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Formation of *p*-cresol:piperazine complex in solution monitored by spin–lattice relaxation times and pulsed field gradient NMR diffusion measurements

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Abstract

A study of the nature of the anthelmintic *p*-cresol:piperazine complex in chloroform solution has been conducted using different NMR techniques: self-diffusion coefficients using DOSY; NOE, NULL, and double-selective T_1 measurements to determine inter-molecular distances; and selective and non-selective T_1 measurements to determine correlation times. The experimental results in solution and CP-MAS were compared to literature X-ray diffraction data using molecular modeling. It was shown that the *p*-cresol:piperazine complex exists in solution in a very similar manner as it does in the solid state, with one *p*-cresol molecule hydrogen bonded through the hydroxyl hydrogen to each nitrogen atom of piperazine. The close correspondence between the X-ray diffraction data and the inter-proton distances obtained by NULL and double selective excitation techniques indicate that those methodologies can be used to determine inter-molecular distances in solution.

Keywords: Molecular complexes; Diffusion coefficients; Inter-molecular distances; Longitudinal relaxation; Selective T_1

1. Introduction

The great importance of molecular recognition in biochemical processes has given impulse to research on inter-molecular interaction phenomena [1]. For example, understanding how supramolecular complexes are formed and how the forces that keep them together are affected by changes in the molecular environment is crucial for the rational design of pharmacologically active compounds [2]. Among the interactions that are involved in inter-molecular recognition, the hydrogen bond is one of the most important ones [1,3]. This kind of interaction is involved in several of the most important biochemical processes, such as base pairing and interactions in nucleic acids [4], substrate recognition by the active sites of several enzymes [5], cell recognition by antibodies [6], protein folding [7], etc. Clearly, understanding how hydrogen bonds are affected by the chemical nature of the compounds involved and by the changes in the molecular environment (pH, viscosity, solvent nature, and salinity, etc.) is of great importance to have a deep insight of such biochemical processes and to be able to manipulate them.

It is clear that the study of hydrogen bonding in biochemical processes, and any other similar interactions, would be better carried out in solution, since those conditions would be closest to physiological conditions. On the other hand, from a technical point of view, the most attractive features of NMR for inter-molecular interaction studies are three. First, the possibility of determination of inter-atomic distances and the topology of the molecular recognition using techniques which are based on nuclear relaxation and related phenomena. Among those techniques are included non-selective, selective, and double selective longitudinal or spin–lattice relaxation rates (R_1, R_1^S, and R_1^{DS}), which allow for the determination of inter-proton distances

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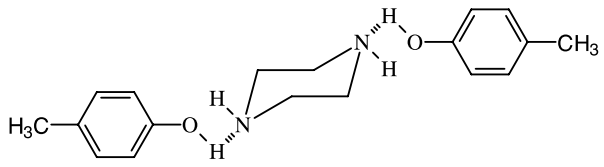

Fig. 1. 2:1 *p*-Cresol piperazine complex.

and correlation times (τ_c) [8,9]. Second, the possibility of determination of molecular self-diffusion coefficients (D) using DOSY and related techniques [10]. Third, NMR allows for the study of the dynamics involved in certain inter-molecular interaction studies [11].

Recently, the X-ray diffraction structure of the simple 2:1 *p*-cresol:piperazine complex (Fig. 1) has been reported [12]. This complex seems to be a very good model for inter-molecular hydrogen bonding studies, as its X-ray diffraction data points out that the two *p*-cresol molecules are attached to the piperazine nitrogens by hydrogen bonds involving the phenolic hydrogens [12].

In this work, we have carried out a nuclear relaxation and molecular diffusion NMR study of this complex in order to determine if it does exist in solution and which would be its nature. This complex would be also used to test the NMR techniques NULL [13] and double selective longitudinal relaxation measurements [14], which will be employed for the first time to determine intermolecular distances.

2. Results and discussion

Initially, it was necessary to determine if this trimolecular complex exists in chloroform solution, and if it does so in a similar manner as in the solid state. For this, we first determined the ^{1}H NMR spectrum of the pure components and their mixtures at different proportions. The results show that the chemical shifts for all the mixtures are quite similar, but different from the chemical shifts of the pure components of the complex, as shown in Fig. 2. Table 1 shows the data for the two extreme conditions.

