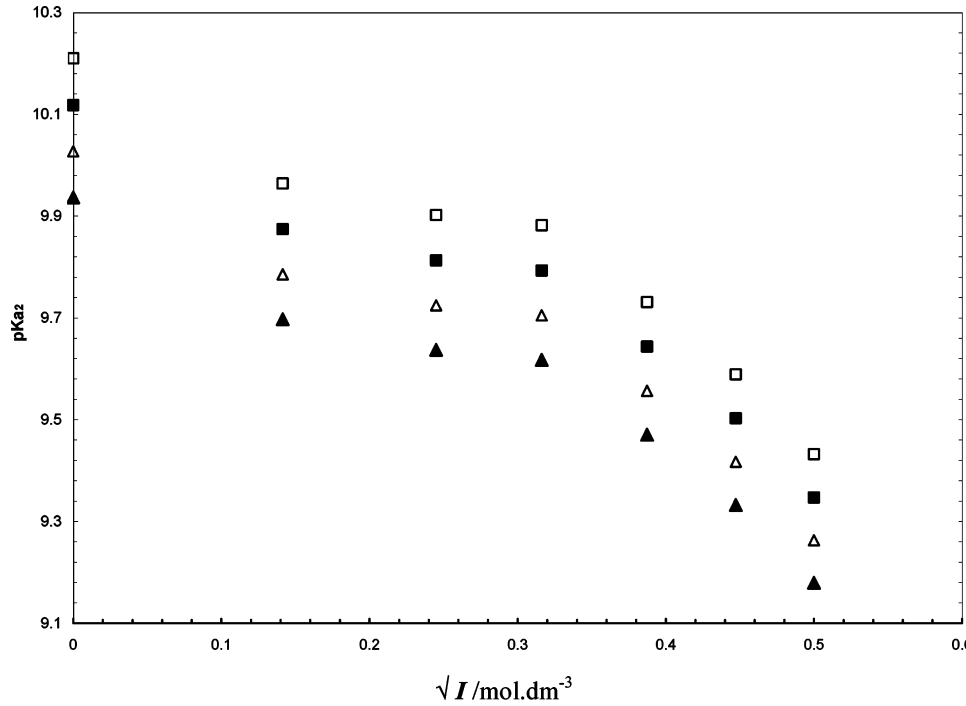

Figure 1. Plot of pKa_2 values of L-norvaline versus $\sqrt{I}$/mol·dm^{-3} at different temperatures: □, 298.15 K; ■, 308.15 K; Δ, 318.15 K; ▲, 328.15 K.

