Biophysical Chemistry 27 (1987) 255–261
Elsevier

BPC 01177

Structural and dynamical characterization of piroxicam by ^{1}H- and ^{13}C-NMR relaxation studies

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Received 5 January 1987
Revised manuscript received 13 April 1987
Accepted 20 May 1987

^{1}H-NMR; ^{13}C-NMR; Piroxicam; Nuclear Overhauser effect; Internal motion

Carbon spin-lattice relaxation rates of an anti-inflammatory drug, piroxicam, have been measured. These results have been used in determining the reorientational rates of the proton carbon vectors. An analysis of internal motions within the pyridinyl moiety of piroxicam was carried out. Selective proton-carbon nuclear Overhauser effect (NOE) measurements were made in order to determine the solution structure of piroxicam. The effect of indirect NOE arising from exchangeable protons has been analyzed and considered.

1. Introduction

Conformational studies of biologically active molecules are of primary importance in order to define structure-activity correlations. In the present paper, the conformational properties of an anti-inflammatory agent, piroxicam [1–3], are analyzed by means of proton and carbon nuclear magnetic relaxation studies.

Anti-inflammatory drugs belong to several classes of chemical structures [4] and their in vivo activity is probably due to interaction with different receptors. Nevertheless, a clearer identification of the conformation in solution and characterization of the internal motions of each moiety of the molecule are extremely important in defining, at least for structurally correlated drugs, molecular active sites and structural requirements for anti-inflammatory activity.

In the present paper several NMR experiments have been carried out in order to investigate the structural and dynamical properties of piroxicam in C^2HCl_3 solution. In particular: (i) two-dimensional NMR spectroscopy has been used in the assignment of the aromatic proton region; (ii) carbon spin-lattice relaxation rates have been employed in determining the reorientational rates of the proton-carbon vectors and (iii) selective proton-carbon nuclear Overhauser effect measurements ({H}C-NOE) allowed the identification of an intramolecular hydrogen bond and the determination of proton–carbon distances in solution.

2. Experimental

Piroxicam (4-hydroxy-2-methyl-*N*-2-pyridinyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide) (fig. 1) was received as a gift from Pfizer and used without any further purification to yield 0.3 mol dm^{-3} C^2HCl_3 solution. A Varian XL-200 spectrometer operating at 200 and 50.3 MHz for pro-

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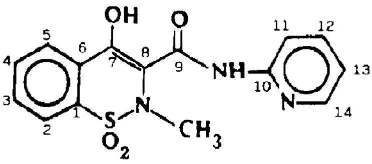

Fig. 1. Structure of piroxicam with the atom numbering used in the present discussion.

ton and carbon nuclei, respectively, was used for recording ^{1}H and ^{13}C spectra. A two-dimensional proton-carbon heterocorrelation spectrum was obtained by using the pulse sequence suggested by Bax and Morris [5]. Spin-lattice relaxation rates of carbon nuclei were measured by using the $(180°-\tau-90°-t)_n$ pulse sequence. R_1 values were calculated by computer fitting of the relaxation curves. Proton-carbon broad-band NOE values were determined by using the equation:

$$\mathrm{NOE} = (I_z - I_o)/I_o$$

where I_z and I_o represent the peak intensities measured under continuous and gated proton decoupling, respectively. Selective proton-carbon NOE measurements were obtained, as previously pointed out [6], by selective perturbation of a single proton resonance and subsequent detection of the fully proton-decoupled carbon spectrum. A 5% experimental error was estimated for both R_1 and NOE measurements. The temperature was held constant at 22 ± 1° C for all measurements.

3. Results and discussion

In order to investigate the dynamical and conformational features of piroxicam in solution, a complete preliminary assignment of both proton and carbon spectra is required. Carbon assignments obtained by means of chemical shift considerations and values of the carbon spin-lattice relaxation rate have been previously reported [7]. On the basis of these reported assignments and by using heterocorrelation spectroscopy, a complete assignment of the aromatic proton region of piroxicam was obtained. In fig. 2, a contour plot of the proton-carbon heterocorrelation two-dimensional spectrum of piroxicam is reported. As shown in this figure it is possible to assign each of eight aromatic protons; even the complex multiplet observed at 7.7 ppm corresponding to H_{12}, H_4 and H_3 protons, respectively, can be resolved and assigned.