It can be observed that the chemical shift changes induced by complex formation are best detected for the acid

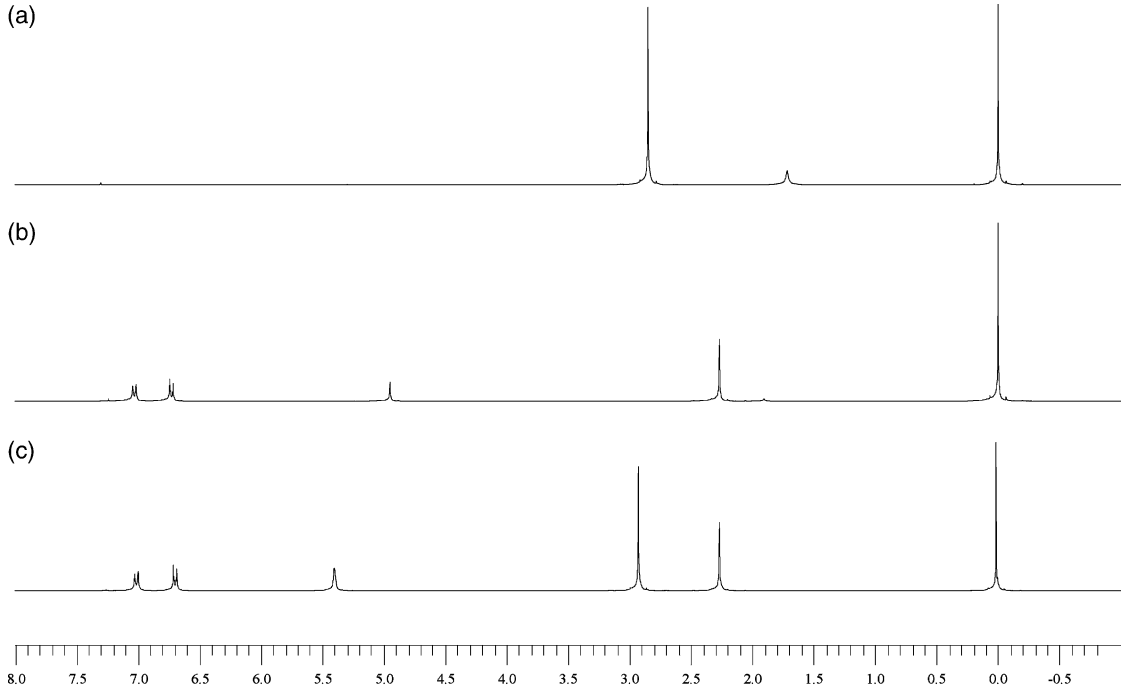

Fig. 2. Comparison between the expansion of the ^{1}H NMR spectra of (a) pure piperazine, (b) pure *p*-cresol, and (c) 2:1 *p*-cresol:piperazine complex.

Table 1
Chemical shifts (ppm) of the different hydrogens of pure *p*-cresol and piperazine and their complex in chloroform solution

Compound	NH (2H)	CH_2 (8H)	OH (2H)	H-2,H-6 (4H)	H-3,H-5 (4H)	CH_3 (6H)
Piperazine	1.81	2.69	–	–	–	–
p-Cresol	–	–	4.78	6.75	7.05	2.29
Complex	–	2.95	5.51	6.70	7.02	2.28

hydrogens of both components and for the methylene groups of piperazine. The NH hydrogens signal, which is observed as a broad singlet at 1.81 ppm in the spectrum of pure piperazine disappears in the spectrum of the complex, possibly because they are broadened to an extent that they can not be detected, even through integration of a 20 ppm spectral window. On the other hand, the OH hydrogen is deshielded by 0.73 ppm and is significantly narrowed upon complex formation, this being an evidence of its participation on plausible hydrogen bonds between piperazine and *o*-cresol, which is not the case for the NH hydrogens of piperazine. As mentioned above, the only other significant spectral change is observed for the signal corresponding to the four CH_2 groups of piperazine, which is deshielded 0.26 ppm. The relative intensities of the complex hydrogens determine that the entity formed includes two *p*-cresol molecules for each piperazine molecule, thus confirming the assumed complex stoichiometry. Also, the fact that the OH signal (2H) remains different from the missing NH signal strongly suggests that this entity is a complex rather than a salt, as in the latter the only acid hydrogens would correspond to the two equivalent $R_2NH_2^+$ groups, which would originate a 4H singlet that is not detected.

