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UV–vis, IR and ^{1}H NMR spectroscopic studies and characterization of ionic-pair crystal violet–oxytetracycline

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ABSTRACT

The present study shows the formation and characterization of the ionic-pair between the antibiotic oxytetracycline and the dye crystal violet in ammonia solution pH 9.0 ± 0.2 extracted into chloroform. The characterization was demonstrated using UV–vis spectrophotometry, ^{1}H NMR, measurement of relaxation times T_1 and IR spectroscopy, using a comparison between the signals of individual pure compounds with the signals with the mixture CV–OTC in different alkaline media. The formation of ionic-pair was also corroborated by new signals and chemical shifts. (2D) NMR spectroscopy experiments show that the interaction is electrostatic.

1. Introduction

The oxytetracycline (OTC), is a wide antibacterial spectrum antibiotic belonging to the tetracyclines (TCAs) family, that acts inhibiting the synthesis of the bacterial proteins for fixation to the ribosomal 30 s subunit [1]. For this reason, the OTC is strongly employed in the salmon and other food industries. Its residual concentrations in the foods could be produce adverse effects in the human [2,3].

Fig. 1 shows the structure of OTC that presents a hydronaphtacene skeleton containing four fused rings. In general, the tetracyclines are chelating agent in which their antibacterial activity and their pharmokinetics properties are influenced by the metallic ions chelation [1,4].

The OTC have acid base properties, due to the presence of hydroxyl and amino groups, containing three pKa values, C_3-OH pKa_1 3.2; C_4-N$(CH_3)_2$ pKa_2 7.5; and C_{10}-OH pKa_3 8.9 [5]. The characterization of OTC has been largely reported by ^{13}C NMR and ^{1}H NMR, in different deuterated solvents [6,7].

On the other hand, the dye crystal violet (CV) belonging to the triarylmethane group (Fig. 1), it is used thoroughly in medicine as antiseptic agent, prescribed for bacterial infections, as dyes in the textile industry [8,9] and it is also used as fungicidal preservative. Due to these properties its inadequate use in aquiculture is a potential risk for the human health. Recently on this respect, the literature has informed the use of CV in the aquiculture [10].

The solid of the crystal violet hydrochloride contains an acid proton (pKa 1.0) [9] (Fig. 1), for this motive CV is a neutral molecule, so this compound is possible to dissolve in organic solvent. However, in aqueous medium it produced a molecular reordering liberating the acid proton and forming a stable cation, with the positive charge delocalized among the three amines groups.

Many organic dyes have demonstrated to be effective agents in the ionic-pairs formation, for wide-ranging and different types of chemical species, including antibiotics [11,12]. The ionic-pairs formation methodology, it is generally used to extract substances from aqueous biological material, pharmaceutical formulations and residual waters, among others, as example in the pentacaine extraction from biological material [13]. In the same way CV has been used for the OTC liquid–liquid extraction (LLE) where Mishra et al. [14] showed that this process is more efficient using chloroform as the organic solvent when using a pH 9–10 solution buffer. Nevertheless, no further proof of this ionic-pair formation or his characterization was presented, and the final concentration of the antibiotic was determined by second LLE procedure with 0.1 mol/L HCl.

The combination of formation of ionic-pair and LLE with spectrophotometric detection has been used because the addition of a chromophere highly conjugated to the system, increases the probability of the π–π^* transition and the molar absorptivities also increase [15].

In this context, the aim of this work is verify and to characterize the CV-OTC ionic-pair formation, by means of nuclear magnetic resonance (NMR) spectroscopy, measurement of relaxation times (T_1) and infrared spectroscopy (IR). This ionic-pair can be useful to develop alternative analytic methodologies for the determination of OTC using different techniques including spec-

