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Tautomeric Ratio and Prototropic Equilibrium Constants of Tenoxicam, a ^{1}H and ^{13}C NMR Theoretical and Experimental Study

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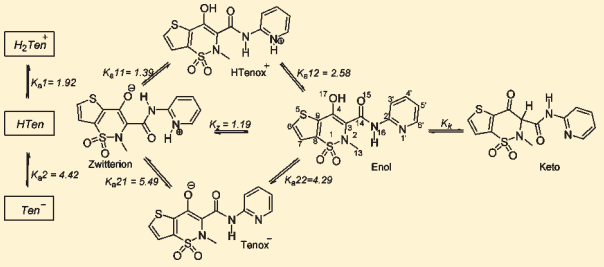
Supporting Information

ABSTRACT: The determination of the micro-equilibrium prototropic constants is often a tough task when the tautomeric ratio favors one of the species or when the chemical exchange is not slow enough to allow the quantitative detection of the tautomeric species. There are just few experimental methods available to reveal the constants of the tautomeric micro-equilibriums; its applicability depends on the nature of the tautomeric system. A combination of experimental and quantum chemistry calculated ^{1}H and ^{13}C NMR chemical shifts is presented here to estimate the population of the species participating in the tautomeric equilibriums of the tenoxicam, an important anti-inflammatory drug. A multivariate fitting of a fraction-mol-weighted contribution model, for the NMR chemical shifts of the species in solution, was used to find the populations of the tautomers of tenoxicam. To consider and evaluate the effect of the solvent polarity on the tautomers' populations, experimental determinations were carried out in DMSO-d_6, in an equimolar DMSO−H_2O mixture of deuterated solvents and in D_2O. Additionally, by employing HYPNMR, it has been possible to refine the acid−base macroscopic constants of tenoxicam.



INTRODUCTION

Tenoxicam is a widely used anti-inflammatory and anti-arthritic drug belonging to the oxicams family, whose pharmacological activity is related to the inhibition of the cyclooxygenase active site of the prostaglandin H synthase (PGH'S) enzyme, by competing with the substrate araquidonate.[1,2] A particular characteristic of tenoxicam, piroxicam, and meloxicam, other drugs of the same family, is to induce membrane fusion at a concentration relevant to the physiological concentration range,[3,4] and this property has never been seen for other families of drugs. Prototropic equilibriums of oxicams are of chemical and biological fundamental interest because drug pharmacological activity and its ability to transfer through the phospholipid membranes of cells depend on their state of ionization and acid−base properties. The prototropic equilibriums of tenoxicam are shown in Figure 1; besides the neutral tautomers (keto, enol, and zwitterion), the acid (H_2Ten^+) and basic (Ten^-) species are also involved.

Knowledge of the prototropic equilibriums constants provides essential physicochemical information on the behavior of oxicams in solution; experimental tools are commonly employed to characterize these equilibriums. There exist a number of reports on the determination of macroscopic acidity constants (K_a1, K_a2, Figure 1) for several oxicams (like tenoxicam, piroxicam, and lornoxicam), for example, by means of UV−visible spectrophotometry,[5,6] zone capillary electrophoresis,[7] and nuclear magnetic resonance (NMR),[7,8] which show consistency, independently of the experimental method employed. The so-called deductive method has been applied to determine the tautomerization constant K_z and the other microscopic constants K_a11, K_a12, K_a21, and K_a22.[9] This method consists of measuring the acidity constant of a derivate in which one of the protonation sites has been previously complexed. For instance, the K_a12 of piroxicam has been deduced from the acidity constant of the *o*-methyl piroxicam, a piroxicam derivative.[9] Assuming that the electrostatic effects enhancing the acidity of the enolic OH group in piroxicam are of the same magnitude as in tenoxicam and lornoxicam, Tsai et al. calculated the tautomeric constant K_z of these two oxicams by UV−visible spectrophotometry.[5] We have not found another experimental study about the values of the tautomerization constants K_z or K_k of tenoxicam.