The ^{13}C NMR chemical shifts for all the carbons of the complex were determined in solution and the solid state, and of pure *p*-cresol and piperazine in solution. The comparison between the different ^{13}C NMR spectra in the solid state and in solution for the complex is shown in Fig. 3. The results are summarized in Table 2.

It is clear that the same complex species that was characterized by X-ray diffraction is present in solution, as the ^{13}C NMR spectra in solution and the solid state are almost identical. For *p*-cresol only C-1 and C-4 suffer a significant chemical shift variation upon complex formation. In the case of C-1, there is a deshielding of 1.4 ppm in relation to free *p*-cresol. On the other hand, the only carbon signal of piperazine is shielded by 1.1 ppm when complexed. In solution, there should be a rapid exchange between the free and the complexed state for each on of the components, but that is not possible in the solid state. Since for the complex both spectra, in solution and solid, are quite similar, it can be concluded that either the complex exists in solution with a minimum exchange or, because the chemical shifts for the free and complexed molecules are very similar, the rapid exchange only produces small variations in chemical shifts, as shown in Table 2. With the evidence obtained at that time we believed that the second alternative was more likely to occur.

As a whole, these observations indicate that there is an interaction between both molecular components, *p*-cresol and piperazine, strongly pointing out to the formation of a complex, but do not allow for a definite clear-cut distinction between this possibility and the formation of a salt.

We then proceeded to determine the self-diffusion coefficients of the pure molecular components and in the 2:1 mixture in chloroform solution using DOSY [10] with the stimulated echo sequence, [15] shown in Fig. 4.

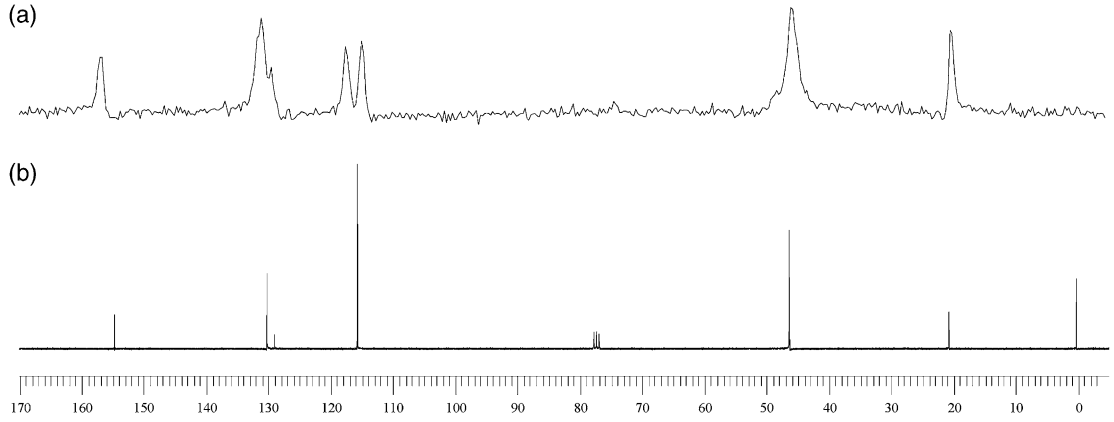

Fig. 3. Comparison between the ^{13}C NMR spectra of the 2:1 *p*-cresol:piperazine complex (a) in the solid state (CPMAS) and (b) in solution in $CDCl_3$.

Table 2
^{13}C NMR chemical shifts for *p*-cresol and piperazine in solution and for the complex in solution and in the solid state

Compound	C-1	C-2, C-6	C-3, C-5	C-4	CH_3	CH_2
p-Cresol	153.3	115.5	130.3	130.4	20.8	–
Piperazine	–	–	–	–	–	47.4
Complex	154.7	115.7	130.2	128.9	20.7	46.3
Complex[a]	157.2	115.2	131.4	117.4	20.3	45.3

[a] Determined in the solid state by CP/MAS.

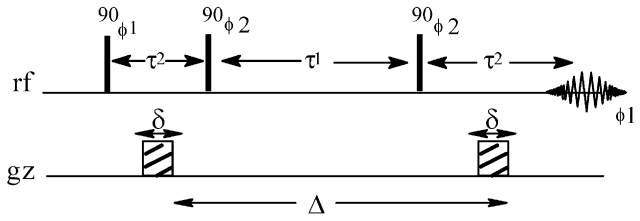

Fig. 4. Pulse sequence for the stimulated echo sequence for DOSY.

