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Physico-chemical characterization of benzocaine-β-cyclodextrin inclusion complexes

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Abstract

Local anesthetics are able to induce pain relief by binding to the sodium channel of excitable membranes, blocking the influx of sodium ions and the propagation of the nervous impulse. Benzocaine (BZC) is a local anesthetic whose low water-solubility limits its application to topical formulations. The present work focuses on the characterization of inclusion complexes of BZC in β-cyclodextrin (β-CD). Differential scanning calorimetry and electron microscopy gave evidences of the formation and the morphology of the complex. Fluorescence spectroscopy showed a BZC/β-CD 1:1 stoichiometry. Phase-solubility diagrams allowed the determination of the association constants between BZC and β-CD (549 M^{-1}) and revealed that a three-fold increase in BZC solubility can be reached upon complexation with β-CD. The details of BZC/β-CD molecular interaction were analyzed by ^{1}H 2D NMR allowing the proposition of an inclusion model for BZC into β-CD where the aromatic ring of the anesthetic is located near the head of the β-CD cavity. Moreover, in preliminary toxicity studies, the complex seems to be less toxic than BZC alone, since it induced a decrease in the in vitro oxidation of human hemoglobin. These results suggest that the BZC/β-CD complex represents an effective novel formulation to enhance BZC solubility in water, turning it promising for use outside its traditional application, i.e., in infiltrative anesthesia.

Keywords: Benzocaine; Cyclodextrin; Drug delivery; Local anesthetic

1. Introduction

Pain is one of the most extensively studied problems in medicine and biology, challenging physicians and other professionals to look for novel ways to deal with it. Anesthetics are compounds that, when administered locally or systemically, are able to induce pain relief. While general anesthetics act on the synapses, local anesthetics (LA) work along the axons, blocking the action potential [1–3]. Today, there is a strong clinical need for long-acting LA, as well as for molecules with decreased systemic uptake that could lead to less toxic side effects.

Benzocaine (BZC, Fig. 1a) is an ester-type local anesthetic used in topical, dermal and mucous formulations, while its parenteral administration is restricted by its low water solubility [3,4]. The interaction of this molecule with model membranes has been previously examined in our laboratory [5], where electron paramagnetic resonance studies have

Abbreviations: BZC, benzocaine; DSC, differential scanning calorimetry; LA, local anesthetics; NMR, nuclear magnetic resonance; SEM, scanning electron microscopy

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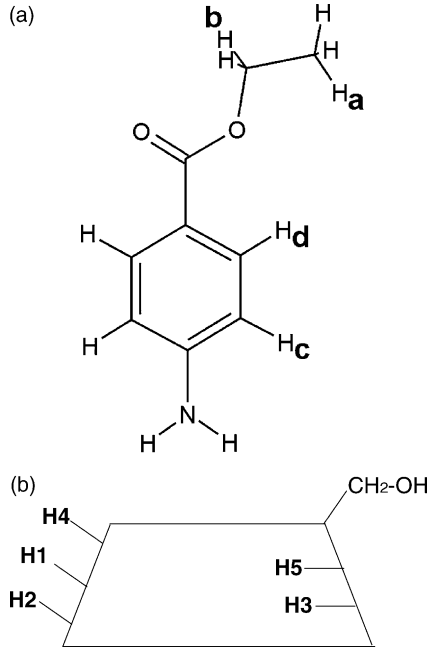

Fig. 1. Schematic representation of chemical structures and NMR peak identification for (a) benzocaine and (b) β-cyclodextrin.

revealed that BZC has intermediate hydrophobicity and a relatively low effect on lipid bilayer organization compared to other LA such as lidocaine and tetracaine.

The toxicity of ester-type LA has also been reported in the literature [1] as they can be hydrolyzed by plasma esterases, generating toxic metabolites such as the *p*-amino benzoic acid derivatives. BZC has a chemical resemblance to the ethyl ether *p*-amine-propyophenone (PAPP), which induces hemoglobin oxidation [6]. The literature reports increased Met-hemoglobin (Met-Hb) levels induced by BZC in different species [6–11].