The determination of the tautomerization constants in solution is often a tough task because the tautomeric ratio frequently favors one of the neutral species, making difficult the quantitative detection of the other tautomer(s). In many cases, the tautomers

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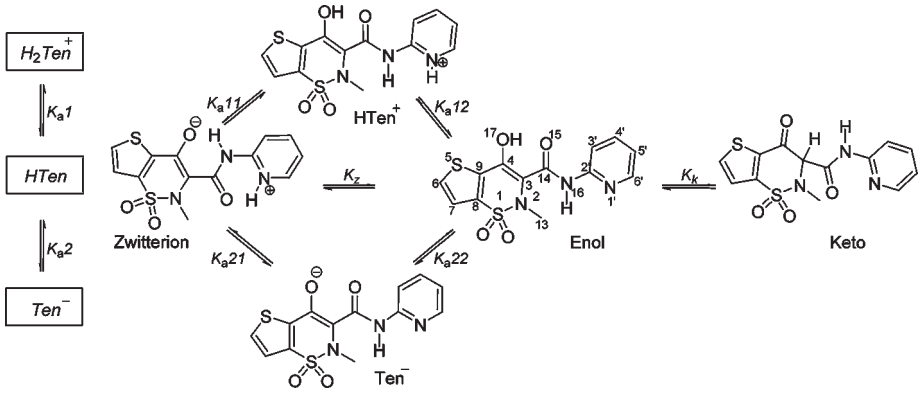

Figure 1. Prototropic equilibriums for tenoxicam.

of a compound display different solubilities in a given solvent. This fact becomes an advantage for obtaining the tautomerization constant. The tautomeric ratio in aqueous solutions is determined by taking the UV−visible spectra of solutions prepared in mixtures of water and some organic solvent at different proportions. Then, extrapolating to zero concentration of organic solvent, the tautomeric ratio in water is found.[9] However, there is no guarantee that the absorption coefficient of a particular tautomer, measured in an organic solvent, remains the same when it is either in water or in the different solvent mixtures; actually, it has been reported that for the oxicams the spectral properties are extremely sensitive to solvent properties.[10,11] Moreover, an unambiguous spectral assignment for the tautomers is necessary in each prepared solvent to be able to calculate the tautomeric constant.[8] Furthermore, there is no guarantee of a linear relationship between the ratio of tautomers and the amount of organic solvent.

NMR is a versatile technique used to investigate the molecular and electronic structure of chemical compounds. A chemical exchange process, approaching the fast limit on the NMR scale, is exhibited as a field-dependent signal broadening. The polarity of the solvent may improve signal differentiation by favoring the predominance of one species over the others. Because of its high sensitivity and its ability to provide detailed information, NMR chemical shifts have been applied to investigate tautomerization equilibria.[12,13] In general, chemical shifts are sensitive to solvent polarity and sensitive enough to geometry, allowing tautomer differentiation by NMR. Geckler et al. studied the tautomeric stability of piroxicam by NMR spectroscopy.[14] At 205 K, a mole fraction of 0.19 for the zwitterion was found in a solution of *N*,*N*′-dimethylformamide (DMF). In the same solvent, but at room temperature, the mole fraction of the zwitterion was reduced to about 0.1, while in dimethylsulfoxide (DMSO), it was about 0.25. In contrast, in chloroform, the solution was shown to be composed mainly by the enol tautomer. Furthermore, they note that addition of small amounts of water to a DMSO solution increases zwitterionization. Results show that the zwitterion mole fraction can be favored by increasing the polarity of the medium. This zwitterionization has also been reported for the polymorphs of piroxicam. Solid-state and DFT NMR spectroscopy studies[15,16] show that the change from the colorless crystal form to a yellow crystal form of piroxicam is due to a conversion of the enol molecular structure into the zwitterionic form by a solid-state transfer of the hydroxyl proton on the thiazine ring oxygen to produce the pyridinium center at the pyridine nitrogen. The colorless crystal has one enol molecule per asymmetric unit in the space group $P21/c$, and the yellow has two zwitterion molecules per asymmetric unit in the space group $P\bar{1}$.