The investigation of the dynamical properties of piroxicam is based on measurements of the carbon spin-lattice relaxation rate. As is well known [8], the ^{13}C relaxation pathway in liquid solution of diamagnetic systems is dominated by intramolecular dipole-dipole interactions. In particular, protonated carbons relax mainly by way of dipole-dipole interactions with a directly bonded proton. The dipolar contribution to the observed carbon spin-lattice relaxation rate can be determined by using the following equation:

$$R_1^{\mathrm{DD}} = R_{1\mathrm{C}} f^{\mathrm{DD}} \tag{1}$$

where R_1^{DD} is the dipolar relaxation rate, $R_{1\mathrm{C}}$ the experimental carbon spin-lattice relaxation rate and f^{DD} the fractional dipolar contribution. f^{DD} can be determined by comparing the experimental broad-band nuclear Overhauser effect, NOE(BB), with the theoretical value for carbon nuclei totally relaxed by a dipolar mechanism. Under the present conditions of molecular motion, the extreme narrow limit $\omega_o \tau_c \ll 1$ holds. In such a limit the theoretical NOE has a value of 1.99. f^{DD} can be calculated by using the following relation: $f^{\mathrm{DD}} = \mathrm{NOE(BB)}/1.99$.

In table 1 the ^{13}C relaxation parameters, obtained using a 0.3 mol dm^{-3} C^2HCl_3 solution of piroxicam, are reported. The effective correlation time τ_c modulating the proton-carbon internuclear vector can be determined by using the following equation [9–11]:

$$R_1^{\mathrm{DD}} = \frac{\hbar^2 \gamma_H^2 \gamma_C^2}{10 r^6} \left(\frac{3\tau_c}{1+\omega_C^2 \tau_c^2} + \frac{6\tau_c}{1+(\omega_H+\omega_C)^2 \tau_c^2} + \frac{\tau_c}{1+(\omega_H-\omega_C)^2 \tau_c^2} \right) \tag{2}$$

where ω_H and ω_C are the proton and carbon Larmor frequencies, γ_H and γ_C the magnetogyric ratios, $\hbar$ Planck's constant and r the proton–carbon internuclear distance. In table 2