The development of local anesthetic formulations in carriers such as liposomes, glucose polymers, dextran, hyaluronic acid, biopolymers, microspheres, etc. could offer the possibility of controlling drug delivery in biological systems, prolonging the anesthetic effect and reducing its toxicity [12,13]. Complexation with cyclodextrins (CD) provides a way to increase the solubility, stability and bioavailability of drugs [14,15]. CD is able to form inclusion complexes with different classes of molecules, modifying their physical, chemical and biological properties [16]. These cyclic polymers are formed by glucose molecules linked through 1–4 bonds. They can have six (α-CD), seven (β-CD) or eight (γ-CD) glucose units and the cone-shaped cavity of CD allows the accommodation of the apolar part of molecules such as local anesthetics. Studies involving the complexation of LA molecules with CD have been reported in the literature [15,17–20] although none of them employed benzocaine.

The aim of the present study was to prepare and to characterize inclusion complexes of benzocaine in β-CD to provide a way to increase the solubility, stability and bioavailability of the drug. The stoichiometry of the inclusion complex was analyzed by fluorescence spectroscopy using the continuous variation method described by Higuchi and Connors [21]. Phase-solubility diagrams were used to evaluate the solubility of BZC and to determine its association constants with β-cyclodextrin. Differential scanning calorimetry (DSC) and scanning electron microscopy (SEM) assays were used to observe the formation and morphology of the solid inclusion complex obtained. Finally, nuclear magnetic resonance (^{1}H NMR) experiments revealed details of the geometry of the complex formed. This particular system has a clinical relevance with the potential of significant impact by the possibility to use BZC in infiltrative anesthesia in vivo.

2. Experimental

2.1. Materials

Benzocaine, β-cyclodextrin (Fig. 1a and b) and D_2O were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade. Deionized water was used throughout the experiments.

2.2. Preparation of solid inclusion complex

Inclusion complexes with 1:1 and 1:2 BZC/β-CD molar ratios were prepared by shaking appropriate amounts of the anesthetic and β-CD, e.g. 1 mM BZC and 1 or 2 mM β-CD, in deionized water at room temperature (25 ± 1 °C) for 1 h. Kinetic experiments revealed that equilibrium was reached after 40 min incubation (data not shown).

After reaching equilibrium, the solution was freeze-dried in a Labconco Freeze-dry system (Freezone 4.5) and stored at −20 °C until further use. Physical mixtures (non-complexed drugs) were obtained by mixing BZC and β-CD powders.

2.3. Phase-solubility studies

This methodology was based on the solubility variation of the guest molecule (BZC) upon increase of the host molecule (β-CD) concentration [21]. An excess amount of BZC was added to an aqueous solution with increasing β-CD concentrations (0–10 mM) at room temperature. The samples were stirred for 1 h, at room temperature and an aliquot was filtered through a 0.45 μm membrane filter (Millipore). The amount of soluble BZC was spectrophotometrically determined at 284 nm [5]. The association constant (K_a) was calculated from the slope of the linear portion of the phase-solubility diagram, according to Eq. (1) [21]:

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

where S_0 is the aqueous solubility of benzocaine. The experiment was carried out in triplicate.

2.4. Fluorescence studies

Changes in the intrinsic fluorescence of BZC upon variation of the BZC/β-CD molar ratios of complexation were monitored with a Hitachi F-4500 spectrofluorimeter, at room temperature. Excitation wavelength was set to 284 nm and the emission spectra were recorded from 300 to 450 nm. The stoichiometry of the complex was determined by Job-plot analysis [22]. Equimolar stock solutions of BZC and β-CD were prepared and mixed in order to obtain the same final value of (M) as follows:

$$[\mathrm{BZC}]_t + [\beta\text{-CD}]_t = M \quad (2)$$

where t = total concentration.

Changes in fluorescence intensity (ΔI) were calculated as the difference in the emission of BZC in the presence (I) and in the absence (I_0) of β-CD. Subsequently $\Delta I[\mathrm{BZC}]_t$ was plotted against R, the molar ratio of complexation:

$$R = \frac{[\mathrm{BZC}]_t}{M} \quad (3)$$

In the biphasic plots obtained, the concentration of the BZC/β-CD complex (measured by ΔI) will reach a maximum corresponding to the point where the derivative d[BZC/β-CD]/dr = 0, and the value of R, independently of M, or the association constant (K_a), can be determined [23].

