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Tautomeric Equilibrium of Pyridoxine in Water. Thermodynamic Characterization by ^{13}C and ^{15}N Nuclear Magnetic Resonance

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A remarkable temperature dependence on the ^{13}C NMR and ^{15}N NMR chemical shifts of pyridoxine in water (pH = 7.0) has been observed. C-3, C-6, and N-1 were the most sensitive nuclei to the temperature effect. This dependence has been explained on the basis of an equilibrium shift thermally induced between the neutral and the dipolar form of this molecule. The thermodynamic characterization of tautomeric equilibria that interconvert quickly on the NMR time scale can be carried out from the observed average ^{13}C NMR and ^{15}N NMR chemical shifts at different temperatures (5−90 °C). We have developed a new method for the estimation of the thermodynamic parameters of a given equilibrium by fitting the experimental data to a theoretical curve. This new method allows us to improve the fitting results on our previously proposed methodology. We show that there are linear correlations between the average chemical shifts obtained from different nuclei at the same temperature. This indicates that the parameters of the pure forms are related among them. We have carried out a simultaneous multiple function curve fitting of all data obtained from the most sensitive signals together using these linear correlations as restricted conditions in order to diminish the number of independent parameters to fit. To test the new methodology, we have studied the thermodynamics of the tautomeric equilibrium of pyridoxine in water. We have obtained $\Delta H°$ values ranging from -23.6 ± 1.3 to -25.8 ± 1.7 kJ/mol for this equilibrium depending on the used data set. This kind of methodology has, among others, the following advantages: It allows the use of a great number of experimental points from different signals in the fitting process, it yields very precise and accurate values of the tautomeric process, and it allows the resolution of the problem with only ^{13}C NMR data in some cases saving NMR time.

Introduction

The study of proton tautomerization processes is a field of great interest considering the importance of tautomerism in many areas of chemistry and biology.[1−4] It has also been the subject of a great number of theoretical studies in the gas phase. But, in many cases, quantum-chemical calculations on isolated molecules can lead to qualitatively incorrect results[5,6] because there is no correlation between the gas-phase theoretical predictions and the solution experimental tautomeric equilibrium constants.[5] The results obtained from theoretical studies in solution[7−9] provide, at best, a qualitative description of this phenomenon, since none of them is able to provide the difference in the free energy of solvation quantitatively. On the other hand, there is little experimental work about these processes in solution, despite the obvious importance of such quantitative thermodynamic data.[1,6,7,10]

The techniques used for the measurement of tautomeric populations are mainly spectroscopic.[11−13] One of the most used techniques is UV−vis spectroscopy, where the resolution of electronic spectra by log-normal curves[14−16] is necessary to obtain the spectra of the pure forms.

The temperature dependence on the NMR signals of compounds undergoing fast chemical exchange can be used as a sensitive probe for the equilibrium. In this way, the use of NMR spectroscopy has the same limits as other techniques used to calculate tautomeric equilibrium constants, because the chemical shifts of the pure tautomeric forms must be obtained indirectly. From NMR data, these calculations are much more reliable, as can be seen in the following text.

Experimental chemical shifts $\langle\delta\rangle$ obtained from the spectra of isomers that interconvert rapidly on the NMR

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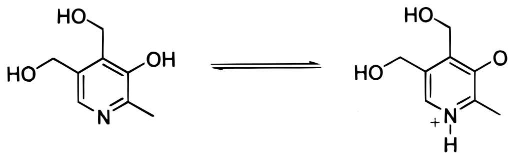
Scheme 1



^{0}**1** $^{\pm}$**1**

time scale are the weighed average of the contributing tautomeric forms. To estimate the tautomeric populations of the isomers, it is necessary to determine the chemical shifts for each pure tautomeric form. The study of this type of equilibrium has special difficulties due to the fact that, normally, it is not possible to measure directly the signals corresponding to the pure isomers. Several authors have determined the chemical shifts of the pure isomers from model compounds such as methyl derivatives, where tautomerism has been avoided by replacing H atoms with substituent groups.[12] However, much more reliable values for the extreme tautomeric forms can be obtained from NMR data by a fitting process, as can be seen in the following text.

In a previous paper,[11] following a methodology similar to what was established by Gutowsky et al.,[17] we described a new method for the thermodynamic characterization of tautomeric equilibria from multinuclear magnetic resonance techniques. This new method allowed us to calculate tautomeric equilibrium constants at different temperatures and the corresponding free energy, enthalpy, entropy, etc. of the tautomerization process from variable-temperature ^{13}C NMR and ^{15}N NMR signals.