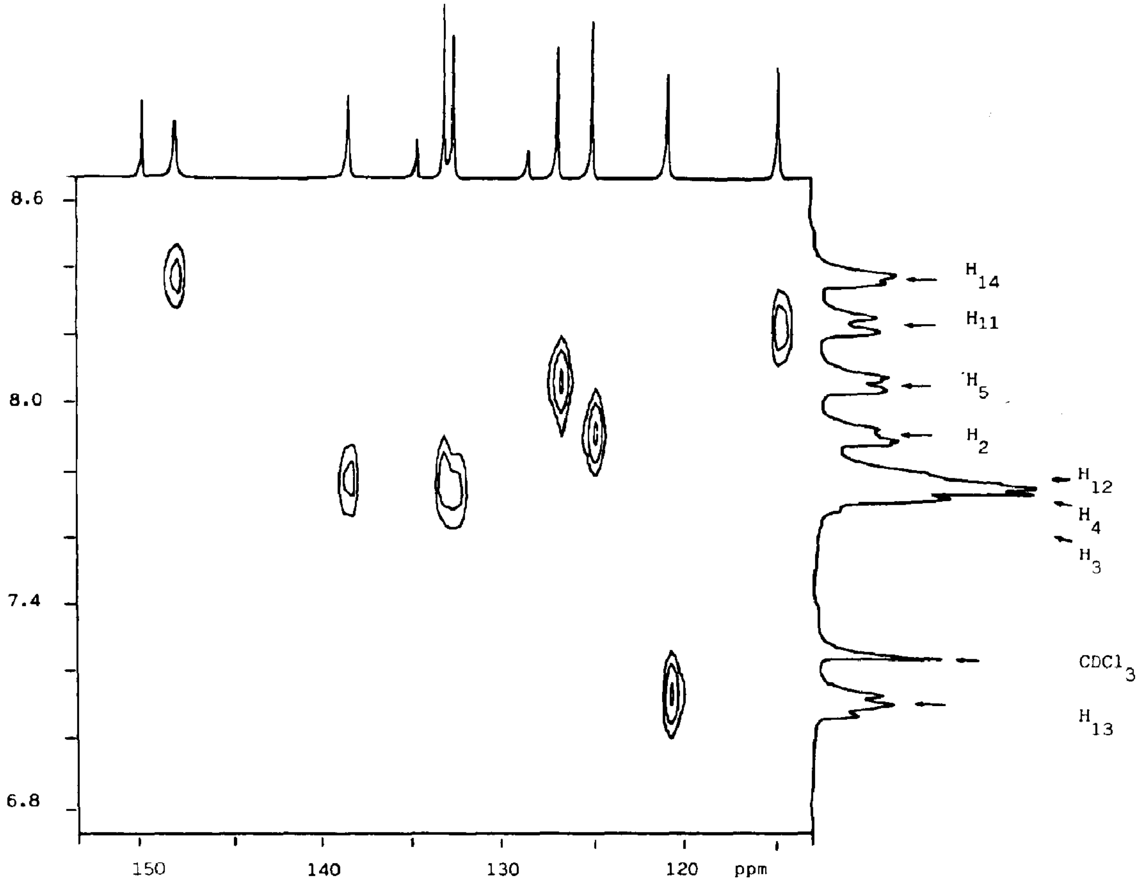

Fig 2. ^{1}H-^{13}C heterocorrelation spectrum of the aromatic region of a 0.3 mol dm^{-3} solution of piroxicam .

the correlation times for all the protonated carbons of piroxicam are reported. These values indicate that piroxicam does not experience isotropic motion; in fact, internal reorientations and anisotropic motions can be inferred. As far as the pyridinyl moiety of piroxicam is concerned, C_{11}, C_{12} and C_{14} carbons show similar τ_c values, whereas the C_{13} carbon shows a much slower τ_c value. This is consistent with the presence of an internal rotation around the NH–C_{10} bond. In fact, in this case the C_{13}–H_{13} vector is aligned with the direction of the internal motion and the ^{1}H–^{13}C magnetic interaction is not modulated by the rotation. If the correlation time determined from C_{13} R_1 measurements is assumed as being the correlation time for the overall reorientation, τ_r, then the correlation time τ_i, describing the internal rotation of the pyridinyl group is given by:

$$\tau_c^{-1} = \tau_r^{-1} + \tau_i^{-1} \tag{3}$$

In table 2 the correlation times determined from C_{11}, C_{12} and C_{14} carbon spin-lattice relaxation rates and by using eq. 3 are listed. The calculated τ_i values signify an internal rotational motion of the pyridinyl group characterized by a reorientational correlation time of about 8×10^{-11} s.

A closer and more detailed inspection of the dynamical features of the pyridinyl moiety of piroxicam can be carried out. In fact, for groups

Table 1

Experimental relaxation parameters of a 0.3 mol dm^{-3} solution of piroxicam at 22° C

