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Complexation of the local anesthetic pramoxine with hydroxypropyl-beta-cyclodextrin can improve its bioavailability

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ABSTRACT

Local anesthetics are widely used in clinical procedures, to eliminate pain during/after invasive procedures. A wide range of drug delivery systems has been developed to improve the effect of local anesthetics and/or to reduce their toxicity. Pramoxine (PMX) is a topical anesthetic agent with an unusual (morpholine ring) structure and low solubility (*ca.* 3 mM at pH 7.4). In this work, a novel formulation was devised for PMX in hydroxypropyl-β-cyclodextrin (HP-β-CD). Host-guest inclusion complex was prepared by the co-solubilization method, with complexation kinetics of 10 h, and 1:1 PMX/HP-β-CD stoichiometry. Complexation promoted 14-fold increase in the solubility of PMX. X-ray diffraction measurements revealed loss of the crystalline PMX pattern in the presence of HP-β-CD, an indication of inclusion complexation. Using ^{1}H NMR (DOSY) experiments the association constant of PMX to HP-β-CD (Ka = 923.1 mol/L) was determined, while nuclear Overhauser (ROESY) analysis confirmed the formation of PMX/HP-β-CD inclusion complex, by detection of spatial proximities between hydrogens from PMX aromatic ring and cyclodextrin's cavity. In two *in vitro* toxicity models (mouse 3T3 fibroblasts in culture and red blood cells hemolysis) pramoxine toxicity was significantly reduced upon complexation into HP-β-CD. These results point out PMX/HP-β-CD as a promising pharmaceutical formulation to improve the bioavailability of pramoxine, allowing its application beyond topical anesthesia.

1. Introduction

Most of the clinically available local anesthetics (LA) agents contain aromatic and ionizable amine groups separated by an intermediate chain. Besides separating the lipophilic and hydrophilic portions of the anesthetic, the intermediate chain classify LA agents into ester and amide families [1]. The clinical importance of this classification is related to chemical stability, fast of metabolization and, especially, risk of allergic reactions and systemic toxicity [2,3].

Pramoxine (PMX), also known as pramocaine, has a distinct structure (Fig. 1), being classified as an amino ether LA. Its functional ether group is much less active at the chemical level, and its free morpholine radical is the cause of the reduced the toxicity and increased pharmacological activity [4].

Pramoxine was said to promote lower systemic toxicity than amides and ester LA [5], its potency being comparable to that of benzocaine for topical administration. Yet PMX produces satisfactory topical anesthesia and is reasonably well tolerated on the skin and mucous membranes, but it is irritating to the eyes and nose [6]. Its pKa (7.1) determines the prevalence of the neutral form at physiologic pH, which low water solubility limits its application by infiltrative routes.

New formulations designed to improve the bioavailability of PMX, leading to improved anesthesia and lower toxicity are highly desirable. In this context, a wide range of drug delivery systems (DDS) has been developed for local anesthetics in microcrystals, liposomes, lipospheres, cyclodextrin complexes, among others [7,8]. DDS allows to modulate the chemical features of the anesthetic agent, improving its therapeutic effects [9].

In the pharmaceutical field, cyclodextrins have been used as complexing agents to increase the aqueous solubility of non-soluble drugs, improving their bioavailability and stability. The stability of the complex arises from non-covalent bonds, such as hydrophobic interactions, steric factors, and van der Waals forces [10]. Many types of modified CDs are commercially available for pharmaceutical purposes. Among

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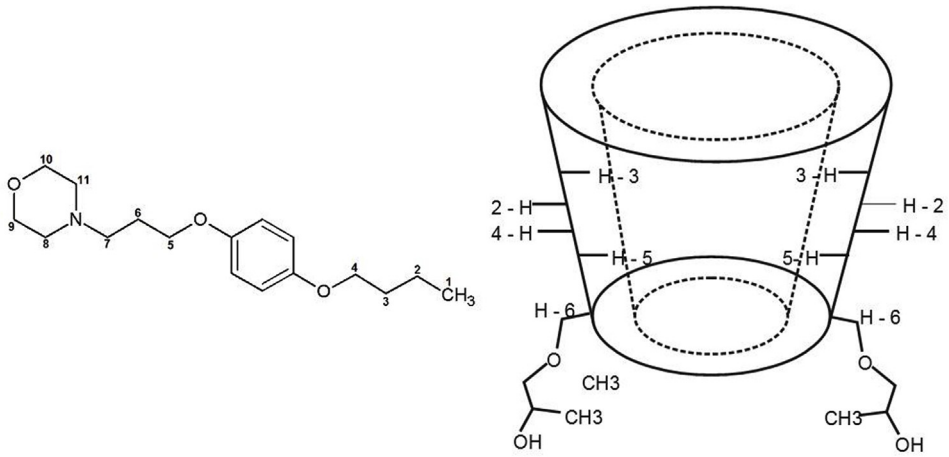

Fig. 1. Chemical structure of pramoxine and hydroxypropyl beta-cyclodextrin. (HP-β-CD). Numbers refer to 1H NMR peak assignment.

them is hydroxypropyl beta-cyclodextrin (HP-β-CD) (Fig. 1), whose application by infiltrative routes is considered safe by the FDA [10,11]. HP-β-CD is quite more soluble and less toxic than the natural β-cyclodextrin, from which it is derived. It contains hydroxypropyl groups attached in the outside face of the macrocyclic cone and has been widely studied as carrier for local anesthetics formulations, promoting improvements in chemical physical properties and clinical potency [7,12–14].