2.5. Scanning electron microscopy (SEM)

The BZC/β-CD inclusion complexes (1:1 and 1:2 molar ratio) were morphologically analyzed by scanning electron microscopy, with a JEOL JSM–T300 Scanning Microscope. Samples of BZC, β-CD and BZC/β-CD physical mixtures (1:1 and 1:2) were also prepared at the same concentration of the inclusion complexes. BZC and β-CD were submitted to liophilization in the same way as the inclusion complexes (item 2.2). The samples were mounted on aluminum stubs, using double-sided sticky tabs and vacuum coated with gold for 180 s, to render them electrically conductive.

2.6. Differential scanning calorimetry (DSC)

The various samples (10 mg) were placed in aluminum pans and the experiments run in a Universal V2.3D TA Instruments calorimeter at a 10 °C/min heating rate over a 30–200 °C range. An empty pan served as reference and Indium was used to calibrate the temperature. Measurements were performed at BZC/β-CD 1:1 and 1:2 (molar ratio) both for the inclusion complexes and the physical mixtures; BZC and β-CD thermograms were also run. Plain BZC and β-CD were submitted to the same freeze drying process of the complexes, to avoid erroneous considerations involving the transitions temperatures of freeze-dried and non-freeze-dried samples.

2.7. Nuclear magnetic resonance (NMR)

One-dimensional ^{1}H NMR spectra of BZC/β-CD complexes were recorded with a 500 MHz Bruker DRX500 spectrometer (Universidade de São Paulo, Brazil) at 25 °C. Samples were suspended in D_2O and degassed by bubbling N_2 directly in the NMR tubes. The chemical shifts ($\Delta\delta$) are reported as ppm and are referenced to the residual water signal (4.7 ppm).

Two-dimensional (2D) rotating frame Overhauser effect spectroscopy (ROESY) experiments were performed on a Varian INOVA 600 MHz spectrometer (CeBiME, Laboratório Nacional de Luz Síncrotron, Brazil) using a spin-lock field of 3 kHz of 300 ms. The spectra were collected using 2048 complex data points in the F2 dimension and 324 increments. The spectral width was 10 ppm in both dimensions and eight free induction decays were acquired per increment.

The spectrum was processed using the VNMR 6.1 software (Varian Inc.). The cross-peaks volumes were directly correlated with inter-nuclear distance, r, of the two observed protons, via the known r^{-6} dependence [24]. The fixed and well-known intramolecular distances between two vicinal aromatic protons (2.48 Å) of BZC – ortho and meta – were used for calibration.

2.8. Molecular modeling

Molecular modeling for the NMR results was performed in an Octane2 workstation (Silicon Graphics Inc.) using the InsightII package (Accelrys Inc.). All the calculations were done with the Discover module using the consistent valence force field (CVFF). The BZC model building was performed with the Builder and Biopolymer modules of InsightII. The β-CD coordinates were taken from the β-amylase/β-cyclodextrin complex crystal structure (PDB 1BFN [25]). Each molecule was first energy-minimized separately through a combination of the steepest descents and conjugate gradient algorithms, until a maximum derivative of 0.001 kcal/mol Å was achieved. The molecules were further manually assembled into five complexes with different relative orientations, followed by energy minimization. No distance restraints were used at any time during the calculations. The final energy-minimized complexes were compared to the experimental results obtained by ^{1}H NMR.

2.9. Methemoglobin formation

Freshly purified (up to 5 days after blood collection) human hemoglobin solutions – 10^{-5} M – were incubated for 30 min with increasing amounts of BZC/β-CD complexes. Met-hemoglobin formation was followed at 630 nm [26] and $K_3Fe(CN)_6$ was used to induce 100% oxidation. The results were compared with the oxidation caused by plain BZC, as published before [5].

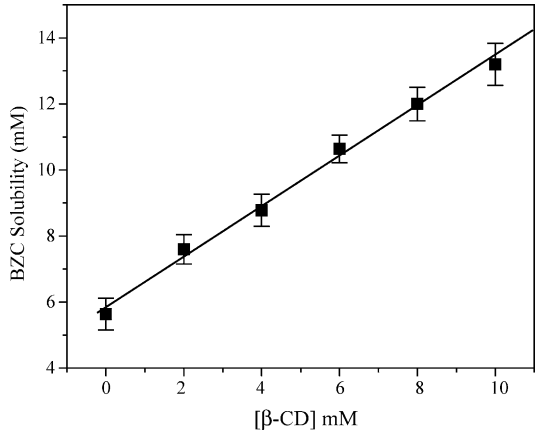

Fig. 2. Phase-solubility diagram for BZC at increasing β-CD concentrations, determined at room temperature.