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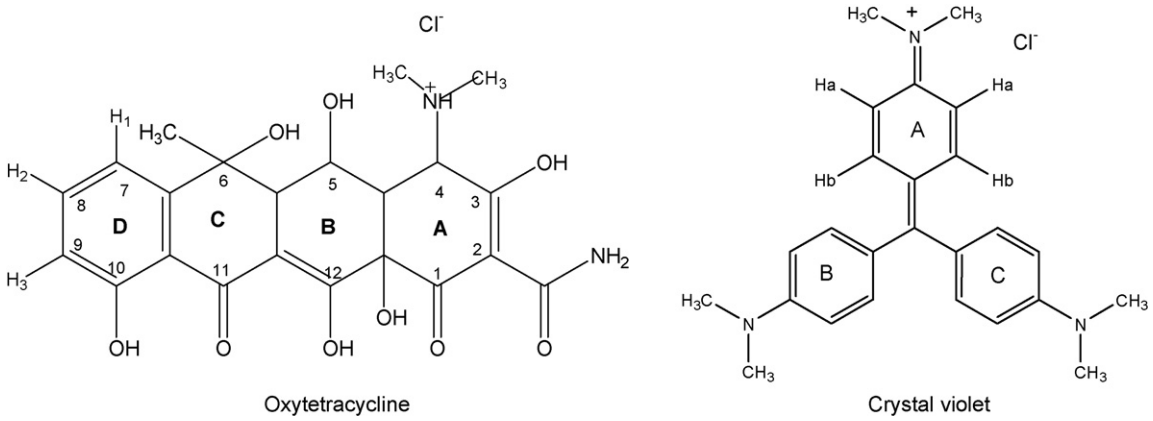

Fig. 1. Structures of oxytetracycline (OTC) and crystal violet (CV).

trophotometry, with this technique is possible to have a simpler and lower cost method in comparison to other more complex and expensive options [16,17].

2. Experimental

2.1. Reagents

Methanol, chloroform, 99.8% deuterated water (D_2O), 99.8% deuterated chloroform ($CDCl_3$), 25% (w/w) ammonia (NH_3) and crystal violet hydrochloride (CV) were purchased from Merck®. Oxytetracycline hydrochloride (OTC) was purchased from Sigma®.

Standard solution 1.0×10^{-3} mol/L of OTC and CV were prepared by dissolving 0.025 g of OTC and 0.020 g of CV to 50 mL with methanol. Both solutions were stored in an amber container and refrigerated at 5 °C.

2.2. Apparatus

The spectral measurements were carried out at room temperature (25 ± 1.0 °C). The UV–vis spectra were recorded with