This study aimed to develop a formulation for the local anesthetic pramoxine by complexing it with HP-β-CD. A detailed characterization of the PMX/HP-β-CD complex was conducted using fluorescence, X-ray diffraction and ^{1}H NMR (Diffusion Ordered Spectroscopy, DOSY, and Rotating frame Overhauser effect spectroscopy, ROESY) approaches, prior to evaluate the *in vitro* cytotoxicity of the formulation.

2. Materials and methods

Pramoxine hydrochloride (329.9 g/mol) was purchased from Sigma Chem.Co (St. Louis, MO, USA). Hydroxypropyl-beta-cyclodextrin was purchased from Roquette Serv. Tech. Lab. (Lestrem, Cedex, France). All other reagents used were of analytical or pharmaceutical grade.

2.1. Solubility and stability of PMX at different pHs

To determine the aqueous solubility of PMX, samples were prepared with increasing concentrations of the anesthetics, diluted in phosphate-citrate-borate (PBC) buffer, at pH 7.4 and pH 10.5. After centrifugation for 5 min at 1680 g, the absorbance of the supernatant was determined at the maximum absorption wavelength of PMX (λ = 286 nm) in a in a DU-70 Beckman® (Brea, CA, USA) spectrophotometer. The experiment was done in duplicate.

The chemical stability of PMX at pH 5.5, 7.4 and pH 10.5 and room temperature was also followed, by changes in the absorbance at 286 nm, for a 24 h period.

2.2. Phase-solubility studies

For the phase-solubility assays, an excess amount of PMX was added to HP-β-CD solutions (in 20 mM PBS buffer) of increasing concentrations, ranging from 0 to 50 mM. The samples were stirred at room temperature and an aliquot was filtered through 0.45 μM membrane filters (Millipore). The soluble PMX concentration was determined by UV absorption, at 286 nm. Solubility data were fitted using linear regression [12]. The association constant (Ka) was determined from the linear relationship between the molar concentration of PMX versus the HP-β-CD molar concentration, according to equation 1, where S_0 is the aqueous solubility of PMX.

$$\mathbf{Ka = slope/S_0(1 - slope)} \quad (1)$$

2.3. Preparation and determination of the complexation time of PMX/HP-β-CD

The PMX/HP-β-CD inclusion complex was prepared by the co-solubilization process, as described in the literature [15]. Both the PMX and HP-β-CD solutions were prepared in PBC buffer, pH 7.4.

The samples were excited at 286 nm and the intrinsic fluorescence of PMX between 300 and 375 nm was followed in a F-4500 fluorimeter (Hitachi, Japan) at time 0, and then every 20 min during the first hour, every 40 min during second and third hour; every 60 min during de next hours. From these data, a plot of fluorescence intensity *versus* time allowed determination of the kinetics of the complexation.

2.4. Determination of the stoichiometry of PMX/HP-β-CD complexation

Job plots were used for determination of the stoichiometry of the PMX/HP-β-CD complexation [16], following changes in the intrinsic fluorescence of PMX at increasing cyclodextrin concentrations. Solutions of PMX and HP-β-CD were prepared in PBC buffer at pH 7.4 and mixed to a final volume of 5 mL, so that the total (PMX + HP-β-CD) concentration remained constant (1 mM), but the PMX/HP-β-CD molar ratio (r) ranged from 0 to 1. The samples were excited at 286 nm and PMX fluorescence in the mixed samples were followed from 300 to 375 nm.

2.5. PMX fluorescence at different dielectric constants

The solvent polarity has a direct influence on the optical behavior of a chemical compound [17]. To verify the influence of the solvent polarity in the PMX fluorescence, PMX (1 mM) samples were prepared at different water:ethanol (9:1 to 1:9, v/v) ratios, as shown in Table 1, to provide different dielectric constants [18].

2.6. X-rays diffraction

X-Rays diffraction measurements were taken in solid samples of HP-β-CD, pramoxine, PMX/HP-β-CD physical mixture and PMX/HP-β-CD complex, using a RINT2100 Rigaku X-ray diffractometer, with λ(kα) = 1.5406 Å copper tube, 1.8 kW radiation source, 40 kV and 30 mA current and SC-50 scintillation detector, at Laboratory of Applied Crystallography and X-rays, Institute of Physics/Unicamp. The X-ray diffractograms were taken between 5° and 50° in 2θ with 1°/

Table 1
Water-ethanol polarity gradient protocol.

Tubes	% water volume	% ethanol volume	dielectric constant
1	100	0	78.4
2	90	10	72.8
3	80	20	67.0
4	70	30	61.0
5	60	40	55.0
6	50	50	49.1
7	40	60	43.4
8	30	70	38.0
9	20	80	32.8
10	10	90	28.1
11	0	100	24.3

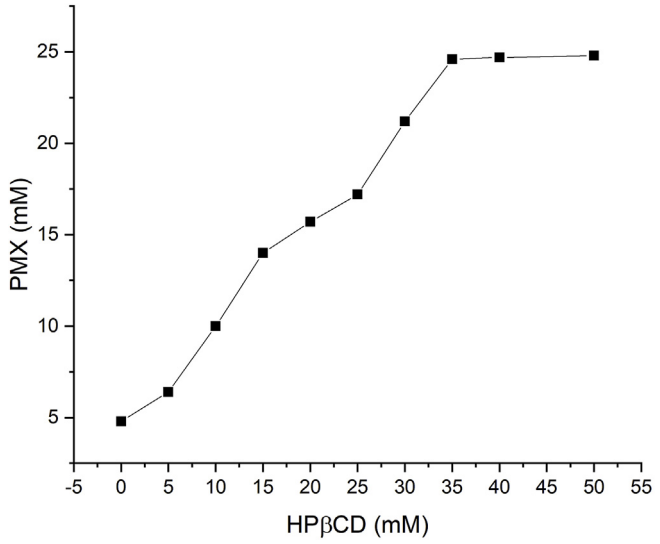

Fig. 2. Phase-solubility diagram for PMX in the presence of increasing HP-β-CD concentrations, determined at pH 7.4 and ambient temperature.

minute steps. Supplementary measurements were performed in the powder diffraction line (XPD-D10) of the National Laboratory of Synchrotron Light (LNLS, Brazil), using 4 + 2 circles diffractometer (HUBER), energy of 8 keV (λ = 1.5498 Å), and a MYTHEN linear solid-state detector (detector-sample distance = 1 m), in the θ-2θ configuration.