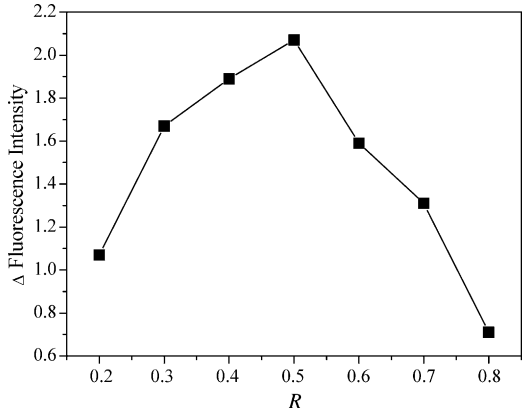

Fig. 3. Continuous variation plot (Job plot) for the BZC/β-CD complex obtained by fluorescence spectroscopy. $\lambda_{excitation}$ = 284 nm, $\lambda_{emission}$ = 300–450 nm, at room temperature.

3. Results

3.1. Phase-solubility studies

The increase in solubility occurred as a linear function of β-CD concentration (Fig. 2) corresponding to the A_L-type profile defined by Higuchi and Connors [21] up to 10 mM. This relationship suggests the formation of a 1:1 BZC/β-CD complex. The association constant (K_a) was calculated to be $549 \pm 42\ M^{-1}$, thus indicating the formation of a stable complex [23].

The increased aqueous solubility of BZC in the presence of β-CD shown in Fig. 2 also, suggests that these molecules are able to form inclusion complexes. Since the complexation occurs at a 1:1 molar ratio (see Section 3.2), the solubility of BZC in water is expected to be enhanced by complexation up to the limit of β-CD solubility, c.a. 16 mM [27]. In fact the results obtained here strengthen this hypothesis since, in the presence of 10 mM β-CD, benzocaine reaches a concentration at least three times higher (Fig. 2) than that of the free drug in water [5].

3.2. Stoichiometry of the complex

Complexation depends on the polarity and size of the guest molecule with respect to the inner cavity of the host CD molecule. Taken advantage of the fact that upon inclusion of a fluorescent guest molecule into the β-CD cavity, generally an enhancement in fluorescence is observed due to shielding from quenching and non-radioactive decay processes [28], the stoichiometry of the BZC/β-CD complexation was obtained by fluorescence spectroscopy. The enhancement in BZC fluorescence upon complexation was quantified by the changes of the integrated fluorescence emission ratio, ΔI, as described in Section 2. The Job-plot obtained (Fig. 3) showed a maximum ΔI at $R = 0.5$ – which is indicative of a 1:1 stoichiometry of the BZC/β-CD complex – in agreement with phase-solubility data.

3.3. Formation and morphology of the complex

Supporting evidence for the complexation of BZC with β-CD was also obtained from SEM, DSC and NMR data. SEM pictures showed that the shape and size of the inclusion complexes were completely different from the ones of free BZC or β-CD (Fig. 4). Typical crystals of β-CD [29] and BZC were found in many different sizes (Fig. 4a and b, respectively). At the two molar ratios of the BZC/β-CD inclusion complexes studied (1:1 and 1:2) a compact and homogeneous powder-like structure was observed, Fig. 4c, whose dimensions were smaller than those of the crystals of BZC or β-CD alone. The physical mixtures of BZC and β-CD powders revealed similarities with the crystals of the free molecules (Fig. 4d), where residual β-CD crystals were easily identified (arrows). On the other hand, some agglomeration of BZC around β-CD crystals was observed both in the 1:1 (Fig. 4d) and 1:2 (not shown) BZC/β-CD physical mixture.

Representative DSC thermograms measuring the rate of heat absorbed by BZC, β-CD, BZC/β-CD inclusion complex and physical mixture, at 1:1 and 1:2 molar ratios, are shown in Fig. 5.