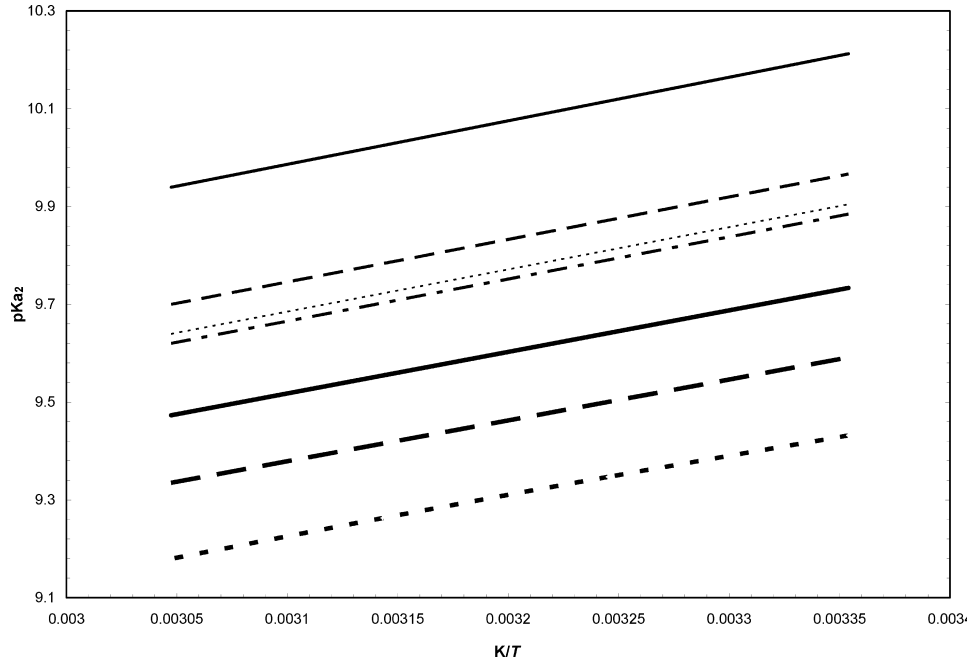

Figure 2. Plot of correlated pKa_2 values of L-norvaline versus K/T at different ionic strengths (I/mol·dm^{-3}): —, 0.00 mol·dm^{-3}; — — — —, 0.02 mol·dm^{-3}; ....., 0.06 mol·dm^{-3}; — - — - —, 0.10 mol·dm^{-3}; —, 0.15 mol·dm^{-3}; — — —, 0.20 mol·dm^{-3}; - - - -, 0.25 mol·dm^{-3}.

aqueous and nonaqueous media to be determined, taking into account the activity coefficient of the species.

Procedure for Equilibrium Titration. The following solutions were prepared (total volume 50 cm^3) and titrated potentiometrically against standard carbonate-free NaOH (0.1 mol·dm^{-3}) solution:

(a) 0.003 mol·dm^{-3} HNO_3 + 0.1 mol·dm^{-3} $NaNO_3$.

(b) Solution (a) + 0.001 mol·dm^{-3} L-norvaline.

The pH-metric titrations were carried out at the desired temperature in a purified nitrogen atmosphere. The temperature, maintained within ± 0.1 K, was controlled by circulating water from an ultrathermostat bath through the jacket. All the test solutions contained an appropriate proportion (w/w) of the different organic solvents studied. The total volume was adjusted to 50 cm^3 by adding double-distilled water. At each solvent percentage, at least four titrations were performed under carefully controlled experimental conditions. Typically, more than 60 pH readings (numbers of potentiometric measurements) were collected and taken into account for each titration.

Calculations. To account for the differences in acidity, basicity, dielectric constant, and ion activities for partially aqueous solutions relative to purely aqueous ones, pH values of the former solutions were corrected by making use of the procedure described by Douheret.[18,19] The pK_a values were calculated by adopting the Irving and Rossotti technique.[20,21]

Table 3. Protonation Constants (pKa_2) of L-Norvaline in a Water (1) + Organic Solvent (2) Mixture at $T = 298.15$ K and $I = 0.1$ mol·dm^{-3} $NaNO_3$

	pKa_2				
100 w_2	methanol	DMF	DMSO	acetone	dioxane
00	9.822 ± 0.007	9.822 ± 0.003	9.822 ± 0.007	9.822 ± 0.007	9.882 ± 0.007
10	9.314 ± 0.002	9.407 ± 0.005	9.501 ± 0.003	9.596 ± 0.006	9.692 ± 0.006
20	9.231 ± 0.005	9.323 ± 0.008	9.417 ± 0.006	9.511 ± 0.003	9.606 ± 0.002
30	9.042 ± 0.004	9.132 ± 0.004	9.224 ± 0.003	9.362 ± 0.006	9.409 ± 0.004
40	8.879 ± 0.004	8.968 ± 0.007	9.057 ± 0.004	9.149 ± 0.007	9.240 ± 0.007
50	8.698 ± 0.008	8.785 ± 0.006	8.873 ± 0.007	8.962 ± 0.008	9.051 ± 0.003

Computations related to the estimation of protonation constants were performed by regression analysis of titration curves using least-squares computer programs ESAB[15,16] and PKPOT.[17] PKPOT has been developed to run on a PC-compatible computer, for the study of ionic equilibria from potentiometric data. It allows for the refinement of equilibrium constants in systems described by up to 5 components and 20 complex species $A_pB_qC_rD_sH_t$. Standard potentials of electrode and reactant concentrations were refined. The program can deal with up to ten titration curves. It provides several statistical tests, as well as graphic presentation of data and residual distribution. The program allows for the determination of stoichiometric formation constants (at fixed ionic strength) or thermodynamic constants. The data analyzed are given as volume/emf or volume/pH for several application examples including titrations in aqueous and nonaqueous media.