In this work, we have further developed our method in order to improve the accuracy of the results, concerning several aspects of both the theoretical approach and the requirement of experimental data. As an example, we have applied this new method to the study of the tautomeric equilibrium $^{0}\mathbf{1} \rightleftharpoons {}^{\pm}\mathbf{1}$ of pyridoxine (Scheme 1) in aqueous solution, where ^{0}**1** and $^{\pm}$**1** represent the neutral and the dipolar forms, respectively. We have measured the ^{13}C and ^{15}N NMR spectra at temperatures ranging from 5 to 90 °C. From this information, the thermodynamic parameters of the tautomeric equilibrium can be easily obtained.

Experimental Section

Measurements were performed either in a Bruker WM-250 or a Bruker ARX-400 spectrometer. Commercial samples of pyridoxine (Aldrich) were used without further purification. ^{13}C NMR variable-temperature experiments were performed in a 10 mm tube with a solution of pyridoxine 0.08 M in H_2O (2 mL) at pH 7.0, adjusted with solid sodium hydroxide and sodium dihydrogen ortophosphate. As external reference, a capillary tube filled with hexamethyl disiloxane (HMDSO) ($\delta = 1.93$ ppm) was used, and the field was locked to external DMSO. The FID was acquired with 32 K points and transformed with zero filling. A delay of 900 s was used to stabilize each temperature. The spectral width was 200 ppm, and the total acquisition time for each pulse was 1 s.

^{15}N NMR variable-temperature experiments were recorded in a 5 mm tube with a 0.08 M solution of pyridoxine in water buffered to pH 7.0 in the same previous way. A capillary coaxial tube filled with a 1:4 mixture of nitromethane and nitromethane-d_3 was used to lock the magnetic field and also as external reference ($\delta = 0$ ppm). Measurements were carried out in the temperature range 5−90 °C. ^{15}N NMR chemical shifts were obtained from the cross-peak between the nitrogen and the methyl protons of a series of HMBC experiments recorded with a spectral width of 7.0 ppm in F2 and 220 ppm in F1. Each experiment was recorded with 1K data points in F2 and a total of 512 data points in F1. Processing in the F1 dimension was carried out with zero filling leading to a digital resolution of 8.705 Hz/pt (0.215 ppm/pt). Each temperature was stabilized for 900 s before the acquisition started.

Theory and Methods

If the relaxation time of an equilibrium $A \rightleftharpoons B$ is small compared with the NMR time scale, the average chemical shift of a particular nucleus, $\langle\delta\rangle$, is given by

$$\langle\delta\rangle = x_A \cdot \delta_A + x_B \cdot \delta_B \tag{1}$$

where x_A, x_B, δ_A, and δ_B are the molar fractions and the chemical shifts of the A and B forms, respectively.

When NMR spectra are obtained at different temperatures, because a shift in the equilibrium is induced, a change of the average chemical shift will be observed. The magnitude and direction of this change will depend on the magnitude and sign of the process $\Delta H°$. Equation 1 will hold at any temperature, but the population and the chemical shifts of the pure forms will be unknown. The method described previously[11] allowed us to estimate these populations, the equilibrium constant as a temperature function, and the standard enthalpy change of the process. This model is based on two assumptions: (a) a temperature independence of the chemical shifts corresponding to the pure forms A and B and (b) a constant value for the standard enthalpy change in the overall temperature range.

The tautomerization equilibrium constant is defined as

$$K(T) = \frac{x_B}{x_A} = \frac{\langle\delta\rangle - \delta_A}{\delta_B - \langle\delta\rangle} \tag{2}$$

Taking a given temperature corresponding to an experimental datum as a reference (T_r, δ_r) and combining the integral form of Van't Hoff equation with eq 2 we can get the following equation

$$\langle\delta\rangle = \frac{\delta_A\Delta_B + \delta_B\Delta_A E}{\Delta_B + \Delta_A E} \tag{3}$$

where

$$\Delta_B = \delta_B - \delta_r,\ \Delta_A = \delta_r - \delta_A, \quad \Delta T = T - T_r$$

and

$$E = \exp\left[\frac{\Delta H° \Delta T}{R T T_r}\right]$$

where $\Delta H°$ is the standard enthalpy change of the process.

Equation 3 predicts for an exothermic process ($\Delta H° < 0$), a nonsymmetrical sigmoid curve with a high-temperature asymptote equal to δ_A (δ_B for $\Delta H° > 0$) and a low-temperature asymptote equal to δ_B (δ_A for $\Delta H° > 0$). Thus, the standard enthalpy change and the chemical shift of the pure forms can be estimated by a nonlinear fitting procedure using eq 3, where the $\langle\delta\rangle$ and T values are the experimental results. For each series of experi-

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mental data, the fitting of the average chemical shifts to eq 3 together with the temperature of any nucleus sensitive to the prototropic exchange must give the same thermodynamic parameters within the experimental error.