In a theoretical and experimental fluorescence spectroscopic study, Ferreira de Souza et al. performed a conformational analysis of the keto and enol tautomeric structures of piroxicam in nonpolar aprotic solvents.[17] Their results suggest that the lower energy structures in the gas phase correspond to the keto and enol tautomers, the last being near 10 kcal/mol more stable than the former. Additionally, they conclude that, although the enol conformer is responsible for the piroxicam electronic transitions, the keto tautomer coexists in solution with the enol in a ratio that depends on the polarity of the medium. Even so, with the enol being the energetically most stable tautomer, it is considered as the most abundant species.

Due to the charge distribution of the tenoxicam zwitterion structure, it will show a higher dipole moment than the keto or the enol species. Consequently, it is expected to be the most abundant tautomer in polar solvents. As the polarity of the medium decreases, the populations of the keto and enol tautomers should increase. However, when the structure of the enol is more stable than the keto, the enol proportion might be higher than the keto in solution.

Lately, advances made in theoretical chemistry methods allow accurate reproductions of NMR shielding constants,[18] the source of the chemical shifts. These can be obtained using theoretical high-level and density functional methods.[19−22] It has been reported that accuracy of ^{1}H chemical shifts between theory and experiment is less than 1 ppm, while for ^{13}C, it is somewhat less, 3−10 ppm, depending on the method and basis set.[20−22] Taking this advantage, some studies have analyzed conformational and tautomeric mixtures of compounds using calculated shielding constants.[23,24] However, the calculation of the populations in a conformational or tautomeric mixture, employing theoretically computed and experimental chemical shifts, has not yet been explored.

The aim of this contribution is to determine the populations of keto, enol, and the zwitterion tautomers at the pH value where the neutral species of tenoxicam are predominant (isoelectric point), using the values of the shielding constants of these species obtained by quantum chemistry methods and the corresponding chemical shifts experimentally measured. To consider and evaluate the effect of the solvent polarity on the species' populations, we have carried out our determinations in DMSO-d_6, in an equimolar DMSO−H_2O mixture of deuterated solvents, and in

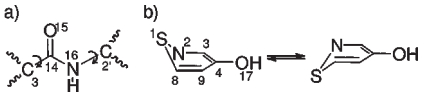

Figure 2. (a) Explored rotations for the tautomeric species of tenoxicam. (b) Thiazine ring half-chair conformations.

D_2O. Additionally, all the macro- and microconstants of the equilibriums displayed in Figure 1 have been calculated. We will employ a multivariate fitting in order to obtain the species' populations in solution.

METHODOLOGY

Experimental Methods. NMR spectra were recorded at 298 K on a Bruker AVANCE III 500 NMR spectrometer, 11.7 T, 500 MHz for ^{1}H and 125 MHz for ^{13}C, using a 5 mm BB-ID *z*-gradient probe. Chemical shifts δ (in parts per million) are given from internal TMS. The HSQC and HMBC spectra allowed the unambiguous assignment of the ^{13}C NMR shifts; assignment was also verified by comparing with theoretical results. Thus, the NMR δ^{13}C of a 10^{-3} M DMSO-d_6 solution of tenoxicam was revealed. For the ^{1}H and ^{13}C NMR tenoxicam spectrum in the equimolar solution of deuterated DMSO−H_2O, 50 mg of tenoxicam was dissolved in 1 mL of DMSO-d_6/D_2O 4:1 (v/v) solution. Aliquots of deuterated HCl or NaOH solutions were added properly for changing the pH of the medium, maintaining the equimolar ratio between solvents. The ^{1}H NMR spectrum of tenoxicam in deuterated water was obtained for a 10^{-3} M solution of the drug in a system composed by D_2O and deuterated perchloric acid (pH 0.56). The pH was increased by adding a solution of 0.3 M sodium deuteroxide. Due to the low solubility of tenoxicam in D_2O, the ^{13}C NMR spectrum was not recorded.

