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Complexation of oxethazaine with 2-hydroxypropyl-β-cyclodextrin: increased drug solubility, decreased cytotoxicity and analgesia at inflamed tissues

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Keywords
bioassay approaches; dosage form design and characterization; pharmaceutical analysis; pharmaceutics and drug delivery

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Abstract

Objectives Oxethazaine (OXZ) is one of the few local anaesthetics that provides analgesia at low pH, but presents poor solubility, cytotoxicity and no parenteral formulations. To address these issues, we aimed to prepare OXZ host-guest inclusion complex with hydroxypropyl-beta-cyclodextrin (HP-β-CD).

Methods The inclusion complex was formed by co-solubilization, followed by a job plot analysis to determine stoichiometry of complexation and dialysis equilibrium analysis (based on UV/VIS absorption and fluorescence profiles of OXZ). Complex formation was confirmed by phase-solubility data, X-ray, Scanning Electron Microscopy and DOSY-^{1}H-NMR experiments. *In vitro* cytotoxicity was analysed by MTT test in 3T3 fibroblasts. In vivo analgesia was tested by Von Frey test (inflammatory wounds – rats).

Key findings Oxethazaine complexed (1 : 1 molar ratio) with HP-β-CD, as indicated by loss of OZX crystalline structure (X-ray) and strong host: guest interaction (NMR, K = 198/M), besides increased solubility. *In vitro* cell survival improved with the complex (IC_{50} OXZ = 28.9 μM, OXZ : HP-β-CD = 57.8 μM). In addition, the complex (0.1% OXZ) promoted *in vivo* analgesia for the same time that 2% lidocaine/epinephrine did.

Conclusion Our results show that complexation improved physicochemical and biological properties of OXZ, allowing its application to inflamed tissues by parenteral routes.

Introduction

In the present days, lidocaine and bupivacaine are the drugs of choice for ambulatory and surgical local anaesthesia procedures respectively. Such amino-amide local anaesthetics have advantages over the formerly synthesized amino-ester compounds (procaine, benzocaine, tetracaine), which present higher toxicity and fast serum metabolisation.[1,2] Oxethazaine (OXZ) is another amino-amide anaesthetic agent, with poor solubility, slow onset and long-term effect.[3] Its peculiar chemical structure ('double-anaesthetic' – Figure 1) comprises a symmetric molecule around the ethanolamine amino group, bound to two amides and two aromatic rings. OXZ maintains analgesia even at low pH, whereas most anaesthetics have no action, in addition to increased potency compared with lidocaine, procaine, dibucaine, and cocaine.[4]

Oxethazaine has a large margin of safety for the intragastrical, subcutaneous, intramuscular and rectal routes, and it is not a primary irritant agent to the skin, cornea or conjunctive tissue. Despite the observed safety, OXZ administration by the intravenous and intrapulmonary routes can

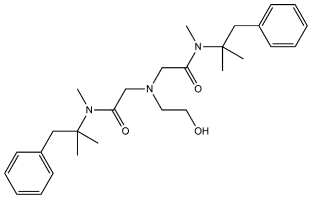

Figure 1 Chemical structure of oxethazaine: two mephentermine molecules combined with ethanolamine through an amide linkage.

lead to CNS/CVS systemic toxicity and even death.[4] OXZ also induces contraction of portal branched veins in perfused liver and *in vitro* haemolysis in the presence of Ca^{2+} ions. Such effects are related to the anaesthetic action on calcium and sodium channels, which impairs nerve stimulus conduction.[5,6] Interestingly, Zhang *et al.*[7] demonstrated that reduction of cytosolic Ca^{2+} levels by OXZ, as a result of blockage of the cytosolic calcium-signalling, inhibited hepatitis B virus replication/capsid assembly. This last finding opens a new application for OZX, besides its anaesthetic role.

Oxethazaine is mainly prescribed for local anaesthesia in the stomach lining, associated with antacids for the relief of oesophagitis, dyspepsia and other gastric disorders.[8,9] Another clinical use is to decrease pain, pruritus and mucous loss in the treatment of haemorrhoids.[10] For the first application, OXZ is vehicled in oral dosage forms, such as tablets, gels, suspensions and syrups; for the latter use, creams and ointments are the available presentations. To the best of our knowledge, there is neither OZX-based medicine for parenteral use, nor studies concerning parental dosage form development. The only clinical study we found focused on minor oral surgery for tooth extraction: 0.1% OXZ in acidic (pH 3) solution successfully achieved analgesia for 2 h, even though a high volume was required (up to 4 ml solution).[11] Probably, higher concentrations were not tested because of its limited water solubility.

Drug solubility can be improved by several excipients, including co-solvents, surfactants and cyclodextrins.[12] Cyclodextrins are cyclic oligosaccharides that can form inclusion complexes with drugs, which increase aqueous solubility and drug stability, but has another advantage as well: less excipient side-effects for parenterals because of its short plasma half-life and systemic absorption mode (excipient distribution in extracellular compartments, no storage in deep compartments).[13] Their glucopyranose units (6, 7, 8 for α, β and γ respectively) linked by α (1,4) linkages form a truncated cone with hydrophobic internal cavity, which accommodates hydrophobic drug moieties compatible with the cavity size (cavity size of 6.0–6.5 Å, in the case of β-CD).[14] Regarding local anaesthetics, complexation with CDs has been shown to prolong the analgesia and decrease drug toxicity (see[15,16] for a review). A recent study showed that OXZ-γ-CD complex largely improve OXZ aqueous solubility,[17] but this type of CD is not recognised as safe for parenteral administration.[18]

Considering the above-mentioned points, we decided to formulate and characterize OXZ as a *host-guest* complex with hydroxypropyl-β cyclodextrin (HP-β-CD), a chemically modified CD with improved solubility and parenteral application.[14] We hypothesised that OXZ complexation with HP-β-CD would increase OXZ solubility, decrease cytotoxicity and prolong infiltrative analgesia in inflamed tissues.