To carry out the fitting process by eq 3, it is necessary to use an experimental data point (δ_r, T_r) as reference, and obviously, the experimental error of these data can affect the reliability of the fitting process. A new equation similar to eq 3 can be developed in order to avoid this problem. From eq 2 and the integrated Van't Hoff equation, it is possible to obtain

$$\langle\delta\rangle = \frac{\delta_A + \delta_B K_{T_r} E}{1 + K_{T_r} E} \qquad (4)$$

where δ_A, δ_B, and E have their usual meaning and K_{T_r} is the equilibrium constant of the tautomeric process at the reference temperature, T_r.

Equation 4 introduces a new parameter in the fitting process, K_{T_r}, which is a disadvantage.

At the same temperature, the average chemical shifts from different nuclei are a tautomer population measurement, and they must allow the same value of the equilibrium constant to be obtained. Therefore, it is possible to equalize eq 2 for two series of average chemical shifts data from two sensitive nuclei obtained at the same temperature. In this way, eq 5 is obtained

$$\langle\delta\rangle_1 = \frac{\delta_{A1}\delta_{B2} - \delta_{A2}\delta_{B1}}{\delta_{B2} - \delta_{A2}} + \frac{\delta_{B1} - \delta_{A1}}{\delta_{B2} - \delta_{A2}}\langle\delta\rangle_2 \qquad (5)$$

where subscripts 1 and 2 refer to data from the two different nuclei. Equation 5 indicates that a plot of $\langle\delta\rangle_1$ versus $\langle\delta\rangle_2$, for data obtained at the same temperatures, is a straight line with a slope $(\delta_{B1} - \delta_{A1})/(\delta_{B2} - \delta_{A2})$. Effectively, we obtain very good linearity among the different signals with a high accuracy (see below). Equation 5 also indicates that δ_{B1}, δ_{A1}, δ_{B2}, and δ_{A2} are not independent parameters.

It is worth indicating that when the fitting of the nucleus 1 data to eq 3 or eq 4 is carried out, only the parameters δ_{B1} and δ_{A1} are obtained but it is not possible to get any information about δ_{B2} and δ_{A2} or any other parameter from other nuclei. Hence, it is only possible to check the coincidence of both results—the fitting process and the slope of the straight line—at the end of the whole process.

To use these linear correlations as restricted conditions, we have carried out a multiple function curve fitting of several signals simultaneously. By means of this calculation, it is possible to analyze together different sets of data from each nucleus. The functions share both the temperature and the standard enthalpy of the process but have individual values for δ_{A1}, δ_{B1}, ... δ_{An} and δ_{Bn}. This kind of methodology has a number of advantages:

(a) It is possible to analyze all or part of the data simultaneously.

(b) It is possible to use a great number of experimental data points in the same fitting process.

(c) Since the value of the standard enthalpy of the tautomerization obtained from the different sets of data must be the same, this parameter is a common one for all data using multiple function curve fitting.

Table 1. Temperature Dependence of ^{13}C NMR and ^{15}N NMR Average Chemical Shifts (ppm) for the Equilibrium ^{0}1 ⇌ $^{\pm}$1 of Pyridoxine in Water (pH = 7)[a,b]

T	N-1	C-3	C-2	C-4	C-5	C-6
278	−171.79	161.04	144.09	138.83	135.55	123.75
283	−170.53	160.93	144.17	138.77	135.48	124.04
285	−170.01	160.89	144.20	138.75	135.47	124.17
288	−169.23	160.81	144.25	138.71	135.42	124.34
293	−166.67	160.68	144.34	138.63	135.36	124.65
298	−164.89	160.54	144.44	138.54	135.30	124.97
303	−162.80	160.40	144.52	138.46	135.24	125.26
308	−160.67	160.23	144.65	138.34	135.17	125.66
313	−158.05	160.05	144.77	138.22	135.09	126.06
318	−155.08	159.86	144.91	138.09	135.02	126.46
323	−152.46	159.67	145.05	137.95	134.94	126.88
328	−149.89	159.43	145.19	137.78	134.85	127.37
333	−147.32	159.26	145.35	137.65	134.80	127.77
338	−144.82	159.04	145.44[b]	137.49	134.71	128.17
343	−141.56	158.85	145.83[b]	137.38[b]	134.63	128.61
348	−138.52	158.59	145.90	137.10	134.53	129.20
353	−137.07	158.41	145.96	136.97[b]	134.47	129.52
358	−134.19	158.26	146.06[b]	136.79[b]	134.41	129.94
363	−131.22	158.08	146.25	136.69	134.36	130.32

[a] Reference: HMDSO, $\delta = 1.93$ ppm. [b] Values determined with a small signal to noise ratio.

(d) The relationship between the chemical shifts of the pure forms of the different nuclei can be included as a restricted condition, which diminishes the number of independent parameters to fit (see below).

(e) This methodology provides very precise and accurate values for all the process parameters.