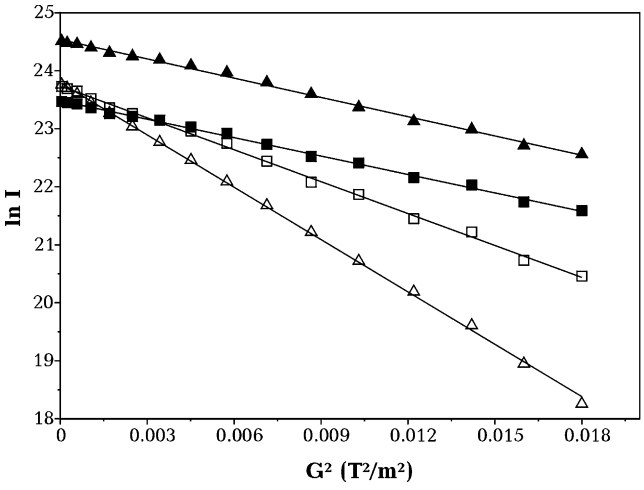

Fig. 5. Signal intensity variation with magnetic field gradient during the DOSY experiments for *o*-cresol, pure (□) and in the complex (■), and for piperazine, pure (△) and in the complex (▲).

Table 3
Self-diffusion coefficients for pure *p*-cresol and piperazine in chloroform and in the complex

Molecule	D (m²/s)	
	Pure in $CDCl_3$	In the complex in $CDCl_3$
p-Cresol	16.7×10^{-10}	9.70×10^{-10}
Piperazine	25.3×10^{-10}	9.91×10^{-10}

In the stimulated spin-echo sequence the echo attenuation for a single diffusing species is given by $I = I_o \exp[-(2\tau_1/T_2) - (\tau_2/T_1)] \exp[-D(\gamma g \delta)^2(\Delta - \delta/3)]$. The first exponential, due to T_1 and T_2 being much greater than τ_1 and τ_2 approaches to unity and the expression can be simplified to $I = I_o \exp[-\gamma^2 g^2 D \delta^2(\Delta - \delta/3)]$, where γ is the giromagnetic ratio and g, δ, and Δ are the amplitude, duration and separation of the single pair gradient pulses, respectively, as shown in the pulse sequence diagram (Fig. 4). The signal intensity variation data from the DOSY experiments is shown in Fig. 5, and the determined diffusion coefficients (D) are shown in Table 3.

From Table 3 it is observed that the self-diffusion coefficients for the free molecular components are, at the least, half their values in the complex. It is observed that piperazine suffers the greatest diffusion change when complexed (a factor of 2.55), a fact that agrees with its participation in the complex as the central molecule, being hydrogen-bonded to two *o*-cresol molecules. The measurements were carried out in dilute solution in chloroform, where viscosity variations due to small sample composition changes are negligible, thus the significant decrease in diffusion for *p*-cresol when piperazine is present, is a strong evidence for the existence of the complex in solution. If the observed phenomenon were salt formation, the self-diffusion coefficients changes would be significantly smaller, as the hydrogen transfer from the phenol to the amine would produce three independent ions, two phenoxides (charge −1) and one piperazonium (charge +2). Despite the Coulombic attraction between the ions, they would move more freely in solution than the molecular components in solutions of the 2:1 complex, in which the molecules would be kept together by means of two hydrogen bonds.

The next step in this study was to characterize the interaction between piperazine and *p*-cresol in solution. Initially, we carried out determinations of non-selective and selective spin–lattice relaxation times for *p*-cresol, piperazine and the complex. The selective T_1 measurements were carried out using a selective version of the inversion-recovery (IR) method, where the initial hard 180° pulse was substituted by a 180° DANTE pulse [16,17]. The results are summarized in Table 4. Although there are modern and efficient ways to achieve selective excitation using shaped RF pulses, older techniques such as DANTE are still very efficient and useful, specially for spectroscopists that do not have access to wave form generators.