Materials and Methods

Hydroxypropyl-beta-cyclodextrin (HP-β-CD – Kleptose HP®) was purchased from Roquette Serv. Tech. Lab. (Lestrem, Cedex, France). Oxethazaine (2-Di(*N*-methyl-*N*-phenyl-tert-butyl-carbamoyl methyl)aminoethanol) and D_2O were obtained from Sigma Chem. Co, St Louis, Mo, USA. All other chemicals used were of analytical grade.

UV–Vis absorption and emission properties of oxethazaine

Oxethazaine solutions were prepared in 5 mM acetate buffer, at pH 4.0. This pH was selected because of the enhanced solubility of the anaesthetic at acid media. The UV/VIS absorption properties of the anaesthetic was determined, as well as its fluorescence (λ_{ex} = 258 nm, λ_{em} = 270–650 nm) at ambient temperature (25 °C), using a F-4500 (Hitachi, Tokyo, Japan) fluorimeter. The fluorescence properties of OXZ were measured in media of different dielectric constants (24.3–72.8), i.e., mixtures of ethanol and water (1 : 0, 2 : 8, 3 : 7, 4 : 6, 5 : 5, 6 : 4, 7 : 3, 8 : 2, and 9 : 1 v/v respectively) according to Deyhimi *et al.*[19]

Preparation of OXZ : HP-β-cyclodextrin inclusion complex

The inclusion complex was formed by mixing equimolar amounts of OXZ and HP-β-CD in water, under constant stirring, at 25 °C[20,21] for 3 h. The samples were used immediately after preparation or freeze-dried and stored at −20 °C for further use. Before freeze-drying, collapse temperatures were determined with a freeze-drying microscope (FDCS 196; Linkam Scientific Instruments, Surrey, UK), calibrated with NaCl 1% (collapse temperature of −21 °C). Samples were frozen to −70 °C, followed by heating under reduced pressure (100 mTorr, 2.5 °C/min) until total collapse of the sample, above its collapse temperature, −37.5 °C). For freeze-drying, samples were frozen with liquid nitrogen and processed in a

Freezone 4.5 Freeze Dry System (Labconco Co., Kansas City, MO, USA) for 48 h.

Inclusion complex stoichiometry determination

The continuous variation method (Job plot) was adopted to determine the stoichiometry of the complex. Changes in fluorescence intensity (I_0/I) at 283 nm were determined for a series of OXZ : HP-β-CD mixtures of different ratios (from 0 to 1), in which the final concentration of both species was kept constant.[22,23] I_0/I were plotted in function of r, the molar ratio of OXZ, defined by (Equation 1):

$$r = \frac{[\mathrm{OXZ}]}{[\mathrm{OXZ}]_{\mathrm{total}} + [\text{HP-β-CD}]_{\mathrm{total}}} \quad (1)$$

Phase-solubility studies

For the phase-solubility assays, an excess amount of OXZ (8 mM) was mixed with an aqueous solution (10 mM acetate buffer, pH 4.0) of increasing HP-β-CD (0, 2, 4, 6, 8, 12, 16, 20, 24, 28 mM) concentrations, according to Connors.[22] The samples were stirred at room temperature for 24 h and an aliquot was filtered through a 0.45 nm membrane filter (Millipore, Darmstadt, Germany). The amount of soluble OZX was spectrophotometric determined, at 258 nm. The association constant (K) was determined from the slope of the linear portion of the phase-solubility diagram according to Equation (2)[24]:

$$K = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (2)$$

Scanning electron microscopy

Oxethazaine, HP-β-CD, OXZ : HP-β-CD physical mixture and freeze-dried complex OXZ : HP-β-CD (1 : 1 mole %) were prepared by metallization with gold under vacuum for 180 s. Images were analysed using a scanning microscope (JSM 5800LV; JEOL, Tokyo, Japan) to observe possible changes on the anaesthetic and cyclodextrin crystalline structure, upon complexation.

X-ray powder diffractometry (X-ray)

X-ray diffraction analysis is one of the best techniques to investigate the complexation of drugs in delivery carrier systems. The technique reveals the complex formation by changes in the diffractograms, when compared with pure compounds. Loss of the crystalline structure of the guest compound accompanies its complexation with the amorphous HP-β-CD solid phase.[25] Solid samples of oxethazaine, HP-βCD, their physical mixture and OXZ : HP-β-CD complex (1 : 1 mole%) were measured in a D10B-XPD Rigaku diffractometer (1.8 kW)[26] at the Brazilian Synchrotron Laboratory (LNLS) using 4 + 2-circle Huber diffractometer and a Mythen detector,[27] at 9 keV. Diffractograms were obtained at room temperature, in a θ–2θ geometry (Bragg-Bretano configuration), with a flat plane sample holder, over a 2θ range of 10–50 with a step size of 0.005 and exposure time of 1 s.