DSC thermograms of β-CD and free BZC detected endothermic peaks at 140.4 and 90.7 °C, respectively (Fig. 5a and b), corresponding to the loss of solvatation water from β-CD cavity and melting point of BZC, respectively [30,31]. The thermogram of the BZC/β-CD inclusion complex lacked the endothermic peaks of both BZC and β-CD and showed a weak and broad transition, extending from 60 to 120 °C for the 1:1 molar ratio (Fig. 5c) and from 80 to 160 °C, for the 1:2 molar ratio (Fig. 5d). The absence of the endothermic peaks in the complex suggests the insertion of the guest molecule inside the β-CD cavity [32]. The increased absorption observed for the 1:2 molar ratio complex is due to some non-complexed, remaining β-CD. Finally, the curves of the physical mixtures resemble the sum of the individual curves of BZC and β-CD (Fig. 5e and f).

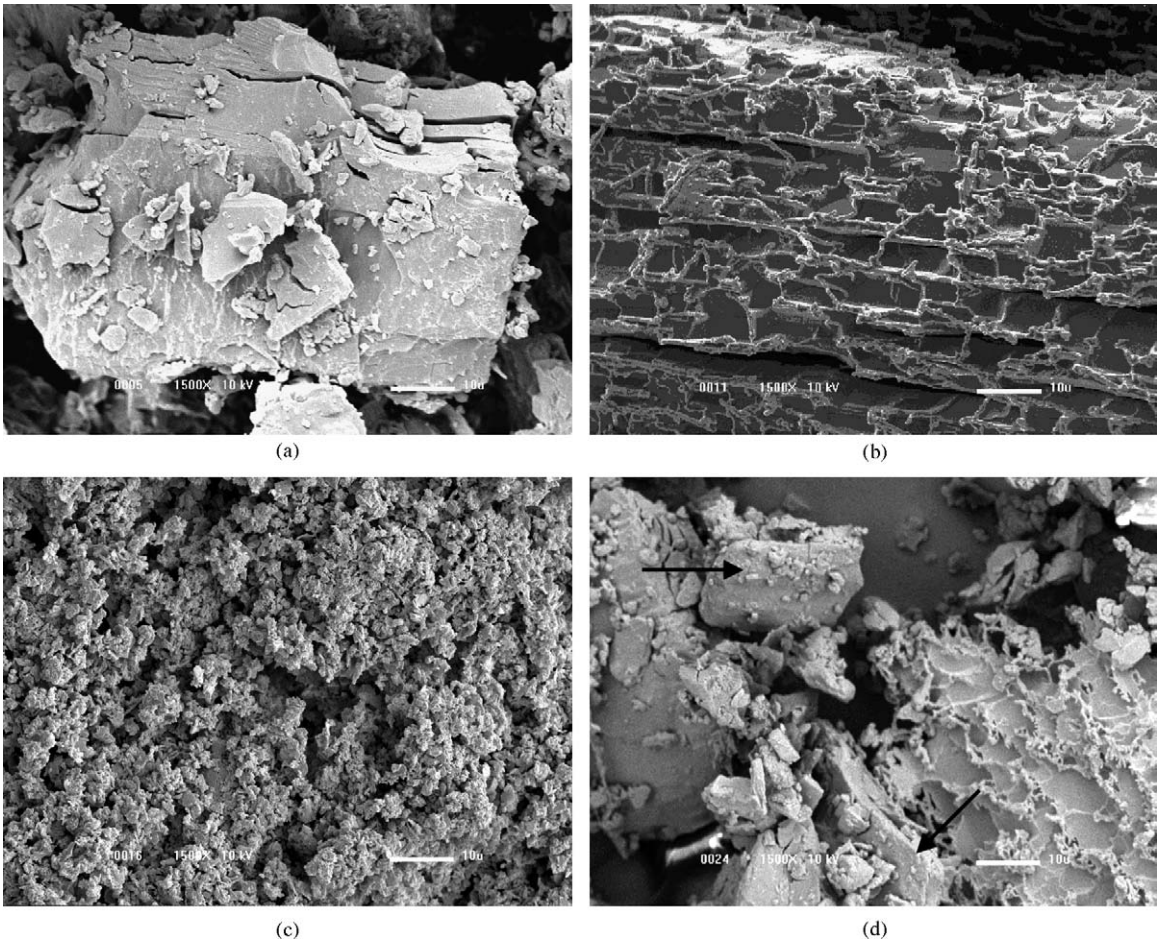

Fig. 4. Scanning electron microscopy of (a) β-CD, (b) BZC, (c) BZC/β-CD 1:1 complex, (d) physical mixture of BZC and β-CD, 1:1, molar ratio. 1500× magnification, bar = 10 μm.