With this data it is possible to calculate the molecular correlation times (τ_c) for all the entities involved. For a pure dipolar mechanism, the relaxation rates $R^{NS}(1/T_1^{NS})$ and $R^{S}(1/T_1^{S})$ for a hydrogen i are give by $R_{1i}^{NS} = \Sigma_{j \neq I} \rho_{ij} + \Sigma_{j \neq I} \sigma_{ij} + \rho_i^*$ and $R_{1i}^{S} = \Sigma_{j \neq I} \rho_{ij} + \rho_i^*$, respectively, where ρ_{ij} and σ_{ij} are the direct relaxation rate and the crossrelaxation term for a pair of hydrogens i and j. According to the literature, [16] for a pure dipolar relaxation mechanism for a pair of hydrogens, if $R_1^S < R_1^{NS}$ and $R_1^{NS}/R_1^S = 1.5$ molecule is under the extreme narrowing conditions, that is $\omega\tau_c \ll 1$, where ω is the Larmor frequency and τ_c is the correlation time. Accordingly, the hydrogens of that molecule present short τ_c values, which are characteristic of small molecules. If $R_1^{NS}/R_1^S \sim 1$, the molecule is in a region where $\omega\tau_c \sim 1$, thus presenting an intermediate correlation time, typical of medium size molecules. Usually, for big molecules $\omega\tau_c \gg 1$.

Using the data from Table 4, it is possible to calculate the ratio R_1^{NS}/R_1^S for each one of the hydrogens in all the present entities. For example, for pure 0.2 M *p*-cresol in chloroform this ratio is 0.82 ($\omega\tau_c < 1$), indicating that it behaves as a small molecule, as it should be expected. On the other hand, the R_1^{NS}/R_1^S for *p*-cresol in the

Table 4
Selective (S) and non-selective (NS) spin–lattice relaxation measurements for 0.2 M *p*-cresol, piperazine, and the complex

H	T_1 (s)					
	Pure *p*-cresol		Pure piperazine		Complex	
	NS	S	NS	S	NS	S
H-2,6	3.56	2.92	–	–	1.70	1.96
H-3,5	3.56	–	–	–	2.15	2.47
OH	1.65	–	–	–	0.52	0.63
CH_3	2.25	1.28	–	–	1.20	1.14
CH_2	–	–	2.38	1.05	0.46	0.48

presence of piperazine is 1.15, which is characteristic of a medium size entity, a fact that is in total agreement with the hypothesis of complex formation. This information is confirmed through the calculation of the correlation times, which are 4.05×10^{-10} s for pure *p*-cresol, and 4.77×10^{-10} s for *p*-cresol in the presence of piperazine, both in a 0.2 M solution in chloroform. The slight increase in the value of τ_c for *p*-cresol when piperazine is present is compatible with an increase in entity size, in agreement with complex formation.

A more accurate form of determination of complex formation and its structural properties would be to measure the distance between the *p*-cresol and the piperazine in the complex in solution, and to compare this information with the previous X-ray diffraction results [12]. If this were possible we would have proof of the complex formation. The methodology used for the intermolecular distance measurement was based on the determination of the crossrelaxation rate between two hydrogens in the complex, one from each component. When the selective relaxation rate, R^S_{1i}, is being determined, the excitation pulse perturbs only the selected hydrogen i and there is not any contribution from crossrelaxation with other nuclei, In this case, the total relaxation rate for hydrogen i is given by $R^S_{1i} = \Sigma_{j\neq I}\rho_{ij} + \rho^*_I$, where ρ_{ij} is the dipolar relaxation term between hydrogen i and hydrogens j and ρ^* is the contribution from all other relaxation mechanisms. This last term is negligible for inter-hydrogen relaxation and can be ignored in this case. When a double selective excitation experiment is carried out, that is two hydrogens i and k are selectively inverted, the total relaxation rate for hydrogens i and k should now include the crossrelaxation term between these two hydrogens (σ_{ki}) and the expressions for the double excitation relaxation rates become $R^{DS}_{1i} = \Sigma_{j\neq I}\rho_{ij} + \sigma_{ik} + \rho^*_i$ for hydrogen i and $R^{DS}_{1k} = \Sigma_{j\neq K}\rho_{kj} + \sigma_{ki} + \rho^*_k$ for hydrogen k. The crossrelaxation term σ_{ki} can be obtained from the difference $R^{DS}_k - R^S_k = \sigma_{ki} = \sigma_{ik} = R^{DS}_i - R^S_i$. Furthermore, in the extreme narrowing region, where $\omega\tau_c \ll 1$, the dipolar relaxation and the crossrelaxation terms are given by $\rho_{ik} = (\mu_0/4\pi)^2 3\gamma^4 h\tau_c/4\pi r^6$ and $\sigma_{ik} = \rho_{ik}/2$, respectively, where r is the distance between hydrogens i and k. Clearly, if the correlation times (τ_c) and the crossrelaxation term (σ_{ik}) are known, it is possible to calculate r. In the present case, to carry out such measurements, it was necessary to choose one hydrogen from each component. It was decided that the best choice for *p*-cresol was its hydroxyl hydrogen, since it is observed under all conditions and because it participates directly in the hydrogen bonding observed in the X-ray diffraction studies [12]. For piperazine, the only choice is the CH_2 signal, since the NH signal disappears when in the presence of *p*-cresol.