Nuclear magnetic resonance

Samples of equivalent concentrations (5 mM) of OXZ and HP-β-CD were prepared in 10 mM acetate buffer pH 4.0 (lyophilized and suspended in D_2O), homogenized for 3 h and transferred to 5 mm tubes for spectrum acquisition. ^{1}H-NMR spectra were recorded at 20 °C with a Varian Inova 500 MHz (11.75 T) instrument at the Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil). The residual water peak (4.78 ppm) was used as internal reference, to avoid addition of external ones, that could possibly interact with HP-β-CD.[28] Spectra using diffusion ordered spectroscopy (DOSY) were recorded at 25 °C, using the DgcteSL (gradient compensated stimulated echo spin lock) HR-DOSY sequence, as described before.[29] The pulsed gradient range amplitudes were 0.1067–0.5334 T/m, at a diffusion time of 0.06 s. The processing program (DOSY macro in the Varian instrument) was run with the data transformed using fn = 32 K.

In vitro drug release – vertical diffusion cell

Drug release experiments were conducted under constant stirring in a two-compartment dialysis system that uses a cellulose membrane (Spectrapore, MWCO 1000 Da) to separate the sample at the donor compartment (1 ml capacity, containing samples of 3 mM OXZ in 10 mM acetate buffer pH 4.0, plain or complexed with HP-β-CD) from the acceptor compartment (100 ml, containing 10 mM acetate buffer pH 4.0, under moderate agitation). Aliquots were withdrawn from the acceptor compartment at regular intervals (0–8 h) and the OXZ concentration was determined at 258 nm. Data were expressed as % of OXZ released for each sample. The experiments were carried out in triplicate.

In vitro cytotoxicity – MTT test

The 3T3 cell line (perpetual Balb/c mice fibroblasts) was routinely grown in DMEM medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2.

Cells (1×10^5 per well) were incubated in 96-well plates for 24 h, until semi-confluence. Then, they were treated for 24 h with OXZ (1–100 μM) free or complexed with HP-β-CD, in a 1 : 1 molar ratio. HP-β-CD was used as a control. After treatment, cell medium was replaced with a MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 1 mg/ml), and the cells were incubated for 4 h at 37 °C. Thereafter, MTT solution was removed, and ethanol (0.1 ml) was added to dissolve the formazan crystals. The formazan absorbance was measured at 570 nm using a microplate reader (ELx800; Bio Tek Instruments Inc., Winooski, USA).[30] The results (mean ± SD) were expressed as a percentage of the value obtained with untreated controls. IC_{50} values were determined by nonlinear regression analysis using a sigmoidal concentration-response equation of individual experiments, at Origin 6.0 (Microcal™ Software, Inc., Northampton, MA, USA).

Anaesthetic efficacy in inflamed tissue – hind paw incision test

This study was approved by the Ethics Committee on Animal Research (CEUA) of the University of Campinas (protocol # 3541, 2014), which follows the 'Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals' of the International Association for the Study of Pain[31] and to the National Institutes of Health guide for the care and use of Laboratory animals.[32]

Sensorial *in vivo* tests were performed in the hind paw incision model in Wistar rats, according to the method proposed by Brennan *et al.*[33] and modified by Grant *et al.*[34] This model provides a challenge to the anaesthetic action once the test is performed in inflamed tissue, induced by incision and suture of the hind paw; tissue anaesthesia is evaluated by force application with Von Frey analgesimeter.

Briefly, the rats were placed in special cages (23 cm wide × 20 cm deep × 18 cm high; Insight Equipment Ltda, Ribeirão Preto, Brazil) with a wire grid floor (0.5 × 5 cm), which allowed performing perpendicular pressure stimulus with von Frey analgesimeter (Insight Equipment Ltda) on their right hind paw plantar surface. After acclimation (15–30 min), the baseline response was recorded as the mean of three measures, being the initial force 0.0073 N, which was progressively increased until the paw withdrawal or the force reached 0.456 N. Following this procedure, under general anaesthesia with isoflurane (Isoforine, Cristália Prod. Quim. Farm.Ltda, Itapira, Brazil), an incision (1 cm long × 0.3 cm deep) was performed and sutured.

After 24 h recovery in plastic cages with soft bedding the animals returned to the special cages for an equivalent period of acclimation and submitted to pressure application. Animals showing a reduction of at least 20% in the mean of three measurements in relation to baseline response were considered as presenting hypernociception and were subsequently submitted to the analgesia test.

The animals randomly received a 0.1 ml injection, laterally to the incision, of one of the following formulations: 2% lidocaine with 1 : 100 000 epinephrine, 0.1% OXZ or 0.1% OXZ complexed with HP-β-CD. The pressure stimulus was performed at five minutes interval. Absence of paw withdrawal to the maximal force (0.456 N) was considered as analgesia success (tissue anaesthetized). The results were expressed as the percentage of animals presenting analgesia in function of time. The period in which the animal did not withdraw the hind paw to the maximal force application was considered as analgesia duration.

Statistical analysis

In vitro drug release results were analysed by two-tailed unpaired *t*-test. *In vivo* anaesthetic efficacy was evaluated by Log-Rank Test (analgesia success) and one way ANOVA and Tukey test (analgesia duration). Statistical significance was defined as $P < 0.05$.

Results

We first determined OXZ properties: dissociation constant of its imine group (measured by titration method) is high, while its water solubility is low (Table 1) when compared with other local anaesthetics. Indeed, OXZ has a rather low pK_a because of its peculiar, 'double-anaesthetic' structure and hydroxyl group close to the amine group. Water solubility is also significantly low (4.7 mM or 0.2% at pH 4.0).