Carbon no.	δ (ppm)	R_{1C} (s^{-1})	NOE(BB)	χ^{DD}	R_1^{DD}
9	166.9	0.06	1.35	0.68	0.041
7	158.6	0.061	1.50	0.75	0.046
10	150.1	0.088	1.20	0.60	0.052
14	148.1	0.88	1.80	0.90	0.80
12	138.4	0.81	1.87	0.94	0.76
1	134.6	0.045	1.03	0.52	0.023
4	133.0	1.0	1.91	0.96	0.96
3	132.5	1.16	1.99	1.0	1.16
6	128.3	0.65	0.85	0.43	0.275
5	126.7	0.83	1.85	0.93	0.77
2	124.8	0.87	1.93	0.97	0.84
13	120.6	1.41	1.99	1.0	1.41
11	114.4	0.78	1.94	0.97	0.76
8	111.5	0.045	1.10	0.55	0.025
CH_3	40.0	0.56	1.98	0.99	0.55

undergoing internal isotropic reorientation, the dipolar relaxation rate is given by the following equation [12,13]:

$$R_1 = \frac{K}{r_{C-H}^6} \chi \tau_r \tag{4}$$

where $K = \hbar^2 \gamma_H^2 \gamma_C^2$ and χ is given by:

$$\chi = 1/4(3\cos^2\vartheta - 1)^2 + 18(5+\rho)^{-1}\sin^2\vartheta \times \cos^2\vartheta + (9/4)(1+2\rho)^{-1}\sin^4\vartheta \tag{5}$$

Table 2

Correlation time and χ values determined for a 0.3 mol dm^{-3} C^2HCl_3 piroxicam solution at 22° C

Carbon no.	τ_c ^{a} (s)	τ_r ^{b} (s)	τ_i ^{c} (s)	χ ^{d}	χ ^{e}
14	3.7×10^{-11}	6.5×10^{-11}	8.6×10^{-11}	0.57	0.79
12	3.55×10^{-11}	6.5×10^{-11}	7.8×10^{-11}	0.54	0.78
4	4.5×10^{-11}	–	–	–	–
3	5.4×10^{-11}	–	–	–	–
5	3.6×10^{-11}	–	–	–	–
2	3.9×10^{-11}	–	–	–	–
13	6.5×10^{-11}	6.5×10^{-11}	–	–	–
11	3.55×10^{-11}	6.5×10^{-11}	7.8×10^{-11}	0.54	0.78

[a] Effective correlation times; [b] overall rotational correlation times; [c] correlation times for internal rotation; [d] χ values determined by using eq. 4; [e] χ values determined by using eq. 5.

ρ reported in eq. 5 is defined as:

$$\rho = \frac{D_\parallel}{D_\perp}$$

where

$$D_\parallel = 6\tau_\parallel^{-1} = 6\tau_r^{-1} + 6\tau_i^{-1}$$

$$D_\perp = 6\tau_\perp^{-1} = 6\tau_r^{-1}$$

here, $D_\parallel$ and $D_\perp$ denote the respective diffusional constants for rotational diffusion, parallel and perpendicular to the axis, for internal rotation and the C-H bond vector. χ can be determined directly from eq. 4 by using the previously determined value of the overall reorientational correlation time τ_r, and the spin-lattice relaxation rate of carbon nuclei experienced by both the overall molecular motion and internal reorientation. The calculated χ values are reported in table 2, however, the χ values can also be calculated by using eq. 5. In fact from $D_\parallel$ and $D_\perp$, ρ can be determined and χ obtained. In this case, a value of $\vartheta = 120°$ is used. As shown in table 2, the χ values of the same carbon, determined by using both methods, are not exactly identical. This is probably due to uncertainty in the determination of τ_r and τ_i. In fact, the C_{13}–H_{13} bond vector could not be exactly aligned with the axis of internal rotation as shown by diffraction studies in the solid state [14]. Nevertheless, this effect does not appear to change values of the correlation time beyond the experimental error limits.