Quantum Chemistry Methods. Chemical shifts calculations for the tautomeric and ionic species (Figure 1) were computed using molecular quantum chemistry methods within the frame of the density functional theory.[25] Calculations were performed with the G03[26] and G09[27] suite of programs. For each tautomer, the conformational stability related to the rotations present in the C3−C14 and N16−C2′ bonds (see Figures 1 and 2), as well as the conformational analysis of the thiazine half-chair (S1−C8, Figure 2), was carried out employing the B3LYP[28] functional and the 6-31+G(d)[29] basis set. Resulting structures were optimized in the gas phase using the same functional but with a larger basis set, 6-311++G(d,p). We confirm that all of the structures corresponded to a minimum on the potential energy surface (PES) by an analysis of vibrational frequencies. Then, the B3LYP/6-311++G(d,p) geometries in water and DMSO were obtained by reoptimization of the gas phase structures, considering the solvent effect with the polarizable continuum model (PCM),[30] using the default G09 settings for the pure solvents. To model the equimolar DMSO−H_2O mixture, the values of 64.02 for the static dielectric constant, 4.9 for the dielectric constant at infinite frequency, and 1098 g/mL for density were employed.[31,32] Moreover, a value of 3.05 Å was used for the molecular radius of the solvent. In order to assess the effect of the functional on the final population of each tautomeric species, we have calculated the shielding constants with the PW91,[33] B3LYP, O3LYP,[34] OLYP,[34] OPBE,[35−37] and OPW91[33,37] density functionals, which have been proven to give the best accuracy in comparative studies for calculating chemical shifts.[38,39] In our calculations of

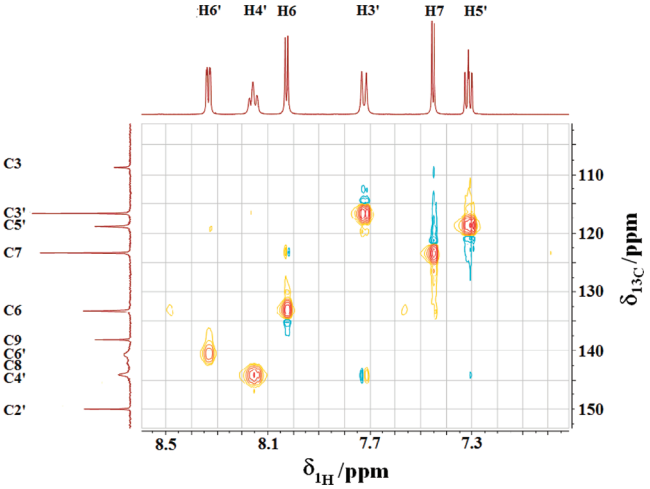

Figure 3. Two-dimensional HMBC gradients for tenoxicam at the pH 3.17 in the equimolar solution of deuterated DMSO−H_2O mixture.

shielding constants, we use the 6-311++G(d,p) basis set and the gauge invariant atomic orbital (GIAO)[40,41] scheme as well as the PCM method to model the solution environment in which the experiment was carried out.

Determination of the Tautomeric Populations of Tenoxicam. If the species in solution are related through fast chemical exchange equilibriums, the overall response to an external perturbation will be the average of the individual responses of those species involved in the equilibriums, with weights equal to the mole fractions of those species. When tenoxicam is at its isoelectric point in solution, we have its neutral tautomeric species: keto (K), enol (E), and zwitterion (Z) in equilibrium. Under such circumstances, the recorded chemical shift for the *i* nucleus (δ_i) comes from the contribution of the individual chemical shifts of each tautomer (δ_i^K, δ_i^E, δ_i^Z) weighted by the corresponding mole fraction (f^K, f^E, f^Z):

$$\langle \delta_i^{KEZ} \rangle = f^K \delta_i^K + f^E \delta_i^E + f^Z \delta_i^Z \quad (1)$$