The absorption properties of OXZ in the UV region (Table 1) are like other amino-amide local anaesthetics, of low quantum yield: ε = 400/M at 258 nm. By exciting the molecule at 258 nm we could measure its intrinsic

Table 1 Physicochemical properties for the protonated form of Oxethazaine and other amino-amide local anaesthetics

Local anaesthetic	pK_a[a]	λ_{max} (nm)[b]	ε (/M/cm)[b]	Solubility (M)[b]
Oxethazaine	5.0[c]	258[c,d]	400[c,d]	4.7×10^{-3}[c,d]
Lidocaine	7.9	263	480	2.30
Prilocaine	7.7	265	280	0.83
Etidocaine	7.7	263	480	0.20
Mepivacaine	7.6	263	550	1.30
Bupivacaine	8.1	263	470	0.07

pK_a = log dissociation constant; λ_{max} = maximum absorption wavelength; ε = molar extinction coefficient. [a]According to Ref. [43]. [b]Determined at pH 5.5, by de Paula & Schreier.[52] [c]Experimental results from this work. [d]Determined at pH 4.0.

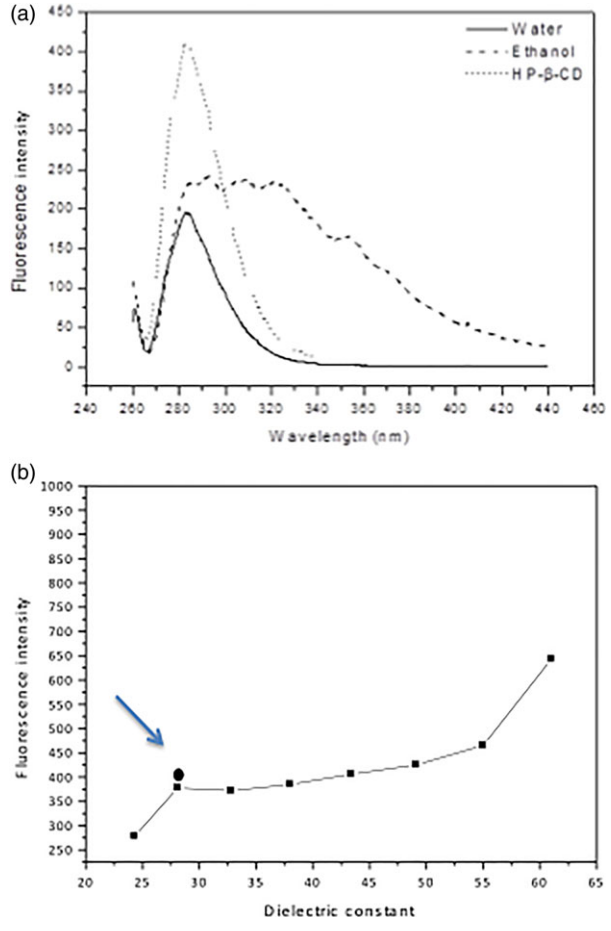

Figure 2 Oxethazaine fluorescence in media of different polarity. (a) Oxethazaine fluorescence in water, ethanol and hydroxypropyl-beta-cyclodextrin. (b) Maximum fluorescence intensity of oxethazaine in the presence of hydroxypropyl-beta-cyclodextrin (black dot indicated by the arrow) and in media of increasing polarity.

fluorescence properties in media of different dielectric constants (Figure 2) from a water-ethanol gradient, and in the presence of HP-β-CD. The quantum yield of OXZ fluorescence increased in the presence of HP-β-CD (Figure 2a) and ethanol. Figure 2b shows the changes in OXZ fluorescence in media of different polarities and in the presence of HP-β-CD. When OXZ is complexed with HP-β-CD its fluorescence is compatible with a medium of dielectric constant = 28 (Figure 2b, black dot indicated by the arrow).

Physical-chemical characterization of the complex

We have employed the Job plot to determine the stoichiometry of complexation of OXZ with HP-β-CD, using 10 mM acetate buffer at pH 4.0 (Figure 3). Changes in

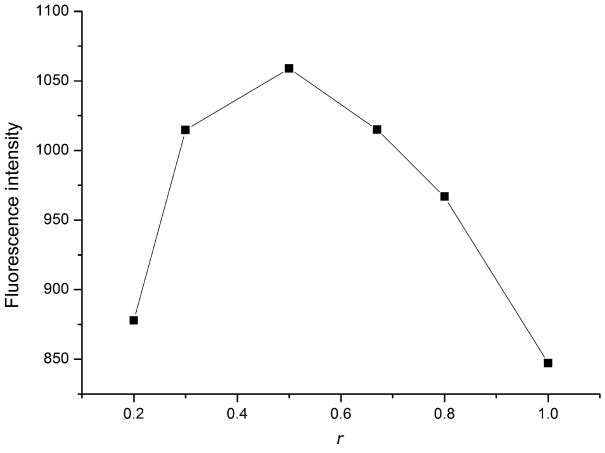

Figure 3 Job plot: changes in fluorescence intensity at different oxethazaine : hydroxypropyl-beta-cyclodextrin molar ratios (*r*), for determination of the stoichiometry of complexation; 10 mM acetate buffer, pH 4.0, ambient temperature.

fluorescence intensity of OXZ were plotted in function of *r*, the molar ratio of OXZ : HP-β-CD, as described in methods. The maximum in Figure 3 occurred at $r = 0.5$ (Equation 1), revealing a 1 : 1 complexation.[22]

Phase solubility

Figure 4 shows the increase in water solubility of OXZ in the presence of excess HP-β-CD, at pH 4. The association constant was calculated from the slope of the phase-solubility plot ($K = 126$/M, Equation 2, Figure 4).