(f) When old fitting procedures are used, it is necessary to obtain ^{15}N NMR data in order to get reliable results due to the relatively low effect of the temperature on ^{13}C NMR chemical shifts. However, the advantages of the multiple function curve fitting together with the linear correlation allow the accurate resolution of the problem with ^{13}C NMR data only.

(g) The possible error induced by the temperature effect on the reference has been minimized because signals obtained from the same measurement are correlated.

The chemical shifts of the pure forms A and B have been considered as constant parameters. It is known that the chemical shifts are intrinsically dependent on the temperature. However, these changes of chemical shifts are small when compared with the change induced by the tautomeric equilibrium. Since the method reliability can be affected by a possible effect of the temperature on the chemical shifts of the pure forms A and B and the references, this topic will be discussed at the end of the next section.

Results and Discussion

We have measured the ^{13}C NMR and ^{15}N NMR spectra of pyridoxine in water (pH = 7) at temperatures ranging from 5 to 90 °C. Under these conditions, the pyridoxine is present in solution in equilibrium between the neutral ^{0}**1** and the zwitterionic form $^{\pm}$**1** in the equilibrium (see Scheme 1).[14,18]

The ^{15}N NMR and ^{13}C NMR spectra show one and nine signals, respectively, being well-defined and sensitive to temperature changes. The measured average chemical shifts of the most sensitive nuclei at different temperatures are given in Table 1. The greatest temperature

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Table 2. Parameters and Standard Errors Obtained by Fitting the ^{13}C NMR and ^{15}N NMR Average Chemical Shifts of Pyridoxine in Water (Table 1) to Eq 3[a]

	C-3	C-2	C-4	C-6	N-1
δ_A	155.58 ± 0.3	147.91 ± 0.7	134.14 ± 0.6	137.27 ± 0.9	−94.5 ± 6
δ_B	161.51 ± 0.05	143.84 ± 0.1	139.07 ± 0.06	122.39 ± 0.2	−180.2 ± 1
$-\Delta H°$	27.1 ± 1	29.4 ± 5	28.7 ± 2	23.7 ± 1	24.9 ± 1
R	0.9998	0.9976	0.9994	0.9998	0.9998
SEF	0.018	0.050	0.025	0.042	0.30
$\Delta\delta$	2.96	−2.16	2.14	−6.57	−40.57
$\Delta\delta_{AB}$	−5.93	4.07	−4.93	14.88	85.69
slope	−0.07355	0.05356	−0.05325	0.16082	

[a] The correlation coefficient and the standard error of the fit (SEF) are also included. Chemical shifts are given ppm, standard enthalpy change $\Delta H°$ in kJ/mol. $\Delta\delta = \langle\delta\rangle_{278} - \langle\delta\rangle_{363}$, $\Delta\delta_{AB} = \delta_A - \delta_B$ ppm

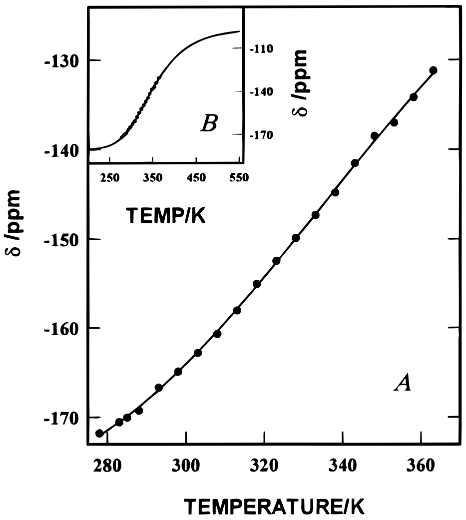

Figure 1. (A) Temperature dependence of ^{15}N NMR average chemical shifts (ppm) for the equilibrium $^{0}\mathbf{1} \rightleftharpoons {}^{\pm}\mathbf{1}$ of pyridoxine in water. The line is the theoretical curve calculated using eq 3 and the parameters given in Table 3. **(**B) The theoretical curve has been simulated in an extended range of temperature.

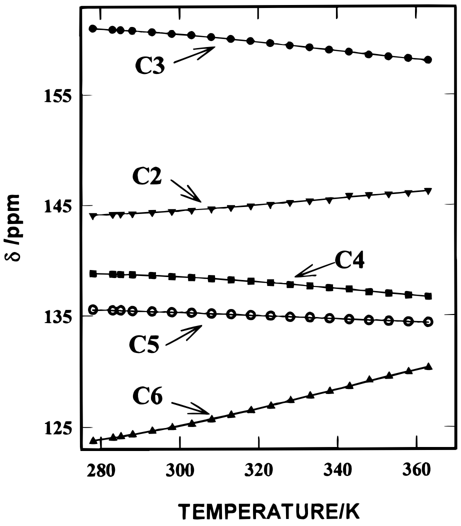

Figure 2. Temperature dependence of ^{13}C NMR average chemical shifts (ppm) for the equilibrium $^{0}\mathbf{1} \rightleftharpoons {}^{\pm}\mathbf{1}$ of pyridoxine in water. The continuous line is the theoretical curve calculated using eq 3 and the parameters given in Table 3.

