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Phase solubility, ^{1}H NMR and molecular modelling studies of bupivacaine hydrochloride complexation with different cyclodextrin derivates

Mario Jug [a,*], Natascia Mennini [b], Fabrizio Melani [b], Francesca Maestrelli [b], Paola Mura [b]

[a] Department of Pharmaceutics, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, Zagreb 10000, Croatia
[b] Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Florence, via Schiff 6, Sesto Fiorentino 50019, Florence, Italy

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ABSTRACT

A novel method, which simultaneously exploits experimental (NMR) and theoretically calculated data obtained by a molecular modelling technique, was proposed, to obtain deeper insight into inclusion geometry and possible stereoselective binding of bupivacaine hydrochloride with selected cyclodextrin derivatives. Sulphobuthylether-β-cyclodextrin and water soluble polymeric β-cyclodextrin demonstrated to be the best complexing agents for the drug, resulting in formation of the most stable inclusion complexes with the highest increase in aqueous drug solubility. The drug-carrier binding modes with these cyclodextrins and phenomena which may be directly related to the higher stability and better aqueous solubility of complexes formed were discussed in details.

1. Introduction

Despite the recent advances in basic and clinical investigation towards new therapeutic agents, the management of acute and chronic pain or regional anaesthesia during surgery is still a challenge. A possible approach is the administration of local anaesthetics, such as bupivacaine hydrochloride (BVP HCl), which is the most extensively used local anaesthetic in surgical procedures worldwide. BVP HCl is a long-acting anaesthetic drug of the amide type which is commonly administered as injectable solution for local anaesthesia, including infiltration, nerve block, epidural and intrathecal anaesthesia, and is particularly effective in the pain management during and after dental procedures [1]. However, the systemic absorption of BVP HCl is not beneficial, because it may lead to a significant toxicity to central nervous and cardiovascular system [2]. Therefore, frequent local administration of a low BVP HCl dose is often required in order to avoid above mentioned side effects, thus reducing the patient comfort. Moreover, the use of acidic solutions is necessary to improve the low aqueous drug solubility, thus resulting in painful applications. This series of obstacles may be solved by the use of a proper drug carrier which would allow both improvement of drug solubility as well as reduction of systemic drug absorption and would also provide prolonged drug release on the site of action [3]. Furthermore, the enhanced therapeutic efficacy could allow a reduction of the drug dose, thus further reducing the risk of side-effects occurrence. Additionally, the use of such carrier could also make possible the development of new drug formulations, as an alternative to the injectable ones, and to extend its applications for the treatment of oral cavity diseases, such as aphtha, mucositis, etc.

Among many possible ways of obtaining above mentioned carrier systems, the use of cyclodextrins, cyclic oligosaccharides consisting of 6,7 or 8 α-1,4 linked glucopiranose units, may provide an easy approach. Because of their bioadaptability and multifunctional characteristics, cyclodextrins are capable of alleviating the undesirable properties of drug molecules in various routes of administration through formation of inclusion complexes [3]. In particular, it has been demonstrated that cyclodextrin complexation of different local anesthetics has resulted in improved drug solubility and increased therapeutic effect in comparison to the plain drug, accompanied by reduced neurotoxicity of the drug [4–6].

The cyclodextrin effectiveness as drug carrier can be significantly influenced by the size of the cavity and the presence and type of substituents present on the cyclodextrin core [7–9]. Therefore, selection of the most suitable cyclodextrin for complexation with a given drug is an important step for the successful development of effective pharmaceutical formulations. Furthermore, the rational design of pharmaceutical formulations based on drug–cyclodextrin complexes requires a determination of the inclusion complex geometry, which is a difficult step and represents one of the highest challenges in the host–guest chemistry [10]. The combination of experimental and computational studies can be a powerful tool for determining the geometry of complexation. Therefore, in this work, as the first phase of a wider study aimed at the development of a new buccal formulation of the drug, we investigated the inclusion complexation of BVP HCl with a series

* Corresponding author. Fax: +38514612691.
E-mail addresses: mjug@pharma.hr (M. Jug), natascia.mennini@unifi.it (N. Mennini), fabrizio.melani@unifi.it (F. Melani), francesca.maestrelli@unifi.it (F. Maestrelli), paola.mura@unifi.it (P. Mura).