X-ray diffraction studies

In search of evidence of complexation, we performed X-ray diffractograms of HP-βCD, oxethazaine, the physical mixture of OXZ/HP-β-CD and OXZ : HP-β-CD complex (Figure 5). HP-βCD (Figure 5a) presented its characteristic amorphous X-ray diffractogram.[35] As expected,[36,37] complexation induced loss of the crystalline anaesthetic pattern and reduction of the total diffraction intensity (Figure 5b), whereas the physical mixture of OXZ and HP-β-CD (Figure 5d) showed superposition of the crystalline pattern of oxethazaine and the amorphous profile of HP-β-CD.

Morphology

More evidences on the formation of OXZ : HP-β-CD inclusion complexes were obtained from scanning electron microscopy.[38] As observed by X-ray, analysis the crystallinity of pure oxethazaine (Figure 6c) was lost after complexation with the cyclodextrin (Figure 6b), whereas the

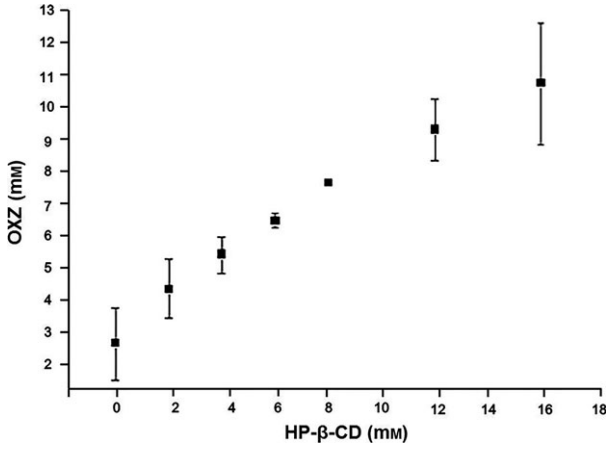

Figure 4 Phase Solubility diagram of oxethazaine in the presence of hydroxypropyl-beta-cyclodextrin, at 10 mM acetate buffer, pH 4.0, ambient temperature.

physical mixture of OXZ and HP-β-CD (Figure 6d) preserved the crystalline pattern of oxethazaine together with the amorphous contribution of the HP-βCD.

NMR

NMR is the most valuable tool for the analytical characterization of host–guest interactions.[16] Investigation of ^{1}H DOSY-NMR revealed a decrease in the diffusion rates of OXZ ($D = 4.48 \times 10^{-10}$ m^2/s) in the presence of HP-β-CD ($D = 2.21 \times 10^{-10}$ m^2/s), as seen in Table 2. The OXZ : HP-β-CD complex association constant (K) was calculated from the DOSY-NMR experiments: $K = 198$/M, in good agreement to the constant determined by the phase-solubility diagram (Figure 4).

In vitro release test

Inclusion complex formation can change drug release rates.[39,40] Complexation of OXZ in HP-β-CD provoked a significant delay on its release from the donor compartment, compared with OXZ in solution (Figure 7). Curves were fitted to the Higuchi model (R^2 of 0.97 for free OXZ and 0.96 for the complex), which considers a linearization using the square root of time, and slopes were significantly different ($P < 0.0001$), which confirms the release kinetic change. Total release (100%) of free OXZ happened after 270 min dialysis, while the complex released only 60% of OXZ at the same time frame.

In vitro cytotoxicity

In vitro cytotoxicity is a mandatory safety assessment to allow clinical applications of drugs; several local anaesthetics can impair cell viability *in vitro*, which limits their application.[41] The results in Figure 8 show that complexation decreased the intrinsic toxicity of OXZ ($IC_{50} = 71$ μM), in comparison to the free anaesthetic ($IC_{50} = 25$ μM) over Balb/C3T3 cells.

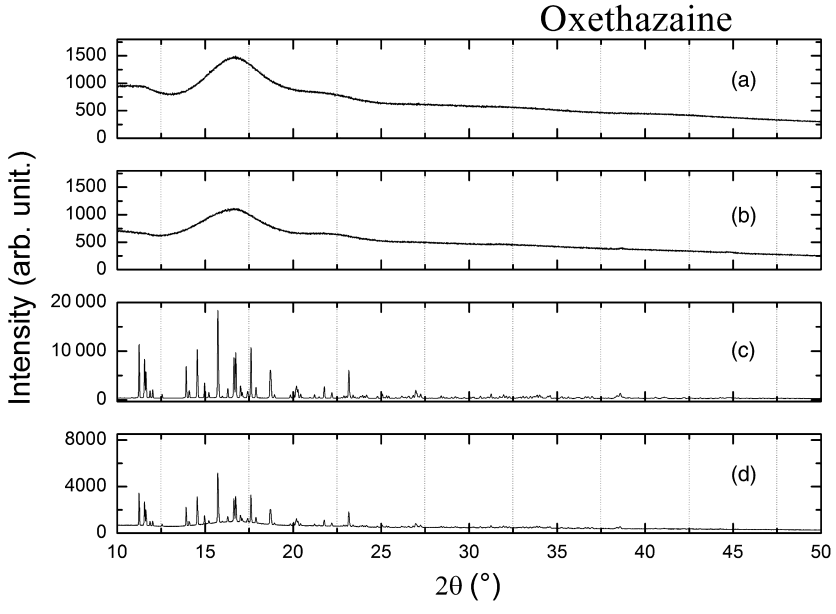

Figure 5 X-ray diffraction patterns of hydroxypropyl-beta-cyclodextrin and oxethazaine. (a) hydroxypropyl-beta-cyclodextrin; (b) oxethazaine : hydroxypropyl-beta-cyclodextrin inclusion complex, (c) oxethazaine and (d) oxethazaine + hydroxypropyl-beta-cyclodextrin (physical mixture).