Clearly, if we could know the individual chemical shifts (δ_i^K, δ_i^E, δ_i^Z) of all the nuclei of the tautomers in a given solvent, and the corresponding chemical shift of the same nuclei in a mixture of tautomers in the same solvent, we could use eq 1 to compute the mole fraction of each tautomeric species by means of a multivariate fitting. However, as it has been discussed, knowing individual chemical shifts is not straightforward. This shortcoming can be overcome by theoretical calculation of the individual chemical shifts, using them as auxiliary information together with experimental measurements to obtain the mole fractions by means of a multivariate fitting based on eq 1. The resulting mole fractions will be those for which the mean of the sum of squared residuals (mSSR) takes its minimum value (eq 2).

$$\min \sum \frac{(\langle \delta_i^{KEZ} \rangle_t - [f^K \delta_i^K + f^E \delta_i^E + f^Z \delta_i^Z + \beta])^2}{N - P} \quad (2)$$

N is the number of magnetically active nuclei considered, *P* is the number of parameters included in the multivariate fitting, and β is the intercept estimator. The estimator β would be zero if the experimental chemical shifts for the individual tautomers were used. In addition, the possible values for the mole fractions should fulfill the conditions $f^K, f^E, f^Z \geq 0$ and $f^K + f^E + f^Z = 1$.

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Table 1. Experimental ^{1}H and ^{13}C Chemical Shifts of Tenoxicam in the Three Studied Media

	δ^1H (ppm)			δ^{13}C (ppm) [a]	
	DMSO	mixture	water	DMSO	mixture
3				108.93	108.76
4				163.07	164.92
6	8.03	7.95	7.91	132.73	133.44
7	7.46	7.41	7.46	122.87	123.50
8				137.65	138.03
9				141.70	143.30
13	2.9	2.89	2.96	39.17	39.98
14				165.60	166.06
2′				149.50	149.60
3′	7.73	7.65	7.52	116.10	116.75
4’	8.16	8.19	8.3	143.56	145.51
5′	7.31	7.34	7.45	118.43	119.16
6′	8.34	8.28	8.26	140.24	139.37

[a] Because of the low solubility of tenoxicam, ^{13}C NMR chemical shifts were not determined in water. The mixture is a DMSO−H_2O equimolar system. All solvents are deuterated.

RESULTS AND DISCUSSION

Tautomeric Equilibriums of Tenoxicam. Since the use of the eq 1 and minimization through eq 2 imply the unambiguous experimental assignment of the chemical shifts of tenoxicam, 2D heteronuclear multiple bond correlation was used to assign the ^{13}C NMR spectra (Figure 3). Table 1 shows the experimental chemical shift values for ^{1}H and ^{13}C nuclei of tenoxicam, obtained in the DMSO, DMSO−H_2O, and H_2O deuterated solvents. In general, changes in the chemical shifts between the different media are small but perceptible, as can be noted from data in Table 1. It can be observed that chemical shifts of different nuclei behave independently of the polarity of the solvent; for example, as the amount of water increases, the chemical shift value of H5′ also increases from 7.31 to 7.45 ppm; in contrast, the shift of H6 decreases going from 8.03 to 7.91 ppm. The lowest value of H13 chemical shift (2.89 ppm) is in the solvent mixture. Both δ^{13}C and δ^1H chemical shifts of the methyl moiety at position 13 in the molecule of tenoxicam have been discarded in our statistical analysis because they are too small in value as compared with to the other δ^{13}C and δ^1H chemical shifts, respectively.

At this point, it is convenient to advance that the tautomeric mole fractions obtained by our statistical treatment are practically the same regardless of the functional used to compute the magnetic shielding constants. Since, in general, the chemical shifts calculated with the O3LYP density functional produce the lowest mSSR, the following analysis of results is based on the chemical shifts calculated with this functional. Similar conclusions are derived with results from the other density functionals considered here (see Supporting Information, Tables S1, S2, and S3).