Overall, the SEM and DSC results indicate the formation of inclusion complex between BZC and β-CD and also demonstrate that no complex is formed in the physical mixture of the compounds.

3.4. Model validation by NMR

Cyclodextrin has six identifiable protons in the NMR spectrum: protons H1, H2, H4 being at the outer surface of cyclodextrin, while protons H3 and H5 sit in the inner surface (or cavity) and are very important for the study of the interaction of guest compounds with cyclodextrins. H6 (CH_2OH, Fig. 1b) is located in the minor border cavity of β-CD [33].

The inclusion of BZC into the β-CD cavity was first evaluated by the changes in the chemical shifts (δ) of the protons in the complex, relatively to free BZC and β-CD (Fig. 6). Table 1 presents the assignment of BZC and β-CD peaks and the chemical shifts deviations due to complexation. All the

Table 1
^{1}H NMR: chemical shift of BZC and β-CD protons before and after complexation. Peak identification as visualized in Fig. 1

Assignments	BZC (ppm)	β-CD (ppm)	BZC/β-CD (ppm)	$\Delta\delta$ (ppm)
(a) Ethyl-CH_3	1.31		1.13	0.18
(b) Ethyl-CH_2	4.30		4.28	0.02
(c) Aromatic-3,5	6.79		6.87	−0.08
(d) Aromatic-2,6	7.82		7.97	−0.15
H_1		5.04	5.04	−0.00
H_2		3.62	nd	nd
H_3		3.93	3.78	0.15
H_4		3.56	nd	nd
H_5		3.82	3.75	0.06
H_6		3.84	3.75	0.09

nd – not determined due to peak overlap.

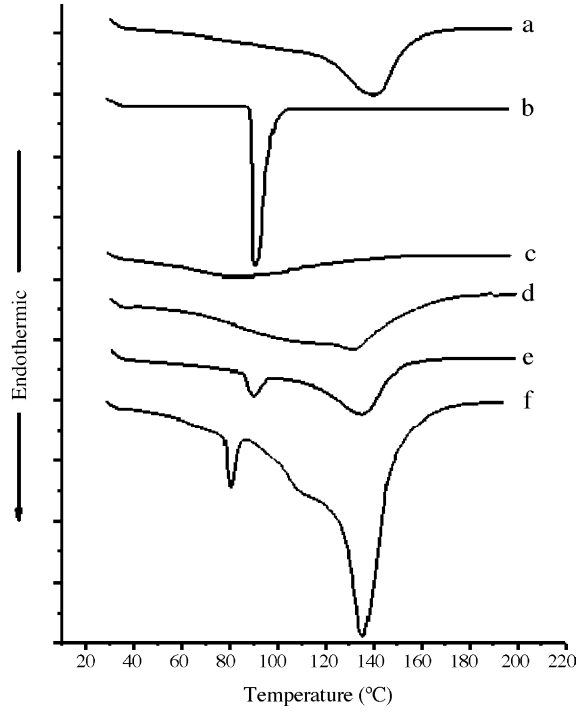

Fig. 5. Differential scanning calorimetry thermograms of (a) β-CD, (b) BZC, (c) BZC/β-CD 1:1 complex, (d) BZC/β-CD 1:2 complex, (e) physical mixture of BZC/β-CD 1:1 and (f) physical mixture of BZC/β-CD 1:2 molar ratio.

peaks belonging to BZC protons were shifted: the ethyl-CH_3 upfield and the aromatics to downfield. In the case of β-CD molecule, only H1 did not change the chemical shifts after complexation with BZC. As for the other protons, the H3 were the most significantly altered ones, followed by H6 and H5. Peak overlap did not allow evaluation of the H4 shift. H3 protons resonance shifted upfield while H5 shifted downfield, leading to an overlap with the peak of H6 protons. Similar changes in the δ of H3, H6 and H5 protons were found for the *p*-iodophenolate/α-CD complex [33], where the aromatic ring of the drug seems to fit near the head of the cavity of CD.