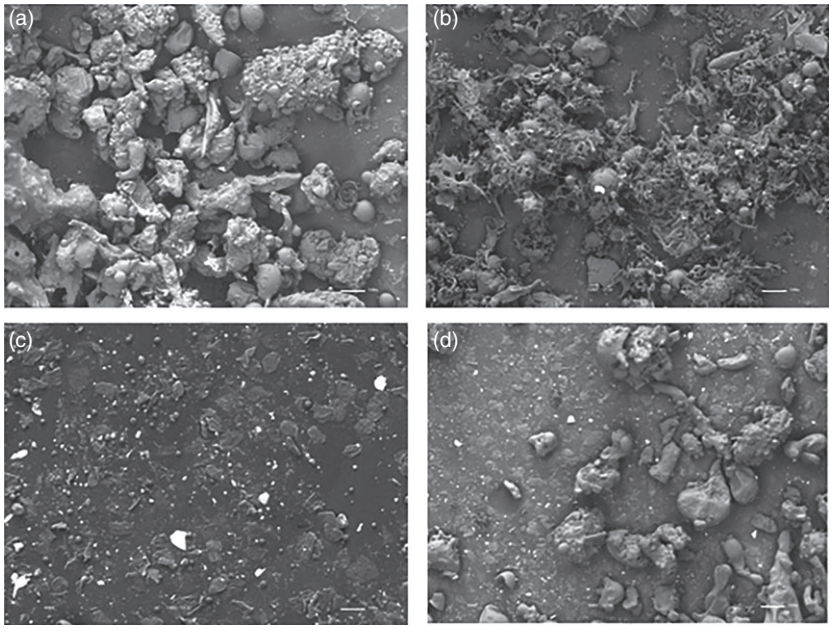

Figure 6 Scanning electron microscopy images of hydroxypropyl-beta-cyclodextrin and oxethazaine. (a) Hydroxypropyl-beta-cyclodextrin; (b) oxethazaine : hydroxypropyl-beta-cyclodextrin inclusion complex, (c) oxethazaine and (d) oxethazaine + hydroxypropyl-beta-cyclodextrin (physical mixture).

Table 2 Diffusion coefficients (*D*) for oxethazaine (OXZ), hydroxypropyl-beta-cyclodextrin (HP-β-CD) and their complex (OXZ : HP-β-CD, 1 : 1 mole %); complex molar fraction (*f*) and association constant (*K*), as determined by DOSY-^{1}H NMR

Samples	D (10^{-10} m^2/s)	Complex molar fraction (f)[a]	K (mol/l)[b]
OXZ	4.48 ± 0.036	–	–
HP-β-CD	2.21 ± 0.047	–	–
OXZ:HP-β-CD	3.61 ± 0.012	38	198

Calculations: [a]$f_{compl} = (D_{free} - D_{complex})/(D_{free} - D_{HP\text{-}\beta\text{-}CD})$; $f = 4.48 - 3.61/4.48 - 2.21 = 0.38$ (38% of complexed fraction). [b]$K = f/(1 - f)$ ([HP-β-CD] − f[OXZ]); $K = 0.38/(1 - 0.38)$ ([5 × 10^{-3} mol/l] − [0.38 × 5 × 10^{-3}] mol/l); $K = 0.38/1.92 \times 10^{-3}$; $K = 198$ mol/l.

Anaesthetic efficacy in inflamed tissue – hind paw incision test

Anaesthetic efficacy test was conducted over inflamed tissue (hind paw incision model), in rats. The effect elicited by the injection of 0.1% OXZ (free or in complex with HP-β-CD) was compared with that of 2% lidocaine with (1 : 100 000) epinephrine. The results of analgesia success are given as percentage of animals presenting anaesthetized paws as a function of time (Figure 9a) and duration of analgesia (Figure 9b). All formulations presented similar analgesia success (log-Rank, $P = 0.7296$) and duration (ANOVA, $P = 0.9853$), stating the potency of OXZ, even at such a low dose compared with lidocaine.