effect becomes visible in the ^{15}N NMR spectra, where $\Delta\delta_{max} = -40.57$ ppm ($\Delta\delta_{max} = \langle\delta\rangle_{278} - \langle\delta\rangle_{363}$ ppm, see Table 2). The ^{13}C NMR chemical shifts are less influenced, and some of them are not feasible for this calculation. In the ^{13}C NMR spectra, the greatest temperature effect arises in the signal that can be assigned to C-6, followed by the signals corresponding to C-3, C-2, C-4, and C-5 (see the $\Delta\delta$ values in Table 2).

We did not apply eq 3 to nuclei with small temperature effects such as C-5 (135.55−134.36 ppm), C-5′-CH_2OH (58.01−58.60 ppm), C-4′-CH_2OH (55.55−56.42 ppm), and C-2′-CH_3 (15.27−16.22 ppm). This low-temperature effect over the average chemical shifts of these nuclei can be logically explained: C-5 is in the position meta to both the nitrogen and the hydroxyl group and therefore must be the least sensitive nucleus inside the ring to the electronic changes induced by the temperature-shifted equilibrium. The other insensitive signals correspond to carbons outside the ring and are too far away to notice the equilibrium changes. We have performed experiments in order to assign the carbon signals. Most of the signals were readily identified. Nevertheless, we could not clearly differentiate between C-4 and C-5 through routine 2D NMR correlations, but they could be assigned on the basis of behavior toward the equilibrium shift. These assignments completely agree with Abbott's paper, one of the previous studies on pyridoxine reported in the literature.[19−21]

Table 2 summarizes the results obtained by nonlinear fitting of the experimental chemical shifts of the most sensitive nuclei to eq 3. To obtain reliable results of the fitting process, we measured a great number of experimental data (19 points) in a broad range of temperatures (5−90 °C). Table 2 also shows the calculated chemical shifts of the pure forms, the standard enthalpy of the process, the correlation coefficient, and the standard error of the fitting (SEF). These two statistical parameters indicate that all the fitting processes are very reliable.

The obtained $\Delta H°$ values must be independent of the considered nucleus within the experimental error (Table 2). The values obtained from the C-2 and C-4 data fitting have a higher standard error and a slightly different value. This fact can be argued on the basis that they are more insensitive to temperature variation. This reason led us to use only chemical shifts from C-6, C-3, and the N-1 signals in further calculations.

Figure 1A displays the raw data of the N-1 signal and the fitted calculated curve using eq 3 and the parameters presented in Table 2. No systematic deviations between the calculated and the experimental values are observed. Furthermore, the residuals are scattered in an interval equal to or less than the experimental error associated with the measurements of the chemical shifts. Figure 1B displays the same experimental points, but the theoretical line has been simulated in an extended range of temperature in order to show that it is necessary to carry out measurements in the broadest possible temperature range. Figure 2 displays the same results for all of the ring carbon signals as a function of the temperature. It can be noticed that the most sensitive signal to the temperature variation, the C-6 nucleus, shows a smooth curvature with a change of −6.57 ppm. It can also be

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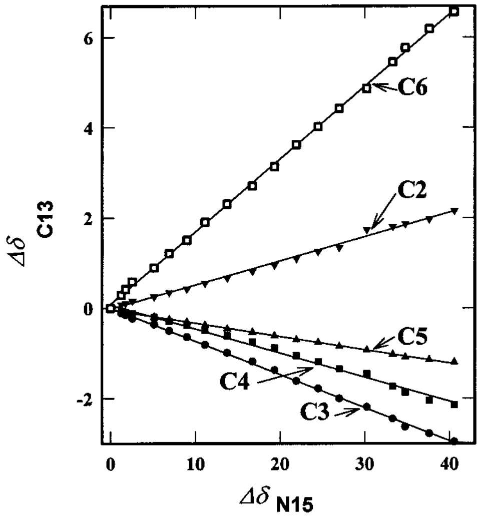

Figure 3. Linear plots of $\Delta\delta_{C13}$ versus $\Delta\delta_{N15}$, defined as $\Delta\delta = \langle\delta\rangle_T - \langle\delta\rangle_{278}$ (Table 1).

observed that, in general, the coincidence between experimental data and theoretical lines is very good.

We also carried out the fitting process using eq 4 in the same way as the one followed with eq 3. In this case, the results did not improve significantly. Possibly, this disappointing result is due to the fact that the new parameter to fit, K_{T_r}, is not independent of the chemical shifts of the pure forms.