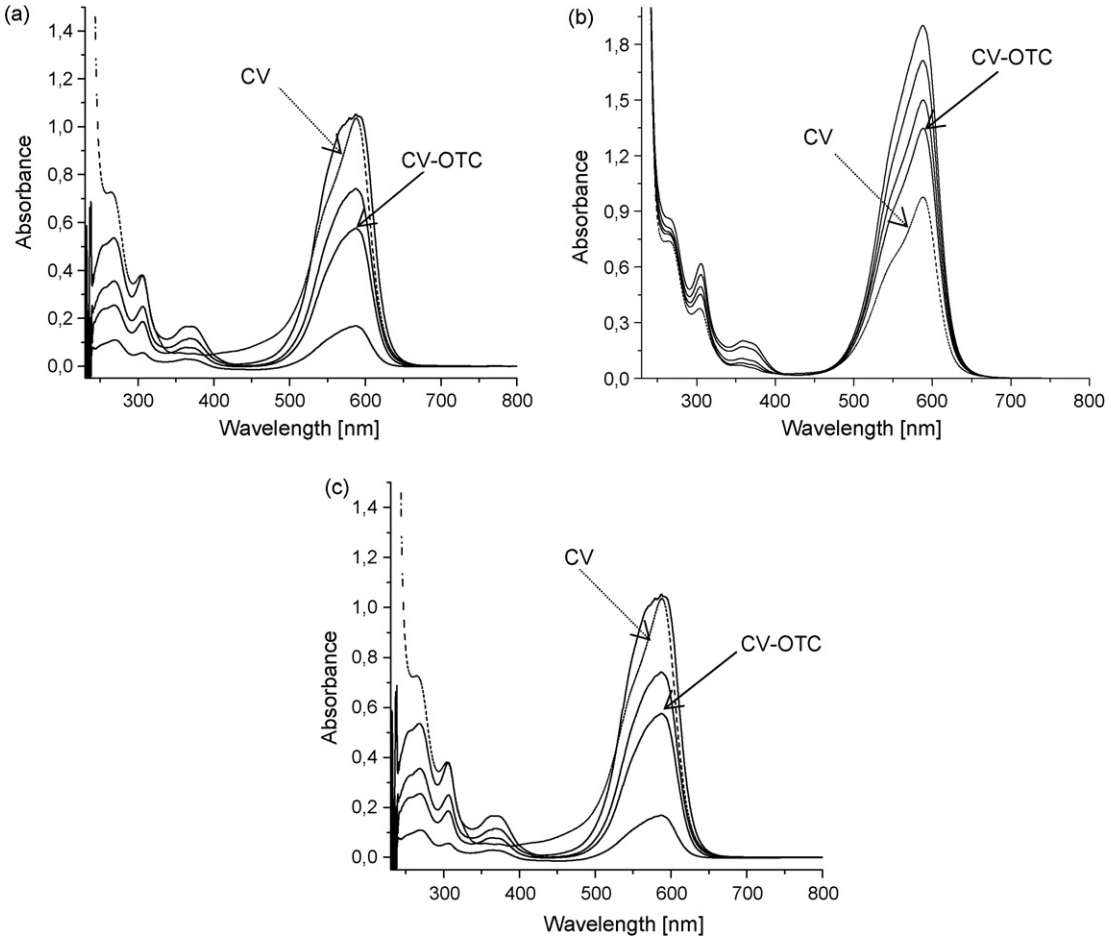

Fig. 2. Absorption spectra of the CV and CV–OTC, in different basic media. (a) Medium NaOH 2.0×10^{-4} mol/L, (b) medium NH_3 2.0×10^{-4} mol/L and (c) medium $Na_2B_4O_7$ 2.0×10^{-4} mol/L. Concentration CV: 8.0×10^{-6} mol/L and OTC: 4.0×10^{-7}, 8.0×10^{-7}, 12.0×10^{-7} and 16.0×10^{-7} mol/L.

1.0 cm quartz cells on a Shimadzu 1603 spectrophotometer in the 190–800 nm range with a sampling interval of 0.5 nm, online connected to a computer Hewlett Packard. The derivative spectra were obtained using a software Kit version 3.7 (P/N 206-60570-04). The IR spectra were obtained using a spectrophotometer FT-IR, Perkin-Elmer System 2000, in the region from 4000 to 600 cm^{-1}. The data were processed with software version ® 386 Grams. The ^1H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer using TMS as internal standard. The data were processed with the software MestReC V4 [1] .8.6.

2.3. General procedure for the determination of ionic-pairs by liquid–liquid extraction

In different 250 mL Erlenmeyer flask were added: Aliquots between 20 and 100 μL of 1.0×10^{-3} mol/L OTC solution to obtain ranges of concentration between 4.0×10^{-7} mol/L and 20.0×10^{-7} mol/L, 400 μL of 1.0×10^{-3} mol/L CV solution and 100 μL of 0.1 mol/L NH_3 solution to obtain pH 9.0 ± 0.2. Then 50 mL deionised water was added. This solution was stirred 1 min and following 6 mL of $CHCl_3$ was added and stirred by 4 min; the mixture was transferred to a 100 mL separating funnel and was wait until phase's separation. Finally, in the organic phase were obtained zero order spectra.

2.4. Preparation of samples for NMR spectroscopy

Samples were prepared from the standard solid dissolving 10 mg of CV and OTC in $CDCl_3$ and D_2O, respectively. The ionic-pair sample was prepared with 12 mg and 10 mg of OTC and CV into 50 mL of ammonia solution (pH 9.0 ± 0.2). Afterwards, were extracted with $CHCl_3$ using a 100 mL separating funnel (three fractions of 20 mL). The respective chloroform extracts were dryness by evaporation and dissolved in 1 mL of $CDCl_3$.

2.5. Preparation of samples for IR spectroscopy

The IR spectra for CV and OTC were measured in potassium bromide tablets. In the case of CV and CV-OTC in the organic extracts, the solvent was evaporated into dismantled cells of NaCl.