Applying the mentioned multivariate fitting procedure using the δ^{13}C chemical shifts of tenoxicam, we observe (Table 2) that both the DMSO and DMSO/H_2O solutions are enriched with the zwitterion tautomer (mole fractions of 0.61 and 0.73, respectively), while the enol species is the second in abundance (0.39 and 0.27, respectively) and keto is not present (since its population is practically zero) in these two media (δ^{13}C was not recorded in the water solution of tenoxicam). The computed tautomeric population ratios of zwitterion/enol that provides the minimum value of mSSR in the DMSO (mSSR = 3.730) and in the DMSO/H_2O (mSSR = 3.864) solutions are 1.56 and 2.70, respectively. Note that, as expected, the abundance of the zwitterion tautomer is increased as the amount of water increases.

Table 2. Estimated Mole Fractions for the Three Tautomers of Tenoxicam in the Studied Media (Results Obtained by Using the O3LYP Functional)

	DMSO			mixture			water		
	K	*E*	*Z*	*K*	*E*	*Z*	*K*	*E*	*Z*
δ^{13}C	0.00	0.39	0.61	0.00	0.27	0.63			
δ^1H	0.35	0.00	0.65	0.26	0.00	0.74	0.07	0.00	0.93
δ^1H[a]		0.28	0.72		0.21	0.78		0.06	0.94

[a] The keto chemical shifts are not considered for the multivariate fitting.

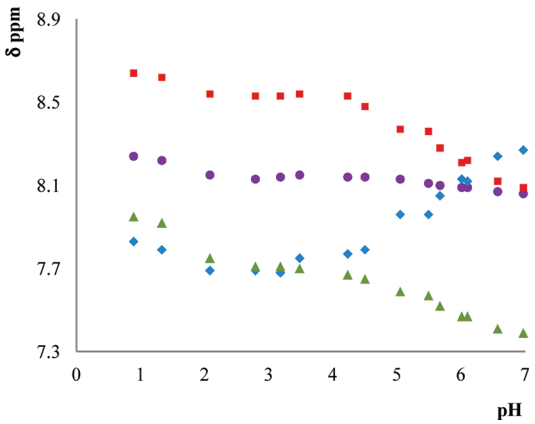

Figure 4. Dependence on pH of some ^{1}H NMR chemical shifts: H6 (purple circle), H3′ (blue diamond), H4′ (red square), H5′ (green triangle).

Now, using the δ^1H chemical shifts of tenoxicam in the statistical analysis, we observe that the population of the zwitterion tautomer is increased with the amount of water, such as is indicated by the δ^{13}C analysis. However, opposite to the results observed with δ^{13}C shifts, the bigger fraction of tenoxicam after the zwitterion is the keto. The predicted tautomeric zwitterion/keto ratios are 1.86, 2.85, and 13.29 in DMSO, DMSO/H_2O, and H_2O, respectively. It is worth noting that, for the applied multivariate fitting, there is two times more data for δ^{13}C than for δ^1H (12 versus 6). Besides that, there are more carbon atoms in the tautomerization site (C3, C4, C14 and all carbons of the pyridyl ring) than hydrogens (only those hydrogens of the pyridyl moiety). For these reasons, we consider that results with δ^{13}C chemical shifts are more significant than those obtained with δ^1H shifts. Therefore, we consider that is the enol instead of the keto that coexists with the zwitterion species. Consequently, we perform the multivariate fitting using the δ^1H chemical shifts, now considering that we have just the zwitterion and enol tautomers in solution. The population zwitterion/enol ratios computed taking this consideration are 2.57, 3.55, and 15.67 in DMSO, DMSO/H_2O, and H_2O, respectively. They differ as much as one part in 10 to those observed when the statistical

Table 3. Comparison of the Micro- and Macroconstants of Tenoxicam in Aqueous Solution and in the Equimolar Mixture DMSO−H_2O, Obtained in This Study with Those Reported by Several Authors

	aqueous solution					mixture	
	ref 5	ref 3	ref 38	ref 39	this study	ref 5	this study
pK_{a1}	1.34 ± 0.07	1.07	1.1	1.08	1.36 ± 0.100	1.92 ± 0.15	
pK_{a2}	5.26 ± 0.03	5.34	5.12	5.31	5.46 ± 0.038	4.42 ± 0.07	
$\log K_z$		2.17	1.46	1.62	1.19		0.10
pK_{a11}		1.07	1.12	1.09	1.39		2.18
pK_{a12}		3.24	2.58	2.71	2.58		2.27
pK_{a21}		5.34	5.1	5.32	5.49		4.68
pK_{a22}		3.17	3.64	3.7	4.29		4.58