Equation 5 indicates that the plot of the chemical shift of a given signal versus the chemical shift of other nuclei at different temperatures must be linear, with a slope value of $(\delta_{B1} - \delta_{A1})/(\delta_{B2} - \delta_{A2})$. Figure 3 shows the plot of all carbon ring signals versus the N-1 signal. The chemical shift values are shown in relative value as increments to the chemical shift at $T = 278$ K ($\Delta\delta = \delta - \delta_{278}$). Straight lines with very high correlation coefficients were obtained in all cases. The slopes for all the lines are given in Table 2. These slopes relate the chemical shifts of the pure forms obtained from each C signal to the N-1 signal.

In addition, we have carried out a multiple function curve fitting of the most sensitive nuclei signals, C-3, C-6, and N-1 (19 × 3 data) in order to improve the results. As a great advantage, this methodology allows a big increase in the number of the data to fit, and consequently, the error of the fitted parameters diminishes greatly. The major drawback is the total number of independent parameters to fit. To avoid problems, this number of parameters must be as low as possible.

Three parameters, δ_A, δ_B, and $\Delta H°$ (Table 2), are necessary to fit the experimental data of an isolated nucleus to eq 3. The number of independent parameters in the multiple function curve fitting for three nuclei simultaneously is seven, six for the chemical shifts and one for the enthalpy, since $\Delta H°$ must be the same. In this way, it is possible to obtain very good fitting due to the high number of experimental data. Therefore, the use of the linear correlations mentioned above reduces the number of parameters and the fitting improves strongly. In fact, the slopes given in Table 2 relate $\delta_A - \delta_B$ for the signal C-3 or C-6 with the corresponding chemical shift for the N-1 signal. Using the two equations as restricted conditions, the total number of independent parameters to fit diminishes from seven to five.

Table 3. Parameters and Standard Errors Obtained from a Multiple Function Curve Fitting of the C-3, C-6, and N-1 Signals to Eq 3 or Eq 4

	C-3	C-6	N-1	F test
δ_A	155.0 ± 1	136.4 ± 1	−94.3 ± 3	6.08e + 6
δ_B	161.6 ± 0.1	122.5 ± 0.2	−180.2 ± 0.6	
$-\Delta H°$	24.8 ± 0.9 kJ/mol			eq 3
δ_A	155.3 ± 0.2	136.4 ± 0.5	−94.2 ± 3	9.4e + 6
δ_B	161.66*[a]	122.53*[a]	−180.2 ± 0.6	
$-\Delta H°$	24.8 ± 0.9 kJ/mol			eq 3
δ_A	155.55 ± 0.3	135.86 ± 0.6	−97.1 ± 4	5.0e + 6
δ_B	161.63 ± 0.1	122.56 ± 0.1	−179.74 ± 0.7	
$-\Delta H°$	25.7 ± 1.2 kJ/mol; $K_{Tr} = 4.6 \pm 0.1$			eq 4
δ_A	155.55*[a]	135.86*[a]	−97.1 ± 3	7.6e + 6
δ_B	161.63 ± 0.06	122.56 ± 0.1	−179.74 ± 0.7	
$-\Delta H°$	25.7 ± 1 kJ/mol; $K_{Tr} = 4.6 \pm 0.1$			eq 4

[a] Calculated values, see text. Standard error of the fit = 0.17.

Table 3 contains the calculated parameters and the standard errors obtained from a multiple function curve fitting using eq 3 or eq 4 and different initial conditions. The results obtained by using both equations agree very well within the experimental error. We also calculated an F test as a criterion of the fitting goodness. In the first section of results (Table 3), the seven parameters are considered independent variables, and in the second one, the calculation is carried out with five independent parameters and the two linear equations. The values indicated by an asterisk have been calculated from the two linear equations. Comparison of the results from the two sections indicates that the values of the parameters do not change significantly but the standard errors diminish strongly in the second section. The F test also improves in the second case, indicating that it is a very reliable calculation method. If only one restricted condition is used, the accuracy of the results is intermediate. (Results are not shown.)

The values given in sections three and four have been obtained with eq 4 and the same initial conditions as in sections 1 and 2, respectively. The results show the same behavior, the error of the parameters diminishes and the F test increases when the total number of parameters to fit is small. In conclusion, the best result is obtained by using eqs 3 and/or 4, multiple function curve fitting, and as restrictions, the linear correlations between the chemical shifts of the pure forms in order to diminish the total number of parameters to fit.