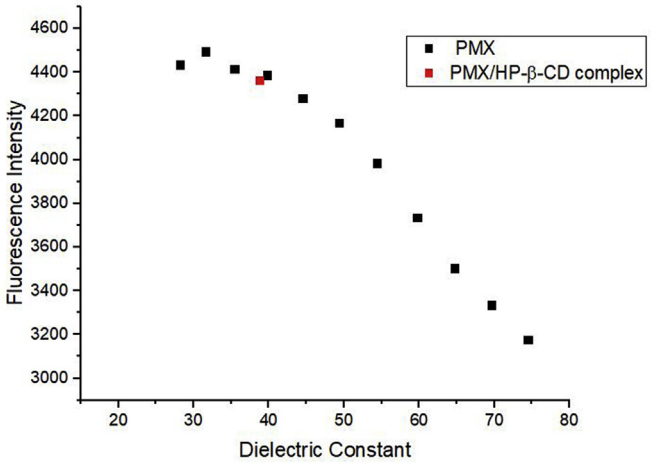

Fig. 4. Intrinsic fluorescence of PMX (1 mM) at increasing dielectric constant, and in the PMX/HP-β-CD complex, at pH 7.4.

2.7. Nuclear magnetic resonance

NMR analyzes were performed in a Varian Inova instrument, at the National Synchrotron Light Laboratory (Campinas, Brazil), at 499.99 MHz for hydrogen frequency. PMX and HP-β-CD samples were prepared in H_2D solvent (10 mM), homogenized for 8 h and transferred to a 5 mm resonance tubes for spectra acquisition. The deuterium signal of the solvent was used as field frequency lock and adjustment of the magnetic field homogeneity.

2D-ROESY, a homonuclear Nuclear Overhauser Effect (nOe) measurement experiment under spin-locked conditions, provided information regarding spatial proximities between the protons. The 2D-ROESY spectrum was acquired with a continuous spinlock during the mixing time (300 ms).

DOSY experiments were obtained by the pulse sequence *DgcteSL* (*Gradient Compensated Stimulated Echo Spin Lock*). For all experiments, 25 different gradients amplitudes were used, and time diffusion was 0.06 s. The gradient pulse amplitudes had a decrease in resonance intensity of approximately 90–95% for the higher intensity gradients. The processing program (DOSY macro in the Varian instrument) was run with data transformed using fn = 32 K. The diffusion coefficient and standard deviation of each species involved in the analysis was given by the arithmetic mean of all coefficients of the same species.

The complex diffusion coefficient is determined based in the exchange between a free and complex state of the ligand. The complexed

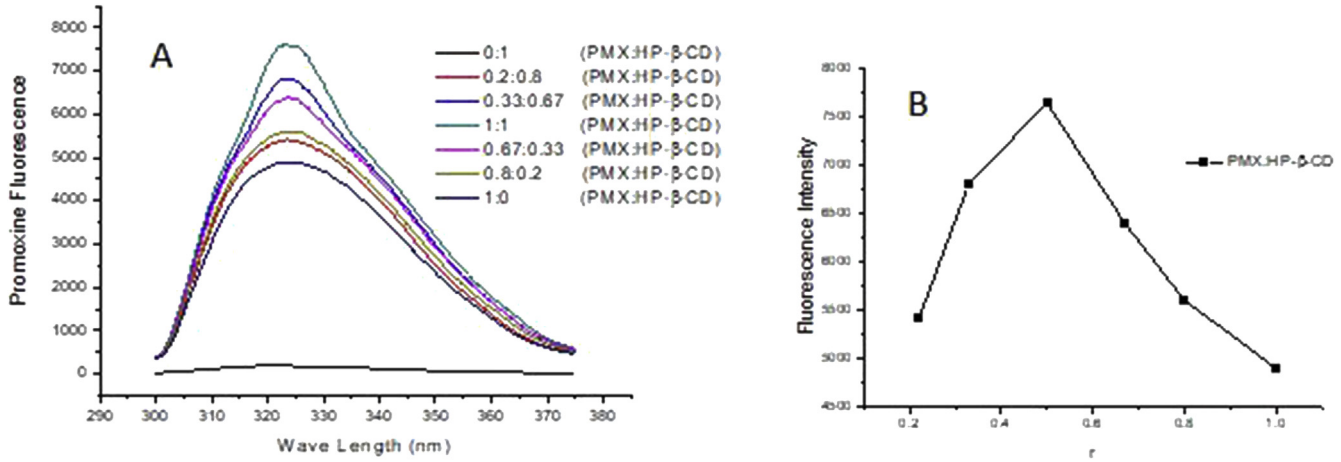

Fig. 3. A) Fluorescence emission spectra of PMX (0.06 mM) complexed with HP-β-CD, at different PMX/HP-β-CD molar ratios (r), at pH 7.4 and room temperature. B) Continuous variation (Job Plot), showing changes in the anesthetic fluorescence as a function of r, used to determine the PMX/HP-β-CD complex stoichiometry.