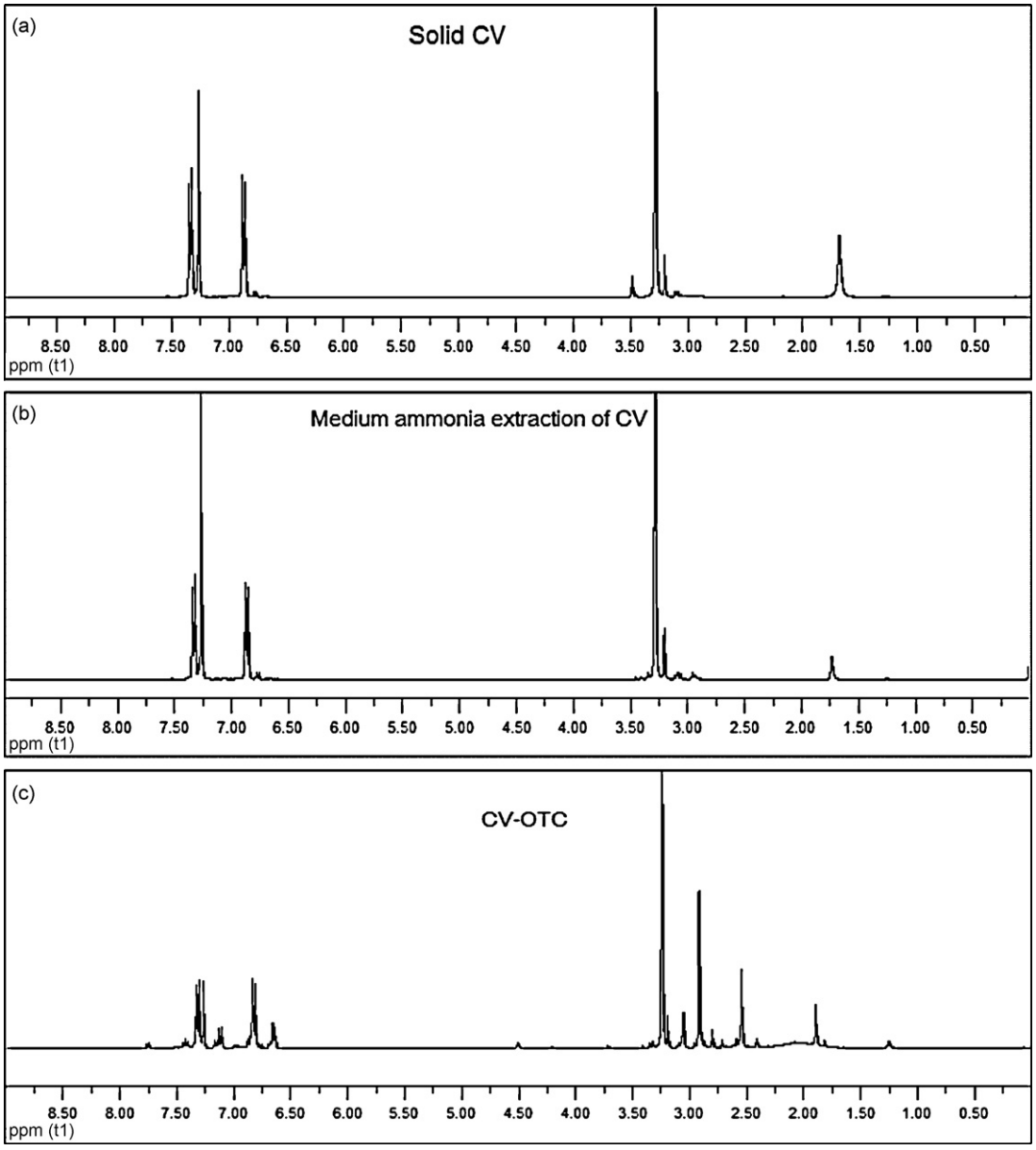

Fig. 3. ^1H NMR spectra in $CDCl_3$ of the (a) CV solid, (b) CV in medium ammoniac and (c) CV–OTC ionic-pair in medium ammoniac.

3. Results and discussion

3.1. Formation of the CV-OTC ionic-pair by liquid–liquid extraction

In this work it is assumed that the formation of CV-OTC in basic medium is based on the electrostatic interaction among OTC and CV in an organic solvent.

The results obtained by UV–vis spectrophotometry, indicating the possible formation of CV-OTC ionic-pair using the following basic media: NaOH, NH_3 and $Na_2B_4O_7$ (Fig. 2). A spectral analysis shows that CV in presence of these media present a principal band at 590 nm; which is broad and depends of the OTC concentration. These bands are assigned to the transitions π–π^* aromatic ring of dye. Furthermore, this ionic-pair presents a band between 250 and 400 nm corresponding to n–π^* transitions of ketones and amide groups of the antibiotic, this drug it is not extract in absence of CV. For these reasons it is possible to postulate in first instance the formation of ionic-pair.