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of native and modified cyclodextrins, in order to select the carriers with the greatest solubilizing and complexing efficacies towards the drug. The selected drug–cyclodextrin systems were then characterized by ^{1}H NMR and 2-D NMR (ROESY) spectroscopy, to confirm the actual inclusion complex formation and determine the structure and dynamics of the complexes. We propose a novel approach, which simultaneously exploits molecular modelling studies and NMR data, to build more reliable three-dimensional models of the complexes, and to shed light on possible stereoselective binding, due to the presence of a chiral centre on the BVP HCl molecule.

2. Materials and methods

2.1. Materials

Bupivacaine hydrochloride (BVP HCl) was kindly donated by S.I.M.S. (Italy). The list of cyclodextrin derivates included in this study comprised α-cyclodextrin (α-CD; Fluka Chemie, Italy), β-cyclodextrin (β-CD; Kleptose 4PC, Roquette, France), hydroxypropyl-β-cyclodextrin with average substitution degree per anhydroglucose unit of 0.6 (HP-β-CD; Roquette, Italy), randomly methylated-β-cyclodextrin with average substitution degree of 1.8 (RAMEB), γ-cyclodextrin (γ-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) with average substitution degree of 0.6 (Wacker Chemie GMBH, Germany) as well as sulphobutylether-β-cyclodextrin sodium salt with substitution degree of 0.9 (SBE-β-CD; CyDex Inc., USA) and soluble β-cyclodextrin-epichlorohydrin polymer (EPI-β-CD, mean MW 4500, Cyclolab R&D Ltd., Hungary). All others chemicals and solvents used in this study were of analytical reagent grade.

2.2. Phase solubility studies

Phase solubility studies of BVP HCl with different cyclodextrins were performed according to the method described by Higuchi and Connors [11]. An excess amount of BVP HCl (50 mg) was added to 10 ml of 0.1 M phosphate buffer solution with pH value adjusted to pK_a of BVP HCl (pK_a = 8.32) or buffered cyclodextrin solutions. The concentration range of the cyclodextrins tested was from 0 to 25 mM, except for β-CD and EPI-β-CD. Due to its limited aqueous solubility, the concentration range of β-CD was from 0 to 12.5 mM while for EPI-β-CD the concentration range was from 0% to 7% (w/v). The prepared samples in sealed glass containers were vigorously stirred at a constant temperature (25.0 ± 0.5 °C) for 72 h until complexation equilibrium was reached. Aliquots of the samples were filtered through 0.45 μm Millipore membrane filter and drug concentrations in the filtrate were determined spectrophotometrically after suitable dilution with the phosphate buffer solution. The measurements were performed at a wavelength of 262.8 nm using Shimadzu 1601 UV–vis spectrophotometer (Shimadzu Italia S.R.L.). Preliminary studies showed that the presence of cyclodextrins did not interfere with BVP HCl absorbance at 262.8 nm. The apparent stability constant K_s was calculated from the phase solubility diagram using the Eq. (1) [11]:

$$K_s = \frac{\text{slope}}{s_0 \times (1 - \text{slope})} \quad (1)$$

3. ^{1}H NMR experiments

One-dimensional ^{1}H NMR spectra were recorded with a Bruker Avance 400 instrument (Karlsruhe, Germany) operating at 300 K using a 5 mm probe and a simple pulse-acquire sequence. The sample solutions were prepared by dissolving the drug, each cyclodextrin tested, and the corresponding 1:1 mol:mol binary systems in D_2O to obtain the final concentration of 10 mM. The data were collected without an external reference to avoid its possible interaction with cyclodextrins. The signal of residual water at 4.70 ppm was suppressed by the use of WATERGATE technique [12].