To better elucidate the relative position of BZC with respect to β-CD protons we carried out ROESY experiments. Contacts were observed between protons H5 and H6 – belonging to β-CD – and the aromatic and methyl protons of BZC. These cross-peaks are intense and agree with the $\Delta\delta$ data (where methyl and aromatic protons of BZC were mainly changed by complexation, Table 1). The volumes of the cross-peaks were measured and the inter-molecular distances calculated, as described in the Section 2.

The calculated distances suggest that BZC interacts only with H5, and not with H3 protons of the inner cavity of β-CD. They indicate that BZC inserts its aromatic ring nearby the protons H5 (2.5 Å) and H6 (2.5 Å) i.e., towards the minor face (head) of the β-CD cavity. The methyl protons of BZC also get close to H5 and H6, but the distances are not so short (3.5 and 2.6 Å, respectively) as for the aromatic protons.

Molecular modeling studies revealed that a preferred final relative orientation for the BZC/β-CD complex occurs in spite of the different initial configurations arbitrarily imposed. The minimum energy complex obtained is shown in Fig. 7. It is interesting to note that although no fixed distances were imposed during the calculations, the results are in very good agreement with the distances obtained by 2D NMR, mainly for the H5-aromatic protons (2.66 and 2.43 Å).

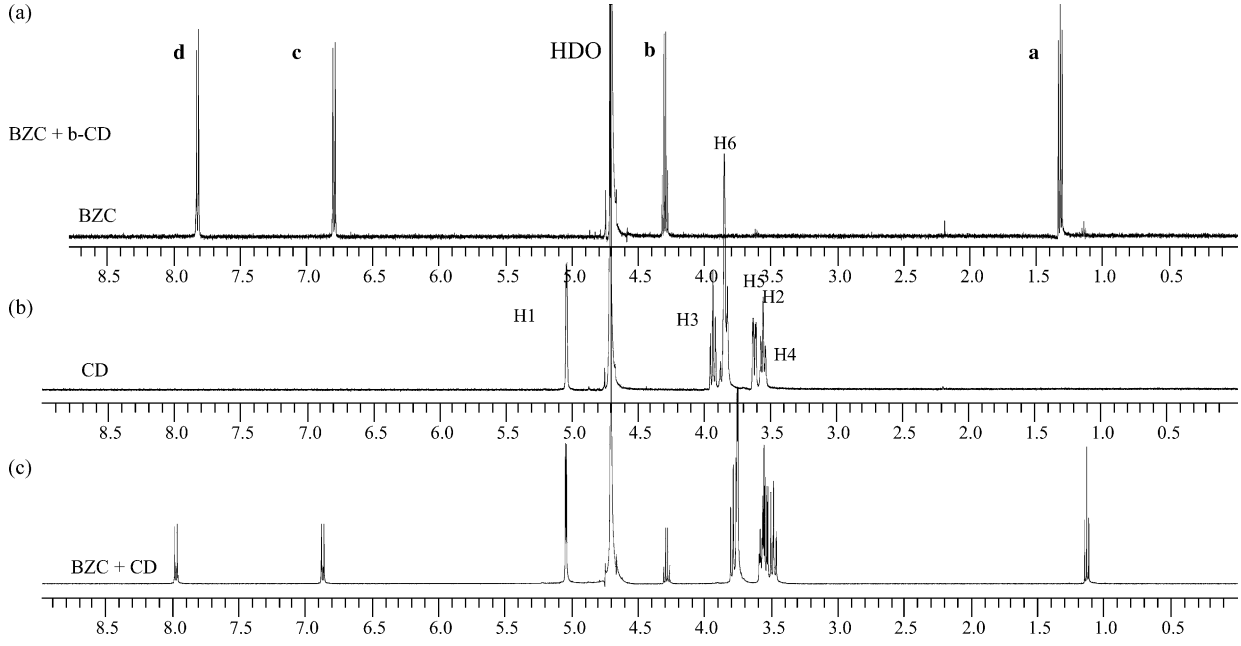

Fig. 6. Mono-dimensional ^{1}H NMR spectra of (a) BZC, (b) β-CD and (c) the BZC/β-CD complex: (a) [BZC] = 3 mM; (b) [β-CD] = 10 mM; and (c) [BZC] and [β-CD] = 10 mM. Samples in D_2O, 25 °C.