The adequacy of a proposed regression chemical model with experimental data and the reliability of parameter pK_a can be estimated and examined by the goodness-of-fit test.[14]

The pKa_2 values were calculated from the relationship[20,21]

$$\bar{n}_H = \beta[H^+](1 + \beta[H^+])^{-1} \quad (6)$$

where β is the proton−L-norvaline formation constant and $\bar{n}_H$ is the average number of protons associated per mole of L-norvaline at several pH values. The following equation[20,21] was used for calculating the $\bar{n}_H$ values from the titration curves corresponding to solutions a and b.

$$\bar{n}_H = \left\{ yC_L + \frac{(V_a - V_b)C_b}{V_o} \right\}(C_L) - 1 \quad (7)$$

where y is the number of dissociable protons ($y = 1$ in the case of L-norvaline). V_a and V_b are the volumes of NaOH consumed to reach the same pH values in titration curves a and b, respectively. C_b and C_L are the concentration of NaOH and L-norvaline, respectively, and V_o is the original volume (50 cm^3).

Thermodynamic quantities ($\Delta G°$, $\Delta H°$, and $\Delta S°$) associated with the protonation equilibria of L-norvaline were calculated by the following equations.

$$\Delta G° = 2.303RT\mathrm{p}K_a \quad (8)$$

and

$$\Delta G° = \Delta H° - T\Delta S° \quad (9)$$

In rigorous thermodynamic calculations, The equilibrium constant (β_t) should be expressed in terms of activities of the component ions at equilibrium. However, for convenience, concentrations are generally used. For a particular species i, $a_i = (C_i)F_i$, where C_i is the concentration of the ion i, a_i is its activity, and F_i is its activity coefficient. This gives a stoichiometric constant β_s provided that the activity coefficients are maintained constant by working in a medium of constant ionic strength.

Results and Discussion

Scheme 1 shows the chemical structure of L-norvaline and its protonation equilibrium. Tables 1 and 2 summarize pKa_2 values and their related thermodynamic quantities evaluated for the protonation equilibrium of L-norvaline studied in 0.1 mol·dm^{-3} $NaNO_3$ in water at different ionic strengths [(0.00, 0.02, 0.06, 0.1, 0.15, 0.2, and 0.25) mol·dm^{-3}] and different temperatures [(298.15, 308.15, 318.15, and 328.15) K]. Certain functional groups found in biological molecules, in particular carboxylic acids or amino groups, can gain or lose H^+ depending on the availability of hydrogen ions (or protons) in the solution. It is worth mentioning that the pKa_1 value of L-norvaline investigated can be associated with the carboxylic acid function. This value is low (≤ 2.40) and exists in acidic solutions which is not in the physiological region and hence is not of interest. Therefore this value is not used in our calculations. The pH-metric data were measured in the range $2.5 \leq \mathrm{pH} \leq 11$. From the chemical structures shown in Scheme 1 and the second proton protonation constant (pKa_2) of L-norvaline that were measured potentiometrically and listed in Table 1, one can conclude that protonation equilibrium constants are controlled by the electronic effects of substituent groups.

The second proton protonation constant (pKa_2) value of 9.730 obtained at $T = 298.15$ K and $I = 0.15$ mol·dm^{-3} $NaNO_3$ agrees with those of other workers in aqueous medium within a very reasonable range.[22] The maximum deviation in pKa_2 values (Table 1) never exceeds more than 0.2 units. Such deviations between the results obtained by different physical methods are not unusual; examples are plentiful in the literature.[22]

The plot of pKa_2 versus $\sqrt{I}$ (Figure 1) fairly agrees with the Debye−Hückel equation.[23] A plot of pKa_2 versus $1/T$ at different ionic strengths gives a straight line (Figure 2). The thermodynamic equilibrium constants (at $I = 0.00$) were determined by applying linear regression analysis.

The thermodynamic quantities ($\Delta G°$, $\Delta H°$, and $\Delta S°$) associated with the protonation of L-norvaline were also studied at each ionic strength, and the values are presented in Table 2. The enthalpy changes for the protonation process are positive (endothermic). The positive values of $\Delta G°$ for the protonation equilibria of L-norvaline denote that the process is not spontaneous. In addition, the negative values of entropy changes point to increased ordering due to association.