When the same methodology is only applied to the ^{13}C NMR values, another interesting conclusion becomes visible (Table 4). In this example, we include four signals (C-2, C-3, C-4, and C-6, 19 × 4 data points). As before, the results improve when the restricted equations in the calculations are included and there are not significant differences using either eq 3 or eq 4. In the same way, there are not significant differences with the results given in Table 3. In conclusion, we arrive at the same results only with the ^{13}C NMR spectra, and it is not necessary to measure the ^{15}N NMR chemical shifts, which is a time-demanding experiment. This conclusion is valid only in systems where the enthalpy of the process is high enough; when the enthalpy is low, it would be possibly necessary to obtain experimental results from both techniques together, to obtain reliable results.

Metzler et al.[14] have reported for this equilibrium, obtained by resolution of UV−vis spectra in log-normal curves, a value of approximately $\Delta H° = -13.2$ kJ/mol.

Table 4. Parameters and Standard Errors Obtained from a Multiple Function Curve Fitting of the C-3, C-2, C-4, and C-6 Signals to Eqs 3 and 4

	C-3	C-2	C-4	C-6	F test
δ_A	154.58 ± 0.5	149.16 ± 0.5	132.11 ± 1	137.23 ± 0.8	948135
δ_B	161.67 ± 0.07	143.67 ± 0.05	139.20 ± 0.04	122.41 ± 0.1	
$-\Delta H°$		23.6 ± 1.1 kJ/mol; SEF = 0.038			eq 3
δ_A	154.68 ± 0.5	148.74*[a]	134.25*[a]	137.81*[a]	1.07e + 6
δ_B	161.68 ± 0.07	143.62 ± 0.05	139.35 ± 0.05	122.50 ± 0.1	
$-\Delta H°$		23.6 ± 1.3 kJ/mol; SEF = 0.045			eq 3
δ_A	155.27 ± 0.4	148.27 ± 0.3	134.72 ± 0.3	136.46 ± 0.9	612296
δ_B	161.58 ± 0.07	143.68 ± 0.05	139.29 ± 0.05	122.67 ± 0.1	
$-\Delta H°$		25.8 ± 1.7 kJ/mol; K_{Tr} = 5.1 ± 0.1; SEF = 0.045			eq 4
δ_A	155.27 ± 0.4	148.29*[a]	134.70*[a]	136.46*[a]	957943
δ_B	161.57 ± 0.07	143.68 ± 0.05	139.29 ± 0.05	122.68 ± 0.1	
$-\Delta H°$		25.8 ± 1.6 kJ/mol; K_{Tr} = 5.1 ± 0.1; SEF = 0.043			eq 4

[a] Calculated values, see text. Number of experimental data points = 76.

In our study, we have obtained from the different nuclei values ranging from −23.6 to −25.8 kJ/mol. This disagreement can be explained if we consider that both values are calculated with different methodologies, which have very different approximations. We think that our results are more reliable since we have used a broader range of temperature, and all nuclei have led to the same result.

Equation 3 has been obtained under the assumption that $\Delta H°$ is constant within the studied temperature range.[11] Equation 3 can be slightly modified by taking into account the possible existence of a significant heat capacity, defining the E term as

$$E = \exp\left[\frac{\Delta H°}{RTT_r}(\Delta H°(T_r) - \Delta C°_p T_r) + \frac{\Delta C°_p}{R}\ln\left(\frac{T}{T_r}\right)\right] \quad (6)$$

The parameters obtained in the fitting process have low standard errors that suggest a small value for $\Delta C_p°$ (Table 3). We have carried out a multiple-function curve fitting of the C-3, C-6, and N-1 data to eqs 3 and 6 with $\Delta C_p°$ as a new parameter to fit. As a result, the values of the parameters do not change and a low positive value of $\Delta C_p°$ = 14 J/K·mol with a high standard error is obtained. When we include this new parameter the F test diminishes, indicating that this parameter is not statistically significative.

Tautomeric equilibrium constant values as a function of the temperature (Figure 4) were obtained from data C-3, C-6, and N-1 by using eq 2 and the parameters given in Table 3. Figure 4 clearly shows a great coincidence in the results obtained from the different signals. In fact, it is difficult to distinguish them in a broad range of temperature. Tautomeric equilibrium constants have also been calculated (results not shown) in the same way as the parameters given in Table 4, and they agree very well with those shown in Figure 4. This fact confirms that, in this case, it is possible to obtain reliable thermodynamic data only with the ^{13}C NMR experimental results. The standard entropy change, $\Delta S° = (-70.3 \pm 0.8)$ J·K^{-1}·mol^{-1} was obtained from the tautomeric constant values given in Figure 4. The values of $\Delta H°$ and $T\Delta S°$ are negative. The values of the entropy change agree with a larger order of the solvent, caused by the dipolar form in all cases.