In the final part of this paper the conformational properties of piroxicam are analyzed. As shown previously selective proton-carbon {H}C-NOE can be used for proton–carbon distance determinations [15–18]. In this procedure, a single proton resonance is selectively perturbed and the increase in intensity of dipolar coupled carbons is correlated to the proton–carbon distances by the equation [6,19]:

$$\{H\}\text{C-NOE} = \frac{\hbar^2 \gamma_H^3 \gamma_C}{10 r_{C-H}^6 R_{1C}} \left(\frac{6\tau_c}{1 + (\omega_H + \omega_C)^2 \tau_c^2} - \frac{\tau_c}{1 + (\omega_H - \omega_C)^2 \tau_c^2} \right) \tag{6}$$

where R_{1C} is the experimental carbon spin-lattice relaxation rate, $r_{C\text{-}H}$ the proton–carbon distance and τ_c the correlation time modulating the ^{1}H-^{13}C magnetic interaction. Alternatively, when the saturation of a proton H_a produces Overhauser effects on two or more carbon resonances, internuclear distances can be calculated from the following relationship:

$$\frac{\mathrm{NOE}_{\mathrm{Ca(Ha)}} R_{1\mathrm{Ca}}}{\mathrm{NOE}_{\mathrm{Cb(Ha)}} R_{1\mathrm{Cb}}} = \frac{r_{\mathrm{ab}}^6}{r_{\mathrm{aa}}^6} \tag{7}$$

In order to calculate r values from this equation, knowledge of correlation time is not required but one of the two distances must be used as a calibration. Moreover, it was assumed that the same correlation time modulated both the C_a-H_a and C_b-H_a dipolar interactions.

From data obtained by selective irradiation of piroxicam protons, two classes of selective NOEs and, hence, information on related proton–carbon internuclear distances are possible:

(Class I) These NOEs refer to strong geminal

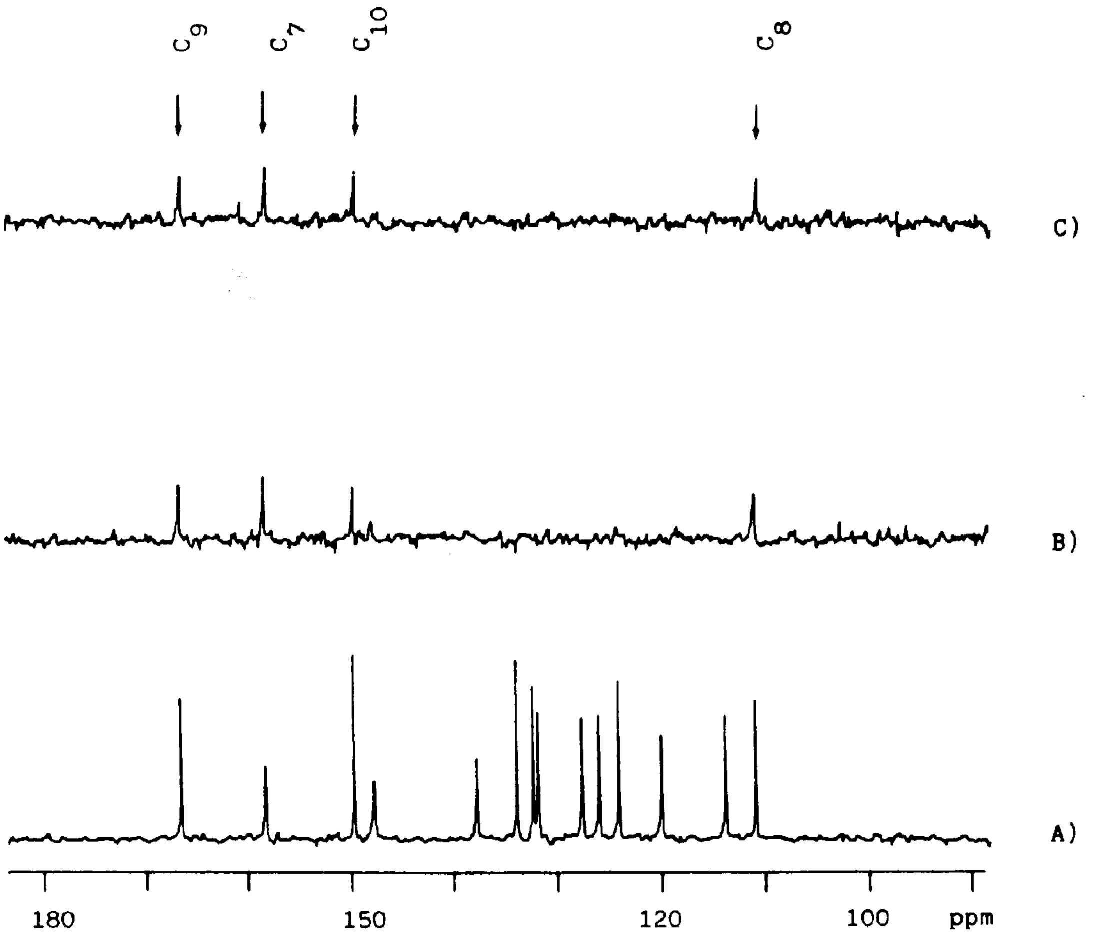

Fig. 3. (A) ^{13}C-NMR spectrum of piroxicam in C^2HCl_3 obtained with the selective low-power decoupler set 'off-resonance' for the proton region; (B) difference spectrum by subtracting the off-resonance spectrum from the spectrum recorded during selective decoupling at the amide proton frequency; (C) as in B, in this case the hydroxyl proton was selectively decoupled.

dipolar coupling which occurs between an aromatic proton and its bonded carbon. The agreement between experimentally derived (1.08 Å) and expected distances found for these conformationally independent heteronuclear interactions indicates a good choice for the correlation time determined from R_1^{DD} values and reported in table 2.

Both eqs. 6 and 7 were used in order to calculate heteronuclear distances between the quaternary C_1 and C_6 carbons and their vicinal H_2 and H_5 protons. The conformationally independent distances obtained by using both eqs. 6 and 7 demonstrate the good reliability of this structural approach.

(Class II) These selective heteronuclear effects arise from selective saturation of amide and hydroxyl protons and contain information about the molecular structure in solution. Nevertheless, in the present system, a proton chemical exchange between these two protons occurs. In this case, eq. 6 must be modified taking into account the presence of indirect {H}C-NOE due to partial saturation of a second proton (via saturation transfer). In a previous paper [20], the presence of a saturation transfer contribution to the observed selective heteronuclear NOE has been discussed and a modified version of eq. 6 (in which the exchange contribution was considered), proposed. The present situation represents a limiting case for proton exchange: in fact, by selective saturation of one of the two exchangeable protons (i.e., the hydroxyl or amide proton), complete saturation of both proton spin populations is induced (see fig. 3) and eq. 6 becomes:

$$\{\mathrm{H}\}\text{C-NOE} = \frac{K'}{R_{1\mathrm{C}}r^6_{\text{C-H hyd}}}f(\tau_c) + \frac{K'}{R_{1\mathrm{C}}r^6_{\text{C-H am}}}f(\tau_c) \qquad (8)$$

where $K' = \hbar^2 \gamma_{\mathrm{H}}^3 \gamma_{\mathrm{C}}/10$, and $r_{\text{C-H hyd}}$ and $r_{\text{C-H am}}$ denote the distances between the C_i carbon and the hydroxyl and amide proton, respectively. As the distances between both amide-benzothiazine and hydroxyl-pyridinyl groups are large, the indirect term due to saturation transfer can in some cases be neglected and eq. 8 reduces to eq. 6. In table 3, the experimental {H}C-NOE, the calculated proton–carbon distances and the distances previously determined in diffraction studies [14] are reported. A preliminary discussion of proton–carbon distances unaffected by conformational changes is reported. By using eq. 6 and the experimental {H}C-NOE values, both the OH–C_8 and NH–C_{10} distances were calculated. Correlation times of 3.6×10^{-11} s (obtained from the C_5 carbon) and of 6.5×10^{-11} s (obtained from the C_{13} carbon), respectively, were used. The distances obtained are in good agreement with results in diffraction studies. The following conclusions can be drawn: (i) both the hydroxyl and amide protons show a large predominant residence time bonded to the O_7 and N_9 nuclei, respectively, and (ii) the hydroxyl proton–O_7 distance is larger than the normal value. This suggests the hypothesis of a conjugate system that involves hydrogen bonding between the hydroxyl proton and the O_9 oxygen.