Discussion

The quantum yield of OXZ fluorescence increased in the presence of HP-β-CD (Figure 2a), reflecting the decrease in polarity of the milieu, or in the collisional probability of the fluorophore. A similar increase in the fluorescence

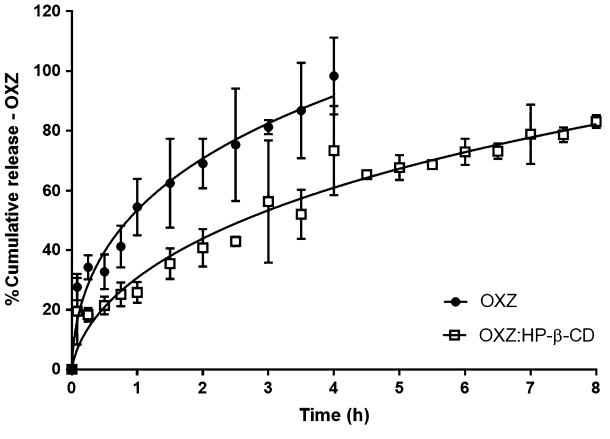

Figure 7 Cumulative release of free oxethazaine (●) and OXZ complexed (□) with hydroxypropyl-beta-cyclodextrin (OXZ:HP-**β**-CD,1 : 1 molar ratio) at 10 mM acetate buffer, pH 4.0 and ambient temperature ($n = 3$). Curves were fitted to the Higuchi model (R^2 of 0.97 for free oxethazaine and 0.96 for the complex), which considers a linearization using the square root of time, and slopes were significantly different ($P < 0.0001$), confirming the release kinetic change.

intensity was observed for tetracaine[20] and other guest molecules, when inserted in the CD cavity. OXZ fluorescence reveals its insertion in an environment of reduced polarity, compatible (Figure 2b) with the effective dielectric constant of the CD's cavity, which may vary between 2.2 and 55.[42]

The solubility profile of OXZ in the presence of HP-β-CD (Figure 4) followed the A_L Higuchi & O'Connors,[24] confirming the 1 : 1 stoichiometry of complexation of OXZ : HP-β-CD determined by Job plot analysis (Figure 3). X-ray and scanning electron microscopy (SEM) data (Figures 5 and 6) provided evidences of OXZ insertion within the cyclodextrin's hydrophobic core, since both demonstrate the loss of OXZ crystalline pattern in the complex but not in the simple physical mixture of OXZ and HP-β-CD. Similar results were reported for the complexation of tetracaine with either β-CD or HP-β-CD[20] and ropivacaine with HP-β-CD.[23]

NMR results allowed determination of the OXZ : HP-β-CD association constant (K = 198/M), in good agreement with that determined by phase solubility (K = 126/M, Figure 4), revealing a strong interaction between OXZ and HP-β-CD. Oxethazaine is the amino-amide anaesthetic agent of higher lipophilicity (log P 3.64)[1]; since K is directly correlated with lipophilicity (as demonstrated for complex non-steroid anti-inflammatory drugs),[43] we expected the highest association constant for OXZ among the amino-amide group. In accordance, the association constant of OXZ with HP-β-CD (pH 7.4 – DOSY-NMR) is higher than S-bupivacaine (K = 91/M[16]), S-ropivacaine (K = 55/M[44]) and prilocaine (K = 41/M[29]).

Currently, there are no compendial methods to effectively evaluate the release profiles of modified release parenterals.[45] We then decided to perform *In vitro* release studies with vertical diffusion cell, using a fast flow cellulose membrane, which is one of the most common methods described.[46] The test demonstrated that complexation delayed drug release to the acceptor compartment in a significant manner. We believe that *in vitro* drug release delay reflects the low solubility of OXZ in water, as observed for other low soluble anaesthetics,[47] besides host-guest complex formation/breaking. The designed method was discriminative (0–6 h), as shown in Figure 7, but did not

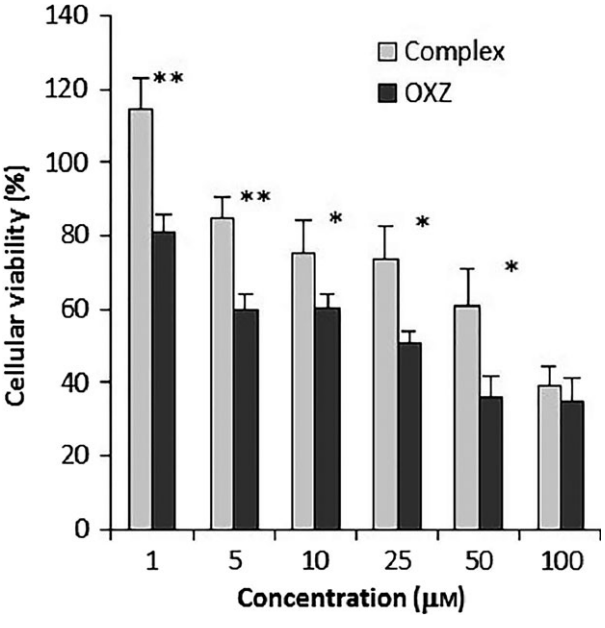

Figure 8 Cell viability tests in Balb/C 3T3 fibroblasts after treatment with free ozethazaine, and complexed with hydroxypropyl-beta-cyclodextrin (oxethazaine : hydroxypropyl-beta-cyclodextrin, 1 : 1 mole %), as assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. Results in percent cell survival, n = 3 experiments. Statistical analysis: non-paired T-test: $^{**}P < 0.01$, $^{*}P < 0.05$.