analysis is carried out using the $\delta^{13}C$ shifts. Therefore, the tautomeric zwitterion/enol ratios in DMSO (1.56) and DMSO/H_2O (2.70) were taken from the multivariate fitting using the $\delta^{13}C$ chemical shifts. It was not possible to record the $\delta^{13}C$ shifts of tenoxicam in water because of its low solubility. Thus, the tautomeric mole fraction zwitterion/enol ratio in aqueous solution was taken from results using the $\delta^{1}H$ shifts (15.67) in this solvent.

Determination of the Macroscopic and Microscopic Constants of Tenoxicam in Aqueous Solution. Figure 4 shows the experimental dependence of ^1H NMR chemical shifts with respect to the pH variation in aqueous solution for the H6, H3′, H4′, and H5′ hydrogen nuclei. The response is the typical sigmoidal curve observed in an acid−base titration. Consequently, we use the chemical shifts of H3′, H4′, and H5′ nuclei, measured at different pH values, to obtain the macroscopic acidity constants of the drug (K_a1 and K_a2, Figure 1) by using the statistical adjustment program HYPNMR.[42] Results are shown in Table 2. It is noteworthy that our results (pK_a1 = 1.36, pK_a2 = 5.46) are very close to those previously reported in the literature by other experimental methods.[5]

Since the tautomerization constant K_z can be obtained from the tautomeric ratio value, it is possible to know the value of the microconstant equilibriums involved in Figure 1 (considering that the keto tautomer is not present in the solution) using eqs 3a−3c. These results are shown in Table 3, where we have included those obtained in other studies for comparison. Additionally, using the K_a1 and K_a2 values of tenoxicam reported in the DMSO−H_2O equimolar mixture in a previous study,[7] we calculate the values of the microconstants in the solvents mixture.

$$K_{a1} = K_{a11} + K_{a12} \tag{3a}$$

$$\frac{1}{K_{a2}} = \frac{1}{K_{a22}} + \frac{1}{K_{a21}} \tag{3b}$$

$$K_z = K_{a11}/K_{a12} = K_{a22}/K_{a21} \tag{3c}$$

It should be noted that only the microconstant values of ref 5 were obtained through experimentation, by the assumption that the electrostatic effects enhancing the acidity of the enolic OH group in piroxicam are of the same magnitude as in tenoxicam. The values of $\log K_z$ here obtained are closer to those in refs 43 and 44, which were obtained by theoretical and computational methods. In general, a good agreement is observed between the microconstant values of refs 5, 43, and 44, as well as with that obtained in this study; however, the tautomeric constant K_z of ref 3 is higher by almost 1 order of magnitude.

CONCLUSIONS

The results of this study suggest that the zwitterion is the most abundant tautomer of tenoxicam in the three considered solution media. The enol tautomer is the second in abundance, and the keto tautomer probably is absent in these media. Additionally, we have seen a clear dependence of tautomeric population of tenoxicam calculated by means of eq 1 to the polarity of the media. This dependence is consistent with that initially expected in this study and with the results previously reported for other oxicams like piroxicam.[14] An important feature in the use of eq 2 is that populations obtained this way are almost the same regardless of the functional employed to the calculation of the shielding constants. The observed dependence of the chemical shift to the pH allows us to obtain the macroconstants of the drug in aqueous solution, and with the tautomeric ratio obtained as well as a previous report of the macroconstants in the DMSO−H_2O mixture, we were able to compute the microconstants of tenoxicam in these two media, obtaining consistent results with previous reports.

ASSOCIATED CONTENT

Supporting Information. Statistical analysis and tautomeric populations obtained with the PW91, B3LYP, O3LYP, OLYP, OPBE, and OPW91 density functionals. This material is available free of charge via the Internet at http://pubs.acs.org.

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