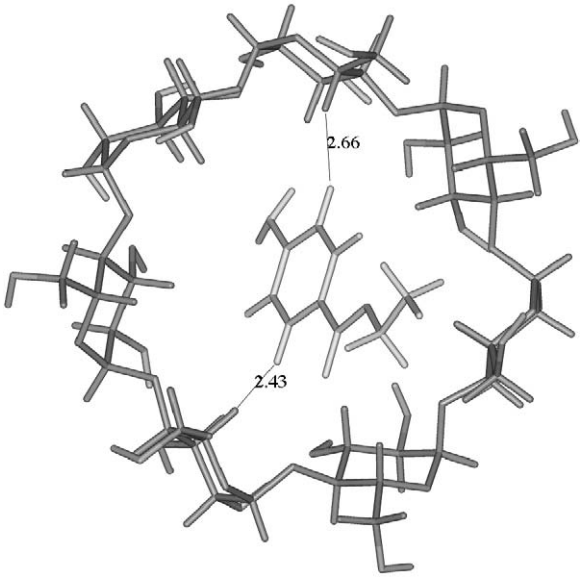

Fig. 7. Model of BZC insertion into the β-CD cavity, proposed by molecular modeling. Distances between aromatic BZC protons and H5 protons are shown.

Taken together, these convergent results coming from independent techniques support the existence of a unique spatial organization for the BZC/β-CD complex, in a 1:1 stoichiometry.

3.5. *In vitro toxicity*

In order to test the toxicity of this novel formulation, an essay using purified human hemoglobin was performed. In a previous work, we have observed that the free anesthetic at high concentrations was able to promote Met-hemoglobin formation [5]. Here, we used the same protocol to check hemoglobin oxidation induced by the BZC/β-CD complex. No significant increase in Met-hemoglobin content was detected after incubation of oxygenated-hemoglobin with the 1:1 complex, up to 6 mM (Fig. 8), indicating that complexation could offer an additional advantage for the infiltrative administration of BZC, diminishing its toxic effects.

4. Discussion

BZC is an interesting local anesthetic molecule with unique features – in comparison to the commercially used LA – such as the lack of an ionizable amine group nearby physiologic pH [1,5] keeping it always uncharged and the absence of use-dependent block [34]. Nevertheless, it is only used in anesthesia of the skin and mucous membranes (such as transesophageal echography, bronchoscopy, fiberotic intubation) due to its limited water solubility [1].

The present results show that a stable BZC/β-CD complex could be prepared at a 1:1 molar ratio. This novel formulation

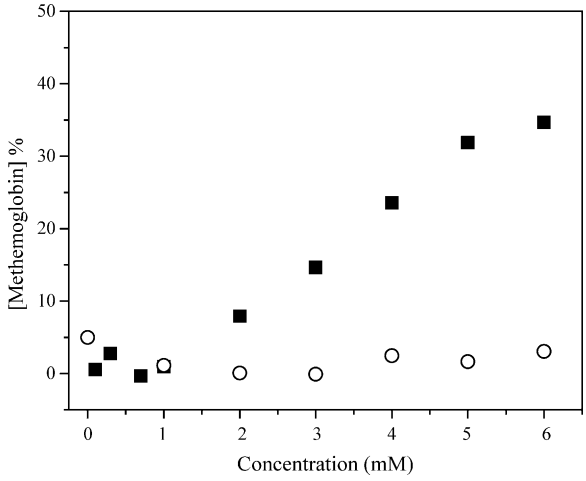

Fig. 8. Met-Hb formation induced by (■) BZC and (◯) BZC/β-CD complex (1:1 molar ratio) in a purified Hb solution, 5 mM PBS buffer, pH 7.4, at room temperature.

effectively enhanced the solubility of benzocaine in water, increasing its local availability in the site of injection.

The complexation with β-CD generated a less toxic BZC formulation, as seen by the decreased oxidation of hemoglobin. These in vitro findings should be not taken as representative of the in vivo conditions where the concentration of cyclodextrin will be much lower than in vitro and moreover, there will be a substantial competition from endogenous molecules such as cholesterol for the binding to the cyclodextrin's cavity. Instead, they show the controlled release of benzocaine from the BZC/β-CD complex in a model system, decreasing its well-known oxidative effect on hemoglobin. Complexation could then delay the uptake of BZC at the site of injection, increasing the duration of anesthesia, as seen for other – liposomal – LA drug delivery systems [13]. In vivo sensory block tests are being carried out with this formulation and will be published in due time.

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