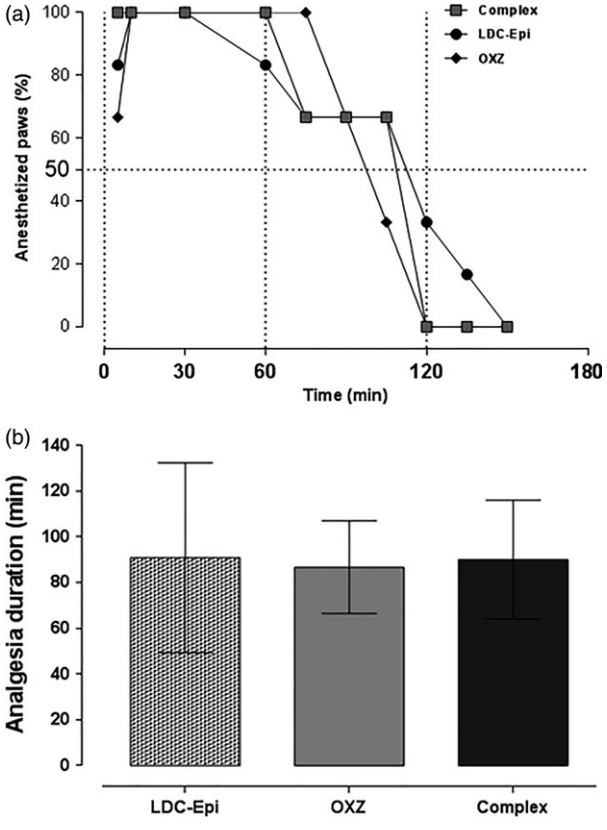

Figure 9 Antinociceptive tests: (a) Analgesia success (percentage of animals with anaesthetized paws) and (b) Analgesia duration after subcutaneous injection laterally to the incision of 2% lidocaine with 1 : 100 000 epinephrine (LDC-Epi), 0.1% oxethazaine (OXZ) or 0.1% oxethazaine complexed with hydroxypropyl-beta-cyclodextrin (complex) (n = 6).

achieve 100% release. Although 100% release is not a requirement for dissolution test of modified release formulations, FDA recommends up to 80%.[48] Therefore, further improvements in the release test should be done to apply it as a complete screening test, such as addition of surfactants in the acceptor compartment.

In vitro cytotoxicity of OXZ was evaluated in 3T3 fibroblast cells (IC_{50} = 25 μM or) by MTT test, which is based on mitochondrial activity.[30] MTT test results were in accordance with the neutral red test, which is based on lysosome integrity[49] (data not shown). Other researchers also reported similar results, but with different cell types, which confirms 3T3 as a suitable cell model (10^{-7} to 10^{-5} M range for PC12 prostate cancer cells[5] and 28.9 μM for HepAD38 liver cells[7]). Complexation with HP-β-CD was found to decrease the *in vitro* toxicity of OXZ (IC_{50} = 71 μM), probably because of the observed delayed release. Although presenting a high cytotoxicity, when compared with other anaesthetics (bupivacaine IC_{50} ~ 2 mM or 0.06%, lidocaine ~4 mM or 0.1%[50,51]), OXZ is the most potent of them (solution concentration: OXZ – 0.1%, bupivacaine 0.5%, lidocaine 2%). If we compared the ratio concentration (solution)/IC_{50}, OXZ has also the highest value (80, 8 and 22 respectively); however, OXZ complexation decreases this ratio to 28, which is closer to the lidocaine one. Considering that lidocaine is one of the safest anaesthetics recommended for infiltrative analgesia in buccal surgery, we believe OXZ : HP-β-CD can also be a safe and effective option. Further pre-clinical and clinical studies should be carried out to confirm this hypothesis.

Finally, *in vivo* analgesia tests confirmed the high potency of oxethazaine, even used at low concentration (0.1%) and over an inflamed tissue (hind paw incision model) in rats.[11] The effect of OXZ was equivalent to that elicited by 2% lidocaine plus epinephrine (used as a standard infiltrative anaesthesia formulation). Complexation did not alter OXZ potency, but also did not prolong analgesia duration, as we expected from *in vitro* experiments. Further studies concerning development of IVIVC release could be performed to best predict *in vivo* outcomes.

Conclusion

Hydroxypropyl-beta-cyclodextrin can form host-guest complexes with the local anaesthetic Oxethazaine, improving its (limited) water solubility. The equilibrium time (3 h) and stoichiometry of complexation (1 : 1) were determined. The 1 : 1 stoichiometry found was quite unexpected, taking into consideration the peculiar (double-anaesthetic) chemical structure of OXZ, but we wonder if steric hindrances would curb different stoichiometries of complexation (e.g. 1 OXZ to 2 HP-β-CD). In fact, the mephentermine molecules in the bisacetamide configuration of OXZ are sterically hindered and incapable of forming D-L isomers.[4] Evidences of the inclusion complex formation were provided by different methodologies (fluorescence, X-ray and SEM); phase-solubility diagrams and ^{1}H-NMR experiments revealed a strong association constant.

Complexation with HP-β-CD not only improved OXZ solubility but also delayed *in vitro* release of OXZ, although the delay was not significant in our *in vivo* model. *In vitro* cytotoxicity tests revealed a low IC_{50} (25 μM) for OXZ, but complexation enhanced cell survival by a factor of 3. *In vivo* antinociceptive studies revealed that infiltration of 0.1% OXZ : HP-β-CD or OXZ alone presented similar effects to those elicited by 2% lidocaine with epinephrine. The latter statement confirms that: (1) OXZ is one of the most potent local anaesthetics,[11] (2) it works at low pH (inflamed) tissues, where most local anaesthetic agents fail to reduce pain, (3) its complexation did not change drug potency or anaesthesia duration, but *in vitro* models indicated complexation led to less cytotoxic and therefore desired safety profile. Overall, this first pre-formulation study focused on OXZ parenteral formulations shows that HP-β-CD is an excellent excipient option for parenteral OXZ.

Declaration

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