3.2. Characteristic signals of OTC and CV by ^1H NMR

In order to confirm the quality and characteristic signals of OTC was obtained the ^1H NMR spectrum of OTC standard solid in D_2O. This spectrum presents signals characteristics and similar according to that reported for Schach von Wittenau and Blackwood [6], among them are the signals corresponding to the protons of the aromatic ring between 6.90 and 7.51 ppm, a singlet at 4.25 ppm, 2.95 ppm and 1.75 ppm, corresponding to the C_5–H, C_4–$NH(CH_3)_2$ and C_6–CH_3, respectively.

In this context, the ^1H NMR spectrum of CV standard solid, using $CDCl_3$ was obtained. The signals characteristics were the following: two doublets at 6.88 and 7.32 ppm of aromatic protons and a singlet at 3.23 ppm of the N–CH_3 equivalent groups (Fig. 3-a). These signals were used as a basis for studying the behavior of CV at different pH basic media.

3.3. Assigning signals of CV and OTC in basic media

3.3.1. Basic medium: NaOH

The ^1H NMR of CV and OTC in different basic medium were obtained. The ^1H NMR spectrum of CV in NaOH solution (pH 9.0 ± 0.2) shows a large difference with the main aromatic signals of the CV standard solid, in these conditions appear a new signals of lower intensity assignable to aromatic protons, between 6.80 and 7.20 ppm, and also appear other new aliphatic signals between 2.50 and 3.20 ppm, suggesting CV decomposition or formation of a new species, these are present in lower amount. The new compound could be formed by CV resonant specie, suggesting a deficit of electrons in the tertiary carbon and could be attacked by hydroxyl ions. These facts lead to discard this medium pH, because is not satisfactory to formation of CV-OTC ionic-pair.

The ^1H NMR spectrum of OTC dissolved in D_2O in presence of NaOH (pH 9.0 ± 0.2) shows new signals with different chemical shift, compared with the spectrum of OTC standard solid dissolved in D_2O, suggesting changes in the antibiotic.

In this condition the formation of CV-OTC ionic-pair could be to produce also the formation of new species from the decomposition of both compounds.

3.3.2. Basic medium: $Na_2B_4O_7$

The CV signals identification by ^1H NMR in $Na_2B_4O_7$ medium pH 9.3 ± 0.1 was carried out, in order to verify the possible ionic-pair formation. The spectrum shows a similar spectral behavior to those found in NaOH medium due to the presence of new signals attributable to a different species in smaller proportion or to a possible interaction among the medium with the dye, which makes more difficult to observe the identification signals of the CV aromatic protons in this medium. On the other hand, it appear new signals of small intensity between 1.00 and 4.00 ppm, which also difficult the CV-OTC ionic-pair signals identification, because of OTC also presents signals of aromatic protons with smaller intensity in relation to CV, what could carry to erroneous conclusions. For these reasons, the $Na_2B_4O_7$ as basic medium is also discarded.

3.3.3. Basic medium: NH_3

According to ^1H NMR spectra in ammoniac medium (pH 9.0 ± 0.2), the ionic-pair formation is possible because are not present additional compound. Fig. 3-b shows the spectrum of CV extracted in this medium. It is observed similarity in the signals of aromatic protons with solid CV dissolved in $CDCl_3$, in the whole measurement range. Under these conditions the ammoniac medium results to be the most appropriate to work with the cation species of CV, favoring the interaction with OTC because if the spectral comparison is carrying out, chemical shift are not observed.

In these conditions both OTC spectra dissolved in deionised water and in ammoniac medium were obtained to verify the OTC signals in the anionic form. The results shows that the signals at 5.11 ppm, attributable to the proton of phenol of the antibiotic