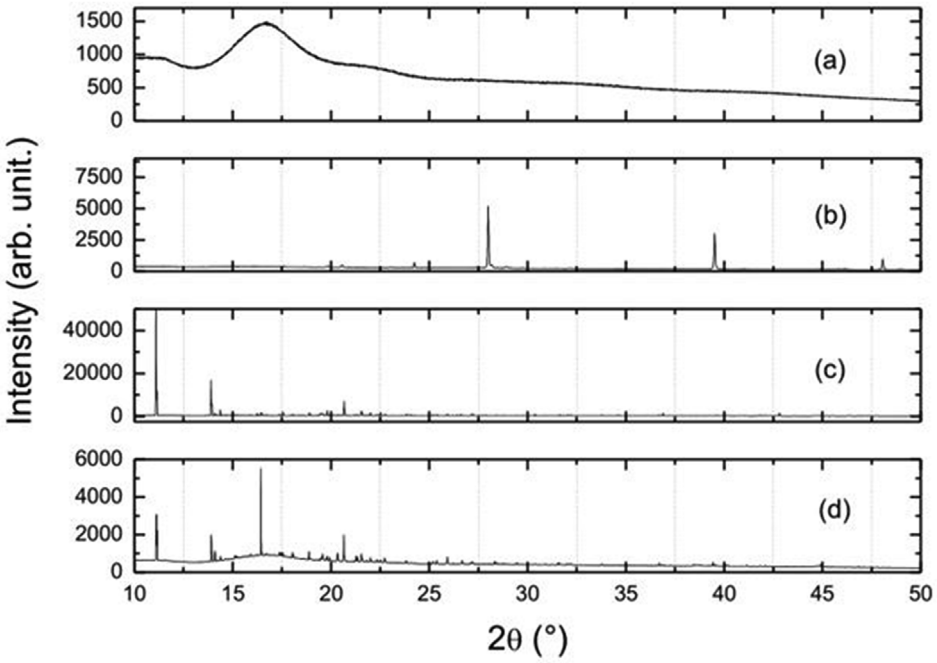

Fig. 5. X-ray diffraction patterns of (a) HP-β-CD, (b) PMX/HP-β-CD complex, (c) Pramoxine and (d) PMX/HP-β-CD physical mixture (1:1 M ratio).

Table 2
^{1}H NMR signals (ppm) of HP-β-CD, PMX and PMX/HP-β-CD complex. Assignments as in Fig. 1.

HP-β-CD	Hydrogen	δ_{free}	$\delta_{at\ complex}$	Δδ
	H_1	5.019	5.029	0.001
	H_2	3.587	[a]	[a]
	H_3	3.908	[a]	[a]
	H_4	3.438	[a]	[a]
	H_5	3.802	[a]	[a]
	H_6	3.768	3.815	0.047
	$CH_{3(hydroxyl)}$	1.105	1.103	0.002
PMX	H_1	0.945	0.921	−0.024
	H_2	1.449	1.430	−0.019
	H_3	1.734	1.734	0
	H_4	4.060	[a]	[a]
	H_5	4.126	[a]	[a]
	H_6	2.132	2.090	−0.042
	H_7	[a]	–	–
	H_8	3.082	2.980	**−0.102**
	H_9	3.912	[a]	[a]
	H_{10}	3.912	[a]	[a]
	H_{11}	3.082	2.980	**−0.102**
	$H_{aromatic}$	6.999	6.868	**−0.131**

[a] Not measured due to spectral overlap.

molar fraction (f_x) and association constant (K_a) can be calculated using equations (2) and (3), respectively [19].

$$f_x = (D_{free} - D_{complex}) / (D_{free} - D_{host}) \quad (2)$$

$$K_a = f_x / ((1 - f_x)([host] - f_x [guest])) \quad (3)$$

Where f_x is the complexed molar fraction and D_{free}, $D_{complex}$ and D_{host} refer to the diffusion coefficients of PMX free, complexed PMX and free HP-βCD, respectively.

2.8. In vitro release tests

Analyses were carried out using Franz diffusion cells to evaluate the *in vitro* release of PMX, free or complexed with HP-β-CD under sink condition. Franz cells have two compartments: the donor, containing 1 mL of the sample (1.5 mM PMX in solution or complexed with HP-β-CD) and the receptor chamber, with 100 mL of 20 mM HEPES buffer in NaCl (150 mM) pH 7.0, kept under agitation [20]. A cellulose membrane (Spectrapore®, 1000 Da pores for molecular exclusion) separated the two compartments. Samples were withdrawn from the recipient compartment (with replacement of the withdrawn volume with the buffer) at the following times: 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450 e 480 min of experiment, and analyzed by UV absorption in a DU-70 Beckman® (Brea, CA, USA) spectrophotometer, at 286 nm.

Absorbance measurements were converted to percentage of drug released using as standard a solution of the PMX diluted in HEPES buffer, pH 7.0. Two systems, one containing the PMX/HP-β-CD complex and the other containing free pramoxine were tested and compared.

2.9. In vitro toxicity of free PMX and PMX/HP-β-CD complex: assays in 3T3 fibroblast cell cultures

The viability of 3T3 cells (Balb/c mice fibroblasts) exposed or not to PMX was determined by the MTT reduction test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The test principle consists of the uptake of MTT by cells and reduction to formazan (purple compound) by the mitochondrial dehydrogenases, resulting in accumulation of this compound in viable cells. The cells solubilization allows the release of formazan, which can be easily detected at 570 nm [21].