Another method to determine the desired OH–CH_2 distance is by means of the NULL method [13]. In this case a selective 180° pulse is used to invert a chosen hydrogen i. Immediately after that pulse, a non-selective 180° pulse is applied to the spin system in order conduce hydrogen i to its equilibrium condition, that is, aligned with B_o, and simultaneously invert all the other hydrogens (k, j) of the system in study. This sequence is followed by a variable delay τ, a non-selective 90° pulse and acquisition. The variation of τ allows for the intensity modulation, and T_1 determination for all the hydrogens k, j, but without crossrelaxation to hydrogen i. Accordingly, if all the hydrogens but i are inverted, the NULL relaxation rate for any of the other hydrogens (k, j), for example j, would be given by $R^{NULL}_{1j} = \Sigma_{k\neq j}\rho_{jk} + \Sigma_{k\neq j,i}\sigma_{jk}$, where the first term refers to the contribution of the dipolar relaxation between the all the spins, including the non-inverted spin i, and the second term describes the crossrelaxation between all hydrogens but i. Since the non-selective relaxation rate for hydrogen j is given by $R^{NS}_{1j} = \Sigma_{k\neq j}\rho_{ik} + \Sigma_{k\neq j}\sigma_{ik}$, the difference between both relaxation rates gives the crossrelaxation contribution between hydrogens i and j: $R^{NS}_{1j} - R^{NULL}_{1j} = \Sigma_{k\neq j}\sigma_{ik} - \Sigma_{k\neq j}\sigma_{jk} = \sigma_{ij}$, thus allowing for the calculation of the distance r_{ij}.

Also, the distance r_{ij} was calculated using the nuclear Overhauser effect (NOE) observed in a series of difference NOE experiments [18]. The selective saturation of the CH_2 signal ($\delta = 2.95$ ppm) while monitoring the signal intensity of the OH signal at $\delta = 5.51$ ppm, led to the calculation of the OH–CH_2 distance as being 3.6 Å.

For comparison with the structure of the complex in the solid state, since the X-ray diffraction data does not include coordinates for the hydrogen atoms, it was

necessary to obtain a theoretical molecular model of the complex. A crude model of the complex was built manually and minimized with a constraint for the O–N distance to 2.70 Å, which corresponds to the O–N distance from the X-ray data. The model was subjected to minimization, using the Gaussian 98/GaussianView 2.1 [19] package and the method HF-3.21G, until a gradient of 10^{-9} kcal/molÅ was reached. In order to validate the OH–CH_2 distance obtained in this way, several experimental (X-ray) inter-atomic distances were compared to the calculated distances. These data are shown in Table 5.

From Table 5 it is easily seen that the experimental (X-ray) and the calculated inter-atomic distances agree quite well, with errors lower than 1%, thus validating the calculated 3.16 Å distance for the OH–CH_2. The results for this distance as obtained by the different methods are shown in Table 6.

Analyzing the data from Table 6 it is possible to conclude that the expected 2:1 complex of *p*-cresol with piperazine does exist in chloroform solution. The NMR measured OH–CH_2 distance in solution shows a very good agreement between the double selective T_1 and the NULL methods, and a greater variation for the result obtained by NOE. This seems to be normal, since the T_1-based methods are more accurate. The OH–CH_2 distance obtained by molecular modeling for the complex in the solid state is significantly smaller than the experimental values in solution. This seems to be likely as it could be expected that the complex be more compact in the solid state than in solution, where the interactions of the individual components of the complex with the solvent would keep them further apart.