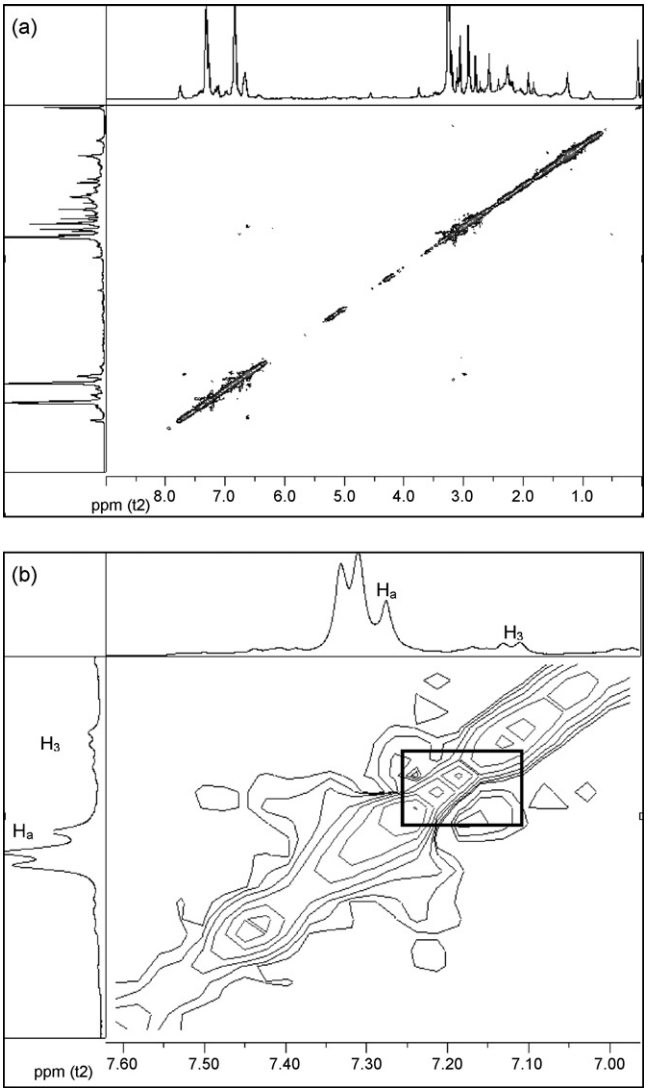

Fig. 4. Two-dimensional ROESY spectrum of CV–OTC ionic-pair in $CDCl_3$. (a) Plot between 0.0 to 9.0 ppm and (b) plot between 7.0 and 7.6 ppm.

Table 1
T_1 values for CV and CV–OTC.

CV		Peak 1		Peak 3		Peak 5		
Signal (ppm)		7.34		6.87		3.29		
T_1 (s)		1.26		0.92		0.78		
CV/OTC	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8
Signal (ppm)	7.77	6.99	6.82	6.69	3.22	3.06	2.90	2.83
T_1 (s)	2.89	1.09	0.24	1.24	0.19	1.41	1.59	1.29

dissolved in distilled water disappear when the OTC is dissolved in ammoniac solution (pH 9.0 ± 0.2), thus confirming the loss of the third proton (pKa_3) of OTC in this pH values. This situation originates an excess of electrons, making possible the electrostatic interaction with CV.

The comparison between the signal of the CV and CV–OTC are shown in Fig. 3-c. It was found that the mixture presents displacements chemical shift in the signals of the aromatic protons of CV, indicating a possible interaction with OTC. Similarly the OTC in relation to CV–OTC signals presents the chemical shift. It is postulated that the interaction between CV and OTC is of electrostatic type. As confirmation, an experiment based on the measure of the relaxation times (T_1) of the protons for CV, OTC and CV–OTC were carried out; the results are shown in Table 1.

According to results, the peak 3 CV and CV–OTC show a great difference of T_1, indicating that the aromatic protons of the CV relax in smaller time in presence of OTC, due to the impediment in the relaxation for the interaction between CV and OTC. Similar results were found for the 3.29 ppm signal, corresponding to groups N–CH_3 equivalent. In all cases, the decrease of T_1 is evident, except in peak 1 which presents a light increase in their relaxation, since it are probably the (H_b) protons of the aromatic-A ring (Fig. 1). Similarly, the signals of OTC aromatic protons were compared with those of the CV–OTC ionic-pair. These results show a notorious difference in the aromatic proton, due to the decrease of T_1, corresponding to the H_3 proton of phenol outlined in Fig. 1. In all the cases a significant decrease of T_1 is shown, concluding that the D ring of OTC is the one that interacts with CV, where the positive charge is located in the