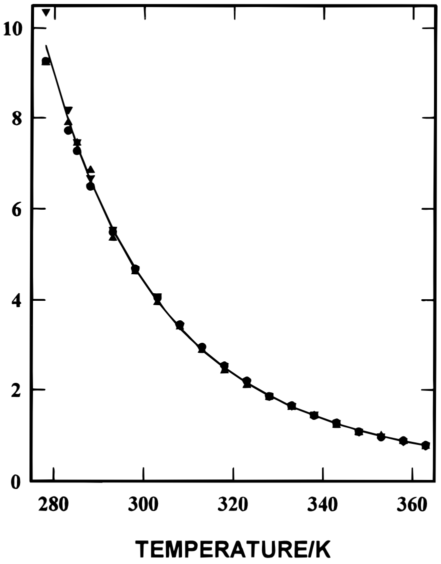

Figure 4. Tautomeric equilibrium constant versus temperature. Data obtained from the (●) C-3, (▼) C-6, and (▲) N-1 signals and the parameters given in Table 3.

As it is pointed out in our previous paper,[11] the method used here is mainly limited by three factors: (1) The narrow temperature range which is experimentally accessible. (2) The value of $\Delta H°$ itself. (3) The possible temperature dependence of both the chemical shifts of the pure forms A and B and the references. In the first case, to increase the reliability of the results, we have carried out the experimental measurements in a temperature range as broad as possible. Concerning the second limitation, it is obvious that to quantify a thermically induced process accurately enough strongly depends on its own $\Delta H°$ value. The third limitation deserves some comments since there are a few reasons that indicate that the intrinsic variation of the chemical shifts with the temperature does not affect substantially the reliability of the method.

On one hand, the magnitude of the intrinsic variation of ^{13}C NMR chemical shifts for several systems has a reported value of ca. 10^{-3}−10^{-4} ppm/K,[22] which would introduce a 0.1−0.2 ppm deviation in the temperature range we have used. In addition to previous experiments we have done in the past,[11] we have performed several measurements in order to estimate this value for pyri-

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(22) Jameson, A. K.; Jameson, C. J. *J. Am. Chem. Soc.* **1973**, *95*, 8559. Schneider, H.-J.; Freitag, W. *J. Am. Chem. Soc.* **1976**, *98*, 478. For the TMS ^{13}C NMR chemical shift dependence on temperature, see: Schneider, H. J.; Freitag, W.; Schommer, M. *J. Magn. Reson.* **1975**, *18*, 393.

doxine. Assuming that the neutral form greatly predominates in organic solvents, we recorded a variable temperature experiment between 25 and 85 °C in DMSO using HMDSO and nitromethane as external references. Although the tautomeric equilibrium cannot be precluded, the chemical shifts for the ring carbons have small variations being the larger one 0.3 ppm. In this experiment, the variation between both references is 0.07 ppm. This confirms that the intrinsic variations of the chemical shifts of the neutral form and the standards with the temperature are very small when compared with the values measured in water. Nevertheless, this small variation might be, among other causes, responsible of the slightly different values obtained for $\Delta H°$ when the method is applied separately to each nucleus (Table 2).

On the other hand, we have found excellent linear relationships (correlation coefficients $r > 0.999$) in the data analysis presented in this paper between data arising from ^{13}C and ^{15}N NMR chemical shifts (Table 2). Considering that both references, HMDSO and nitromethane, should have a rather different temperature behavior since they have both different structure and resonance frequency, this high linearity between values from ^{13}C and ^{15}N NMR chemical shifts indicates a small effect of the temperature on each reference. We have also found excellent linear relationships in the analysis of ^{13}C NMR data alone (slopes not shown). In this case, the problem caused by the temperature on the external references is eliminated since only experimental ^{13}C NMR chemical shift values from nuclei of pyridoxine are correlated (see eq 5) and, therefore, only the effect of the temperature on the pure forms A and B could influence the analysis. Furthermore, the final results using both data analyses (Table 3 and Table 4) are nearly the same. This coincidence clearly indicates that a possible effect of the temperature on the chemical shifts of the pure forms A and B and the references is meaningless in the final results.

If the system nature does not allow direct measurement of the chemical shifts of the pure forms, it is necessary to use optimization procedures in order to estimate the thermodynamic and NMR parameters. The great advantage of this method is that it allows obtaining this information from a large number of experimental data from different nuclei with a small number of parameters to fit. The fitting process with a large amount of experimental data allows the removal of nonsystematic bias such as the possible effect of the temperature on the intrinsic chemical shifts.

In conclusion, the method can be very reliable if the $|\Delta H°|$ of the equilibrium under study is large enough. In addition, we demonstrate here that the accuracy and reliability of the results can be strongly improved when the data analysis is done with the multiple function curve fitting technique. Finally, the results can further be improved when the relations between the chemical shifts of the pure forms obtained from different nuclei are used as restrictions.

For the application of this method, it is recommended to carry out measurements with different nuclei. However, in our example, pyridoxine, the results obtained only from the ^{13}C NMR signals are reliable enough, and it is not strictly necessary to carry out ^{15}N NMR variable-temperature measurements.

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