Cells were taken from stock cultures (DMEM culture medium supplemented with 10% fetal bovine serum, penicillin/streptomycin 10 000 000 IU/L/10 g/L, pH 7.2–7.4, 37 °C, under humidified atmosphere and 5% of CO_2) and maintained in exponential growth. Viable cells (1 x 10^4) were seeded in 96-well plates, incubated for 24 h, until semiconfluency. Cells were then incubated for 24 h with the samples (PMX, HP-β-CD, PMX/HP-β-CD complex). Thereafter, the culture medium was replaced, and the cells incubated with MTT (0.5 m/mL) for 4 h. The absorbance of the solution in each well was measured at 570 nm and the results were expressed as percentage of viable cells in relation to the control [22,23]. PMX and PMX/HP-β-CD complex samples were used at increasing concentrations (50 μM–1 mM).

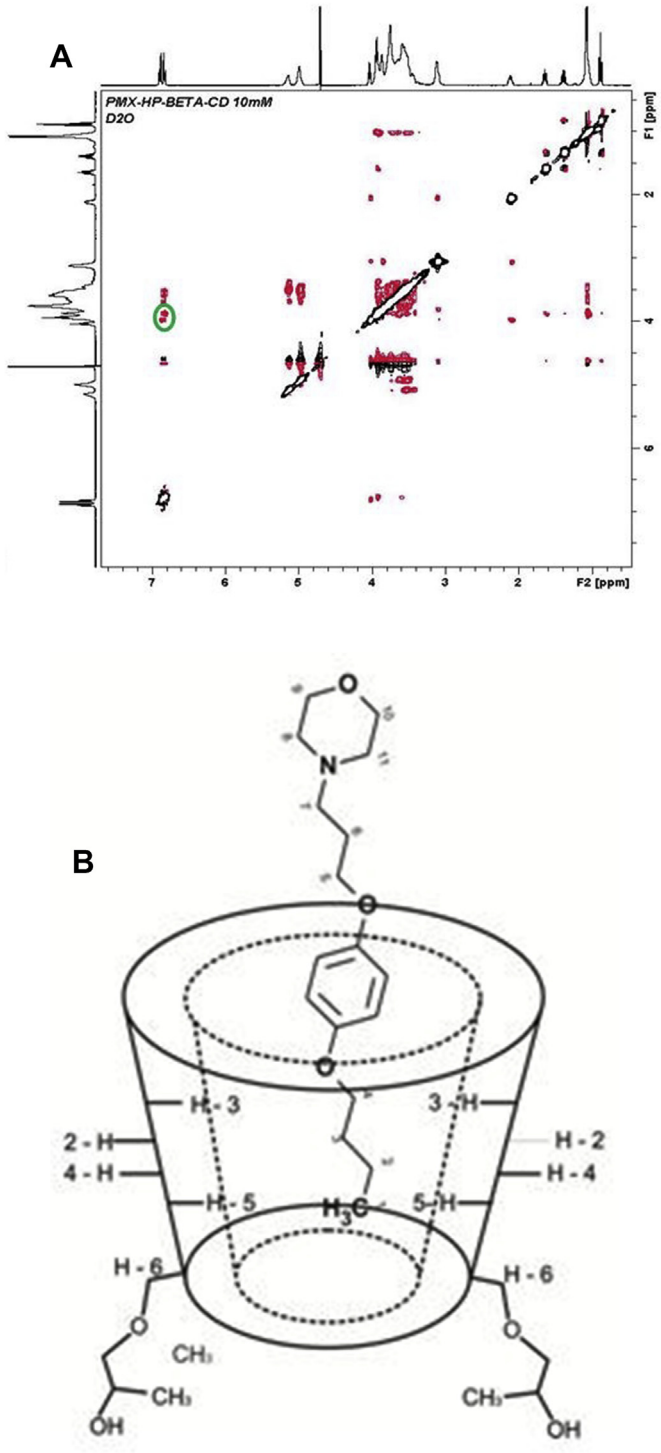

Fig. 6. A) ROESY 2D spectra of the PMX/HP-β-CD complex at 499.89 MHz D_2O/residual H_2O reference was set to 4.7 ppm. B) Suggested topology for the PMX/HP-β-CD complex.

2.10. In vitro toxicity evaluation of free PMX and PMX/HP-β-CD complex: hemolytic assays

For the preparation of the erythrocyte suspension, freshly obtained blood was centrifuged (500 g, 5 min) under refrigeration (4 °C), for plasma, white cells and platelets removal. Then, the red blood cells were resuspended in 40 mM phosphate buffered saline (PBS) pH 7.4 and centrifuged again, under the same conditions. This procedure was repeated 3 times. Hemolytic tests were performed by resuspending the red blood cell concentrate to a final 0.15% hematocrit. The aliquots containing human red blood cell suspensions were incubated with increasing concentrations of free PMX (1.25–1.5 mM) or PMX/HP-β-CD complex (3–20 mM) dissolved in PBS buffer. Samples and controls (buffer and water) were incubated for 15 min (at 37 °C) before centrifugation at 500*g*, 5 min) and the supernatant was used for the hemoglobin determination, at 412 nm [24]. All experiments were performed in triplicate. The results were expressed as percent hemolysis, considering changes in the absorbance of the sample (*S*), erythrocytes in PBS (mechanical hemolysis control, C_1) and erythrocytes in distilled H_2O (total hemolysis control, C_2), according to Ref. [1]:

$$Hemolysis\,(\%) = \frac{Abs\ \ S - AbsC_1}{AbsC_2 - AbsC_1}\ x\ 100 \quad (4)$$

3. Results and discussion

PMX is a local anesthetic that has limited aqueous solubility, so the solubility determination is an important factor and this property can be improved with HP-β-CD complexation. Prior to determine the PMX water solubility tests to analyze the chemical stability of the molecule at different pH (5.5, 7.4 and 10.5) were performed. The absorption readings at 286 nm was followed as a function of time. PMX was found stable at acid pH 5.5 and neutral pH 7.4, with slightly lower stability at pH 10.5, after 1 h (Fig. S1, supplementary material).