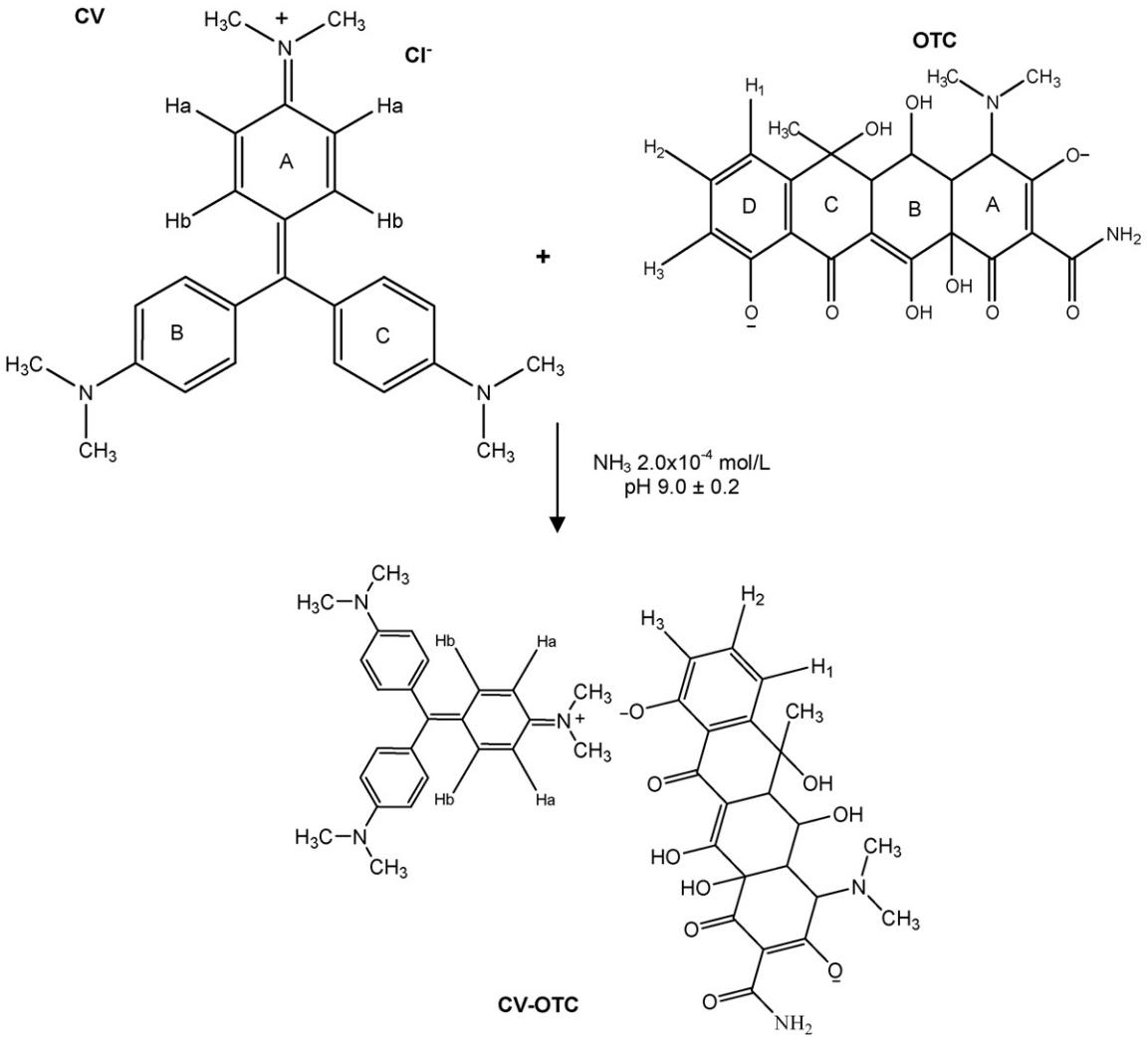

Fig. 5. Scheme of the formation CV–OTC ionic-pair.

nitrogen, confirming the CV–OTC ionic-pair formation. In all the measurements of T_1 the standard deviation ranges was between 3.0×10^{-3} to 9.0×10^{-3}.

3.4. NMR in two dimensions

The (2D) NMR spectroscopy is a significant method for determining inter-molecular and intra-molecular interaction. In order to verify the interaction between CV and OTC, the 2D-COSY for the ionic-pair was obtained. The result of two-dimensional spectrum demonstrates that the interaction is produced through the H–H link of the aromatic protons of the CV. To increase the conformational information, the spectrum 2D-ROESY of ionic-pair was obtained (Fig. 4), where it is possible to observe the interaction of the proton H_a of the CV with H_3 of OTC; which is not observed in the interaction of the aromatic protons of CV obtained by COSY.

This experiment verifies the existence of the electrostatic interaction between CV–OTC, because ROESY it is able to relate the signals through the space at a smaller distance that of 5 Å, this distance is necessary to confirm these interactions. Therefore, through these techniques was possible to confirm that the ionic-pair interaction takes place according to the scheme shown in Fig. 5.