The effects of organic solvents on the pKa_2 values of L-norvaline can be interpreted by using the solvate chromic quantitative values of Kamlet−Taft hydrogen bond acidity and basicity (α,β) and dipolarity polarizability π^* of the solvent.[23,24] These solvate chromic parameters can be used to quantify and

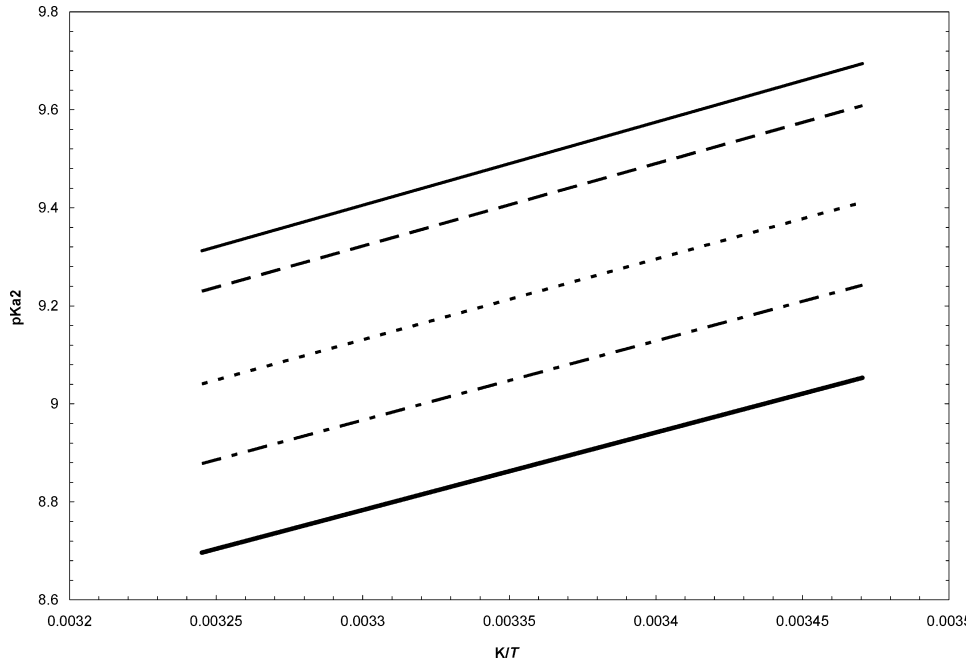

Figure 3. Plot of correlated pKa_2 values of L-norvaline versus K/T in a water (1) + dioxane (2) mixture (100 w_2): —, 10 %; − − − −, 20 %; - - - -, 30 %; - • - • -, 40 %; —, 50 %.

Table 4. Protonation Constants (pKa_2) of L-Norvaline in a Water (1) + Dioxane (2) Mixture at Different Temperatures, I = 0.1 mol·dm^{-3} $NaNO_3$

	pKa_2				
100 w_2	T/K = 298.15	T/K = 308.15	T/K = 318.15	T/K = 328.15	T/K = 298.15
10	9.692 ± 0.006	9.595 ± 0.008	9.499 ± 0.005	9.404 ± 0.004	9.310 ± 0.007
20	9.606 ± 0.005	9.510 ± 0.004	9.415 ± 0.004	9.321 ± 0.005	9.227 ± 0.005
30	9.409 ± 0.007	9.315 ± 0.005	9.222 ± 0.002	9.130 ± 0.005	9.038 ± 0.008
40	9.240 ± 0.003	9.148 ± 0.003	9.056 ± 0.004	8.966 ± 0.005	8.876 ± 0.006
50	9.051 ± 0.005	8.960 ± 0.004	8.872 ± 0.003	8.782 ± 0.003	8.694 ± 0.004

Table 5. Thermodynamic Quantities (Gibbs Free Energy, $\Delta G°$, Enthalpy, $\Delta H°$, and Entropy, $\Delta S°$, Changes) for the Protonation Equilibria of L-Norvaline in a Water (1) + Dioxane (2) Mixture at I = 0.1 mol·dm^{-3} $NaNO_3$