Further evidence of this hydrogen bonding is provided if the {H}C-NOEs observed on C_6 and C_8 carbons and obtained by saturation of the hydroxyl protons, are analysed. A {H}C-NOE of 0.65 is observed on C_8 whereas no detectable effect on C_6 is evident. These results indicate that the hydrogen bond between the hydroxyl proton and C_9 carbon does not allow any rotation around the C_7–O_7 bond. In order to estimate the distances between the exchangeable protons and both C_8 and C_9 carbons, eq. 8 must be used. In fact, as the distances between the exchangeable protons and both C_8 and C_9 carbons are less than 3 Å, both direct and indirect terms contribute to the

Table 3

Experimental selective {H}C-NOE and internuclear distances (Å) calculated for piroxicam

$C_n(H_m)$	R_1 ^{a}	{H}C-NOE	r ^{b}	r ^{c}
C_7(OH)	0.061	0.96	1.88	1.73
C_{10}(NH)	0.088	0.65	2.08	1.90
C_8(OH)	0.045	0.65 ^{d}	2.30	2.25
C_8(NH)	0.045	0.65 ^{d}	2.40	2.40
C_9(OH)	0.06	0.79 ^{d}	2.25	2.25
C_9(NH)	0.06	0.79 ^{d}	2.08	1.9

[a] Spin-lattice relaxation rates; [b] distances calculated on the basis of the observed {H}C-NOE; [c] distances obtained from diffraction studies [14]; [d] {H}C-NOE values obtained as a sum of two contributions arising from exchangeable protons.

observed {H}C-NOE. On the basis of both distances previously determined by way of X-ray diffraction and by using a correlation time of $\tau_c = 3.6 \times 10^{-11}$ s, a theoretical {H}C-NOE for both C_8 and C_9 carbons was determined. The theoretical {H}C-NOE values of 0.74 and 0.86 for C_8 and C_9, respectively, were calculated. These values are in agreement with the experimental values listed in table 3. The small differences observed are probably due to: (i) incomplete saturation of both exchangeable protons, (ii) the presence, as a minor species, of tautomeric structures [1,2] with larger proton–carbon distances, and (iii) the presence of anisotropic motions that induce small differences in the values of the correlation times used.

In conclusion, we suggest that under the present experimental conditions, piroxicam exists in the predominant chemical structure and conformation depicted in fig. 4. Our experimental results do not exclude the presence, as a minor species, of a tautomeric isomer; their importance in order to explain the NMR properties of piroxicam can be overlooked. Nevertheless, the presence of tautomeric forms in rapid kinetic equilibrium with the main structure shown in fig. 4 can be of primary importance in explaining, the anti-inflammatory activity. In fact, due to the structural flexibility induced by proton chemical exchange, piroxicam can adapt its conformation to the receptor site structure, the pharmacologically active form thus expressing its biological activity.

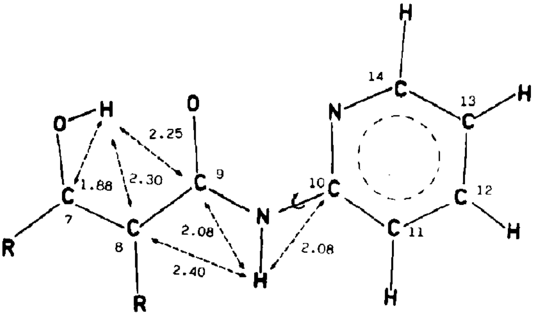

Fig. 4. The proposed solution structure of piroxicam as determined by selective {H}C-NOE measurements. The arrow indicates the detected dipolar couplings and the calculated distances.

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