3.5. Assignment of signals for IR spectroscopy

To characterize the ionic-pair, measurements by IR spectroscopy were made, taking into account the comparison criterion of the compounds signals in separated form with those of the mixture CV–OTC. In first instance the IR spectra were obtained of the standard solids of CV and OTC assigning the characteristic signals of the functional groups of each one of them. The results are presented in Table 2.

In order to compare the spectral signals between CV and CV–OTC, it was necessary to obtain the spectra under the same experimental conditions, which it was necessary to perform the extraction of CV and CV–OTC ammoniac medium in $CHCl_3$ and to evaporate the solvent. Fig. 6 shows the comparison of the IR spectra of CV and CV–OTC signals, where it is observed that the main bands remain. Nevertheless, a displacement of frequencies is observed in this range, associated to angular deformations in the plane of the aromatic ring, between 1.225 and 950 cm^{-1} in the mixed solution. The CV band at 816 cm^{-1} shows a major displacement at 828 cm^{-1} in presence OTC, indicating that this band associated to aromatic angular deformations outside of plane, is susceptible to the interaction of electrostatic type between CV and OTC.

The CV–OTC ionic-pair formation by means of LLE could be applied for the quantitative determination of OTC. In this context, it is necessary that the extractions are carried out using an excess and constant amount of CV and variable concentrations of OTC, so that the ionic-pair extraction depends only of OTC concentration. Preliminary studies, show the feasibility of the quantitative OTC determination in the organic phase by second order derivative spectrophotometry at 380.0 nm, this point correspond a zero crossing of CV.

Table 2
The principal signals of CV and OTC.

Compounds	IR KBr (ν cm^{-1})
CV	Ar–CH 3450; N–Me 2854-2923; Ar–C=C 1586; N–Ar 1365; Ar-angular deformations outside of plane, under 900; Ar-angular deformations in the plane 1225-950
OTC	O–H 3440–3330; N–Me 2854–2923; O=C–NH_2, C=O 1672–1587; Ar–C=C 1587; Ar–OH 1250–1150; Ar-angular deformations outside of plane, under 900; Ar-angular deformations in the plane 1225-950

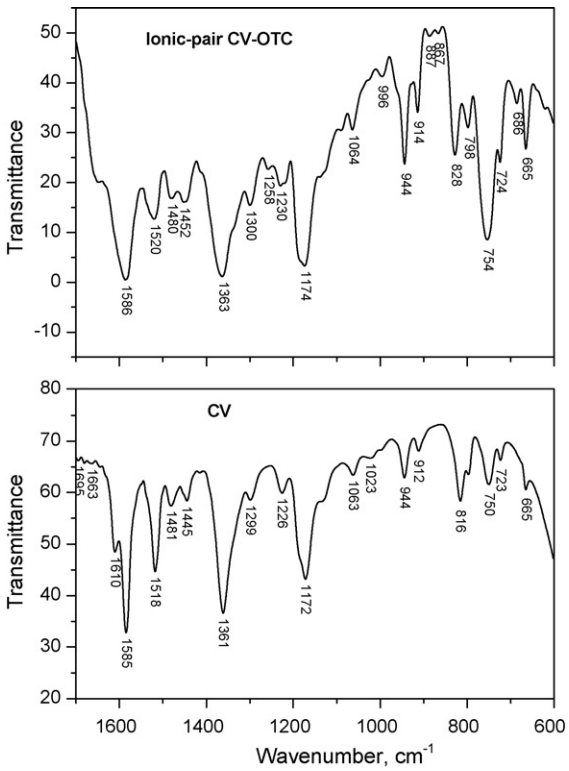

Fig. 6. Spectra IR in medium ammoniac. (a) CV–OTC ionic-pair and (b) CV.

When OTC and CV are present together it is possible to extract the ionic-pair and all the analytical signal is attributed only to CV–OTC. This signal is proportional with OTC concentration.

4. Conclusions

The aim of this work is the structural characterization of CV–OTC ionic-pair, using different spectroscopic techniques. The experimental evidence given by the comparative analysis of the spectral signals of CV, OTC and CV–OTC performed by the techniques of UV–vis, IR and ^{1}H NMR; the measurement of the relaxation times (T_1) by means of ^{1}H NMR and two-dimensional experiments, confirm the postulate of the formation of an ionic-pair in ammoniac medium between CV and OTC in chloroform.

Furthermore, the CV–OTC ionic-pair characterized could be used for the determination of OTC or CV for different techniques.

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