	$\Delta G°$	$\Delta H°$	$-\Delta S°$
100 w_2	kJ·mol^{-1}	kJ·mol^{-1}	J·K^{-1}·mol^{-1}
10	50.216 ± 0.003	25.432 ± 0.034	87.328 ± 0.113
20	50.306 ± 0.004	24.729 ± 0.053	95.763 ± 0.094
30	51.887 ± 0.003	23.357 ± 0.044	100.573 ± 0.078
40	52.851 ± 0.004	21.823 ± 0.092	114.712 ± 0.113
50	54.061 ± 0.007	19.782 ± 0.109	119.289 ± 0.124

rationalize multiple interacting solvent effects on the protonation equilibria of L-norvaline. The results are presented in Table 3.

The observed slight changes in the pKa_2 values of L-norvaline as the solvent is enriched in methanol can be mainly interpreted as resulting from the following two factors. The first is the relatively high stabilization of the conjugate bases by donor hydrogen bonds in a pure aqueous medium relative to that in the presence of methanol. This is due to the greater tendency of the water molecule to donate a proton in a solvent-to-solute hydrogen bond (α = 1.17). Considering only this effect, an increase in methanol proportion in the aqueous medium will result in an increase in the activity coefficient of the conjugate base, thereby causing a slight increase in the pKa_2 value.

The second is the greater stabilization of proton in (methanol + water) through (ion + solvent) interaction.[23−25] This effect will generate a low activity coefficient of proton, therefore causing a slight decrease in pKa_2 values.

The observed constancy in the pKa_2 value of L-norvaline in the presence of varying amounts of DMF and DMSO can mainly be explained as resulting from the following two opposing effects. First, (DMF + water) or (DMSO + water) mixtures are considered to be more basic than water.[23,24] This behavior is based on the building up of a strong acceptor hydrogen bond (β = 0.69 for DMF and 0.76 for DMSO) from the ($-^+$NH) group of L-norvaline in the former medium as compared to that in the latter one, thus facilitating the ionization process of the cationic ($-^+$NH) group, i.e., a low pKa_2 value. Second is the expected low stabilization of the conjugate L-norvaline free base by a hydrogen bond donated from solvent molecules in DMF or the (DMSO + water) mixture compared to that obtained in pure aqueous medium. This in turn results in a high pKa_2 value. The observed small increase in the pKa_2 values of L-norvaline when the amount of the organic cosolvent acetone (low basic aprotic solvent) in the medium is increased can be mainly attributed to a low stabilization of the free conjugate bases of L-norvaline by hydrogen bonding interaction. The observed increase in the pKa_2 of L-norvaline as the medium is enriched in the aprotic nonionizing dioxane solvent may be attributed to the fact that the release of the proton is rendered more difficult in the presence of this cosolvent.[25] This behavior is probably attributed to the lower β values of dioxane (β = 0.37).

The second protonation constants of L-norvaline were determined in a water (1) + dioxane (2) mixture [100 w_2 = 10 %, 20 %, 30 %, 40 %, and 50 %] at five different temperatures from T = (288.15 to 208.15) K at intervals of 5 K. A plot of pKa_2 versus 1/T gives a straight line as shown in Figure 3. The values of pKa_2, together with their standard deviations, are listed in Table 4.

As can be seen from Table 5, the slight increase in $\Delta G°$ as dioxane content changes from (10 to 50) mass fraction could possibly be explained by the decrease in the dielectric constant of the mixed solvent, which could then cause an increase in the electrostatic free energies of the various L-norvaline ions in solution produced by the protonation process. The values of $\Delta H°$ decrease with increasing organic content of the solvent mixture, suggesting that the second protonation process is becoming increasingly exothermic. The similarity between $\Delta H°$ values of the protonation processes of L-norvaline in water and (water + dioxane) indicates a similar pattern of solvation in these media. The values of $\Delta S°$ are expected to be negative in these solvents, and in (water + dioxane) they are more negative. This may be due to the fact that the degree of reorientation and partial immobilization of dioxane and water molecules by L-norvaline ions are greater in (water + dioxane) than in pure water.[25]

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