The solubility of the charged LA species is always much greater than that of the neutral form [25]. According to the literature [26], the solubility of PMX hydrochloride is 0.5 M. We determined the solubility of PMX at pH 7.4 and 10.5, within 1 h of sample preparation (Fig. S2, supplementary material). The maximum soluble concentrations of PMX at pH 7.4 and 10.5 were 1.7 mM and 1.3 mM, respectively.

Data at pH 10.5 for lidocaine and prilocaine, which are more hydrophilic local anesthetics, show solubility values in the order of 16.3 mM and 23.1 mM, respectively. However, bupivacaine and dibucaine, more hydrophobic anesthetic agents, have solubilities lower than 0.58 mM and 0.03 mM, respectively [25]. The solubility of the uncharged PMX form (1.3 mM) is closer to that of hydrophobic anesthetics, which agrees with its high partition coefficient value between octanol and water (log oct/w = 1.2) [27,28].

Because of its relatively low pKa (7.1), the solubility of PMX at pH 7.4 (1.7 mM) is quite similar to that observed at pH 10.5, what can limit the bioavailability of the drug, and justifies the use of HP-β-CD to improve it.

The phase-solubility diagram of PMX in the presence of increasing HP-β-CD concentrations (Fig. 2) showed a linear type curve (r^2 = 0.9939):

$$[PMX] = 0.562.\ [HP\text{-}\beta\text{-}CD] + 4.4$$

The association constant (Ka) between PMX and HP-β-CD, calculated from the slope of the phase-solubility curve (Ka = 752.1 M^{-1}) reveals the formation of a stable complex [12]. The maximum enhancement in PMX solubility in the presence of excess HP-β-CD was 24.6 mM, indicating more than 14-fold increase in PMX solubility at pH 7.4.

The PMX/HP-β-CD stoichiometry of complexation was determined using the Job method, by following changes in PMX fluorescence intensity. Fig. 3A shows the maximum inflection value for the fluorescence intensity at r = 0.5 [29]. The data were treated according to Job Plot (Fig. 3B), allowed determination of a 1:1 complexation stoichiometry for PMX/HP-β-CD at physiological pH.

For a better adjustment of the complex equilibrium time, the complexation kinetics was determined from fluorescence data monitored during a period of 24 h. The PMX fluorescence intensity increased significantly (Fig. S3, supplementary material) being compatible with the insertion of the chromophore (PMX aromatic ring) into the

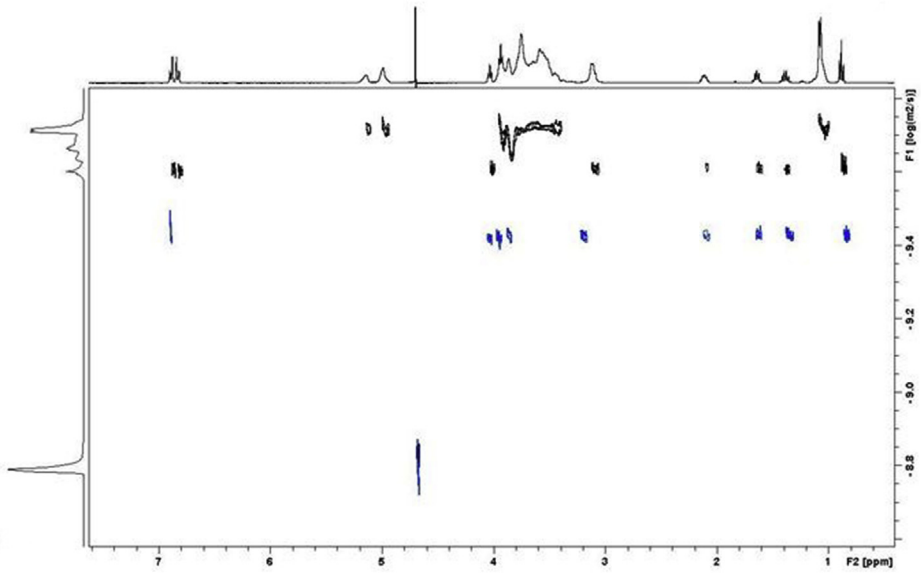

Fig. 7. DOSY spectrum of the PMX/HP-β-CD complex at 499.89 MHz D_2O/reference residual H_2O was set at 4.7 ppm.

Table 3
Diffusion coefficients (D) of PMX, HP-β-CD and PMX/HP-β-CD complex. PMX/HP-β-CD complex molar fraction and association constant (K_a).