Table 5
Experimental (X-ray) and calculated (HF 3.21G) distances (Å) for the 2:1 *p*-cresol:piperazine complex

	Theoretical	X-ray	Error (%)
O–N	2.68	2.70	0.66
O–C-1	1.36	1.37	0.73
C-1–C-2	1.38	1.38	0.22
N–C-2	1.48	1.47	0.95
OH–N	1.68	—	—
OH–CH_2	3.16	—	—

Table 6
Values for the OH–CH_2 distance in the 2:1 *p*-cresol:piperazine complex obtained with the different methodologies discussed in the text

Method	OH–CH_2 distance (Å)
Double selective T_1	3.31
NULL	3.29
NOE	3.6
Molecular modeling (X-ray)	3.16

3. Experimental

The 2:1 complex of *p*-cresol with piperazine was prepared as described in the literature [20], by dissolving both reagents in toluene and refluxing for 10–15 min. After cooling, the crystals formed were separated by filtration and air-dried at room temperature. The prism shaped crystals have a melting point of 90–91 °C (Literature 93 °C [20]).

The simple ^1H and ^{13}C NMR spectra were determined in $CDCl_3$ using TMS as internal reference in a Varian Unity-300 (300 MHz) NMR spectrometer at 19 ± 0.1 °C, using 45° RF pulses (7.2 μs for ^1H and 10.2 μs for ^{13}C). The solid-state analyses were carried out on a Varian XL-300 spectrometer, operating at 75.4 MHz for ^{13}C with CP/MAS and using samples in a 7 mm zirconium oxide rotor rotating at 5.6 kHz. Non-selective T_1 values were measured using the standard inversion-recovery program. For the selective experiments, the 180° selective inversion pulse was achieved by replacement of the hard 180° pulse of the inversion-recovery sequence by a DANTE train (200 pulses, $\tau = 40$–55 μs) with the transmitter power attenuation adjusted to 45 dB (13 dB attenuation in relation to the non-selective pulse). To accomplish double selective excitations, the transmitter offset of the DANTE pulses (50 pulses, $\tau = 84$ μs) was set to excite one of the desired frequencies, while the second desired frequency was perturbed with the first DANTE excitation side band, which frequency was set out by adjusting of the delay between pulses in the DANTE train (τ). The selective 180° pulse for the NULL sequence [13] was obtained using the composite pulse, $\pi/2_x - \pi_y - \pi/2_x$, where each part of the composite pulse was made up of a DANTE sequence (100 pulses, $\tau = 40$ μs). This method gave better selectivity than a single 180° DANTE pulse train.

Pulse field gradient (PFG) NMR spectra [10,11] were acquired at 25.0 ± 0.1 °C on a Bruker DRX-400 spectrometer using a 5 mm broadband inverse probe with a single gradient (Z). A pulse gradient stimulated echo sequence was used [11]. Diffusion coefficients were measured by incrementing the amplitude of the field gradient pulses in 16 steps (0.68–13.62 G cm^{-1}). The duration of field gradient pulse (10 ms) and the diffusion time (20 ms) were constant. The spectra were recorded with eight scans in a 2D mode for each measurement, with a recycle time of 1.5 s between scans.

The NOE difference spectra were determined using the CYCLENOE pulse sequence [20] with a selective decoupling power of 45 dB and 512 transients.

The theoretical structure of the complex was assembled on basis of the X-ray data [12]. The crude model was optimized using the Gaussian 98/GaussianView [19] package at the Hartree–Fock 3-21G level, using consecutively the steepest descent and conjugate gradient algorithms until an energy gradient of 10^{-9} kcal mol^{-1} $Å^{-1}$ was reached.

4. Conclusions

All the tests and measurements that were carried out with the 2:1 complex of *p*-cresol and piperazine indicate that this complex exists in chloroform solution in a very similar way as it does in the solid state. Two *p*-cresol molecules are linked to the nitrogen atoms of the central piperazine molecule through a single hydrogen bond between the piperazine nitrogen and the phenol OH. The fact that it is possible to determine the OH–N distance in solution by different NMR methods is indicative that the complex is very stable under those conditions, as the parameters determined in solution by NMR correspond normally to the isotropic average values. This fact could be ignored only if the measured parameter values were very similar for the free molecules and the complex, which is not the case in this work.

We have shown that NULL and double selective excitation techniques are indeed appropriate for the determination of inter-molecular distances in complexes of this nature. This kind of methodology could be of importance in the study of inter-molecular interactions and molecular recognition processes.

Having established the nature of the complex in solution we are now working on the influence of the phenol acidity on the nature and structure of the complex.

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