Compounds	D (10^{-10} m^2s^{-1})	Molar fraction (f_x.100)	K_a mol/L
PMX	3.8 ± 0.050	–	–
HP-β-CD	2.0 ± 0.020	–	–
PMX/HP-β-CD	2.5 ± 0.030	72	923.1

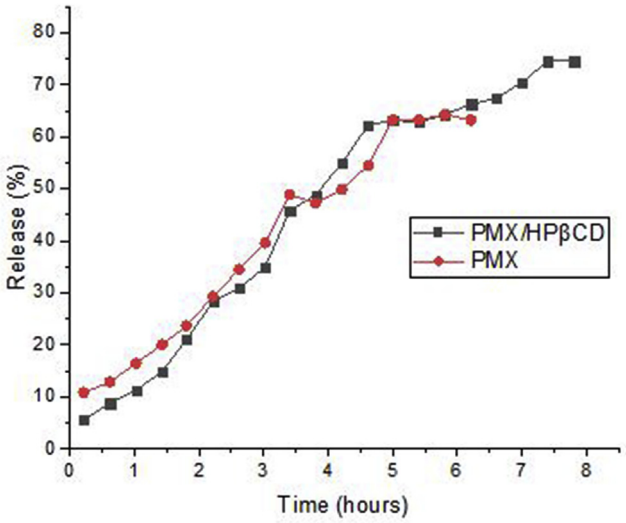

Fig. 8. *In vitro* release kinetics of free pramoxine (%) and the PMX/HP-β-CD complex (%), at pH 7 and 25 °C.

cyclodextrin cavity, as previously observed for the complexation of other local anesthetics and cyclodextrins, such as benzocaine [12] and tetracaine [30]. The final equilibrium was reached within 10 h, which was stablished for further experiments.

Changes in the intrinsic fluorescence of PMX allowed us to get another indication of its inclusion complexation. Fig. 4 shows the fluorescence intensity of PMX submitted to medium of increasing polarity and in HP-β-CD (red point). The emission of PMX is compatible to the polarity of the HP-β-CD cavity which dielectric constant is $\varepsilon = 32.8$ [31,32].

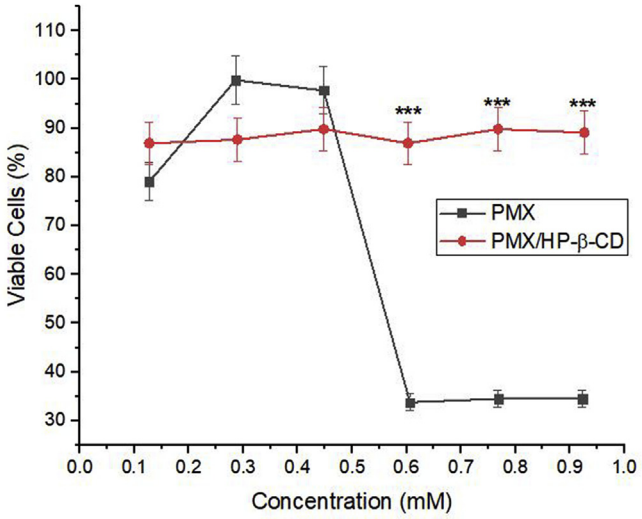

Fig. 9. Cell viability analysis (MTT test) of 3T3 fibroblasts (Balb/C) after treatment (24 h) with free (PMX) and complexed pramoxine (PMX/HP-β-CD). Data expressed as percentage of viable cells in relation to MTT control (mean ± SD) n = 3 experiments. Statistical analysis: PMX *vs* PMX/HP-β-CD. Unpaired T test: ***p < 0.001.

The next indicative of PMX/HP-β-CD complexation was taken from X-Ray experiments. The X-ray diffractogram clearly confirmed the crystalline nature of PMX (Fig. 5c), while HP-β-CD presented as an amorphous structure (Fig. 4a). The diffractogram of the physical mixture (Fig. 5d) showed the superposition of the PMX crystalline pattern with the amorphous HP-β-CD diffraction. By contrast, the inclusion complex (Fig. 5b) showed that the crystalline PMX pattern is lost upon complexation with HP-β-CD.

But in contrast to data obtained with other local anesthetics, e.g. ropivacaine [33], and tetracaine [30], the diffraction pattern of the PMX/HP-β-CD complex is completely different from the pure HP-β-CD diffractogram. The PMX/HP-β-CD complex exhibits novel diffraction peaks that suggest the formation of a new crystalline structure, arising from the ordering of the complex system. In an attempt to get insights on the molecular organization of the PMX/HP-β-CD, NMR experiments were performed.

Nuclear magnetic resonance experiments were carried out in an

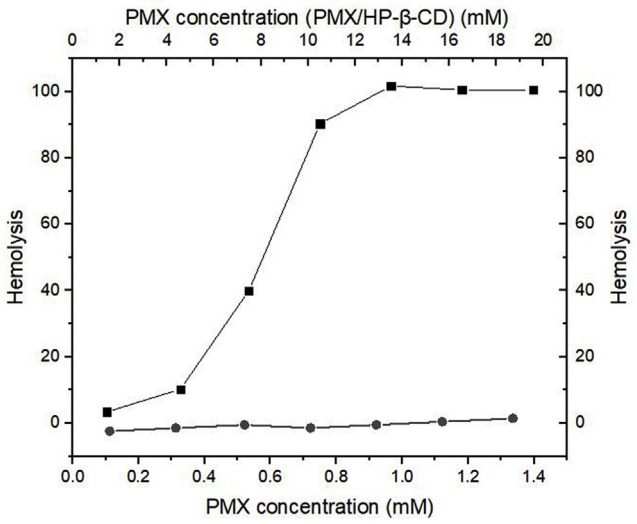

Fig. 10. Hemolytic effect of (■) free pramoxine and (•) PMX/HP-β-CD complex on human erythrocytes (0.15% hematocrit) in PBS buffer, pH 7.4 and 37 °C, incubated for 15 min.

attempt to get information on the energy of the complexation process and to determine of the inclusion complex geometry [34]. Table 2 shows the chemical shift of the hydrogens to PMX, HP-β-CD and PMX/HP-β-CD complex.

According to Table 2, significant variations (> 0.05 ppm) in the ^{1}H chemical shift was detected for PMX (H8, H11 and aromatics hydrogens), indicating a possible change in the PMX chemical environment as a function of complexation. According to the literature, the insertion of the aromatic ring into the cyclodextrin cavity was expected [35], but the chemical shift variation of the morpholine hydrogens ring (hydrogens 8 and 11) was unexpected. This may indicate the formation of hydrogen bonds between PMX and the external OH groups of HP-β-CD macrocyclic ring (perceived by H6 of cyclodextrin, $\Delta\delta$ = 0.047 ppm).

Intermolecular nOe interactions allow identifying spatial proximities between host and guest hydrogens in the bimolecular complex, and they represent an important NMR tool for characterizing interfaces. The green circle in Fig. 6A highlights the intermolecular interaction between PMX aromatic hydrogens and HP-β-CD hydrogens 3 or 5 (due to overlap of signals we could not differentiate between H3 and H5) that are located within the inner cavity of the macrocyclic ring).

Therefore, ROESY measurements clearly indicated that PMX aromatic ring is inserted into the HP-β-CD inner cavity, as observed before for tetracaine [36]. Fig. 6B suggests the topology for the PMX/HP-β-CD complex, based on the molecular model and the nOe experiment.

Further information of PMX/HP-β-CD complex based on ^{1}H NMR spectroscopy was obtained from DOSY experiments. Diffusion-ordered spectroscopy can gather information on a mixture compounds based on their differing diffusion coefficients, depending on the size and shape of molecular species. DOSY can probe intermolecular interactions, and also provide an estimate of the association constant of a complex (Fig. 7).

The diffusion coefficient of the free PMX species (3.8 10^{-10} m^2s^{-1}) was considerably higher than that of complexed PMX (2.5 10^{-10} m^2s^{-1}) which suggests the formation of the inclusion complex. The diffusion coefficients (D) of the free PMX species, HP-β-CD and PMX/HP-β-CD complex were used to determine the molar fraction (fx) and the association constant (Ka) of the complex [19]. The determined association constant (923.1 L/mol) (Table 3) were in good agreement with those from phase solubility studies, confirming the strong association between PMX and HP-β-CD.

3.1. In vitro release tests

Complexation of local anesthetics with HP-β-CD normally provides slower drug release compared to the free anesthetic [14,33,38], decreasing the toxicity and prolonging the anesthetic effect. However, no significant changes in the release rate of complexed PMX was detected, in comparison to free pramoxine (Fig. 8).

Indeed free PMX has a lower release than that observed for other local anesthetics such as oxethazaine, lidocaine, prilocaine, ropivacaine [14,33,37,38] which present 100% release in about 1 h. Interestingly, the total release time of free PMX is similar to that of benzocaine [12], an anesthetic that does not ionize over a wide pH range, which release time is greater than 4 h. The slow release kinetics of PMX (and benzocaine) also correlates with their longer onset time for anesthesia, in comparison to other local anesthetic agents.

3.2. Cytotoxicity tests

In cell cultures the local anesthetics act by inhibiting cell growth, motility and causing morphological changes, which alters the survival rate of cells. These effects are directly proportional to the exposure duration and dose of the drug [21]. The toxic effect evinced by the free anesthetic (PMX) and the complex (PMX/HP-β-CD, 1:1 ratio) was evaluated through the MTT tests in 3T3 Balb/c mouse fibroblasts. The results in Fig. 9 showed an IC50 of 0.41 mM for PMX and an increase in the percentage of viable cells after treatment with the complex. No toxic concentration was detected after treatment with PMX/HP-β-CD, up to 1 mM, evidencing that complexation decreased the intrinsic toxicity of pramoxine.

3.3. Hemolytic tests

Hemoglobin release is one of the best parameters to describe the integrity of erythrocyte membranes [39]. Hemolytic test confirmed the importance of hydrophobic parameters in determining the biological effect of drugs, *i.e.*, the lytic action on erythrocytes [1]. Fig. 10 shows the hemolysis induced by free PMX over human erythrocytes: the onset of hemolysis was observed at the concentration of 1.35 mM and the total hemolysis was reached at the concentration of 1.42 mM. No hemolytic effect was observed with PMX/HP-β-CD complex in the same concentration range, and also up to 20 mM, confirming that complexation with hydroxypropyl beta-cyclodextrin significantly decrease the toxicity of pramoxine.

4. Conclusion

PMX has interesting optical properties that could be used to quantify the drug and measure its complexation with HP-β-CD. PMX formed 1:1 inclusion complex with HP-β-CD that significantly increased drug solubility. The X-ray diffractogram of the complex showed that the crystalline PMX pattern was lost, indicating inclusion complex formation. ^{1}H NMR (DOSY) data confirmed phase-solubility results, revealing that PMX and HP-β-CD form a stable complex (Ka = 923.1 mol/L) while ROESY revealed the insertion of the aromatic PMX hydrogens inside the HP-β-CD cavity.

Complexation decreased the toxicity of the anesthetics, as seen in two *in vitro* models: i) in cultured 3T3 fibroblast cells there was an increase in the percentage of viable cells after treatment with PMX/HP-β-CD, in comparison to free PMX; ii) hemolytic tests revealed that PMX/HP-β-CD complex reverted the hemolysis of PMX in human erythrocytes.

In general, these results are quite promising for the management of pain, indicating that the PMX/HP-β-CD complex may be a sustained release formulation for PMX, increasing the bioavailability of the anesthetic.

Declaration of competing interest

The authors listed below declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2019.101475.

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