Two-dimensional rotating-frame Owerhauser effect spectroscopy (ROESY) experiments were performed with the same instrument using the standard Bruker program, using D_2O as solvent, relaxation delay of 1 s and mixing time of 500 ms under the spin lock conditions.

3.1. Molecular modeling (MM)

The initial molecular geometry of β-CD was obtained using X-ray diffraction data [13], whereas SBE-β-CD was constructed by adding 7 sulphobutyl groups to parent β-CD, according to two different patterns of substituent distribution by replacing all the primary OH groups or by randomly substituting 4 primary and 3 secondary OH groups. The starting geometry of the guest molecule and the sulphobutyl groups were built up using the standard fragment library of the BUILDER module of the INSIGHT II program (Accelrys, Inc., San Diego, CA, USA, software version 2005). The complexes were built-up by inserting the aromatic portion of the drug molecule into the CD cavity from either the wider/secondary rim (type A complex) or the narrower/primary rim (type B complex). Molecular dynamics (MD) calculations have been performed with the AMBER 9 program [14], using FF03 [15] and the General Amber Force Field (GAFF) [16] Atomic charges were obtained by using the AM1–BCC methodology [17,18]. All complexes were solvated with a cubic box of pre-equilibrated TIP3P water molecules [19] with a minimum distance between any CD or guest atoms and the box edge of 10 Å. Periodic boundary simulations PMEMD (Particle Mesh Ewald Molecular Dynamics) and the SHAKE option [20,21] were used to constrain bonds involving hydrogen atoms. The non-bonded cutoff was set to 9.0 Å. All simulations have been performed with 2 fs time step, using the Langevin dynamics algorithm for the temperature control. The negative charge of the complex with SBE-β-CD has been neutralized by adding Na+ ions.

Recording of solvated complexes conformations during MD simulation was preceded by 4 steps, in order to equilibrate the system:

- Conformational minimization of the water shell using steepest descent and conjugate gradient algorithm with constant volume periodic boundaries, by applying a restraint for the complex, with a weak force constant of 2.0 Kcal/mol A^2.
- Conformational minimization of the solvated complex using steepest descent and conjugate gradient algorithm with constant volume periodic boundaries.
- 50 ps of dynamics, with a gradual water heating up to 300 K using constant volume periodic boundaries, by applying a weak restraint to the complex (force constant 2.0 Kcal/mol A^2).
- 50 ps of dynamics to equilibrate the water shell at 300 K using constant pressure periodic boundary with an average pressure of 1 atm. Isotropic position scaling to maintain the pressure and a relaxation time of 1 ps were used, always applying a weak restraint to the complex (force constant 2.0 Kcal/mol A^2).

Finally, the solvated complex is subjected to a simulation of dynamics at 300 K under constant pressure for 1 ns and, during the simulation, 500 conformations are collected.

The stability of complexes was evaluated by the calculation of binding free energy (ΔG). The binding free energies were calculated by using MM-PBSA method, as implemented in the AMBER package. Five hundred snapshots were extracted from the MD simulation, and the binding free energies between the guest and the

CD molecules were calculated. The average binding free energies were calculated every 10 snapshots, getting 50 values of energy for MD trajectory. The binding free energies in condensed phase can be calculated according to the following equations:

$$\Delta G_{bind} = G_{complex} - (G_{guest} + G_{host}) \quad (2)$$

$$G = E_{gas} + G_{solve} - TS \quad (3)$$

$$E_{gas} = E_{bond} + E_{angle} + E_{torsion} + E_{vdW} + E_{ele} \quad (4)$$

$$G_{solve} = G_{PB} + G_{SASA} \quad (5)$$

where $G_{complex}$, G_{guest} and G_{host} are the free energies of the complex, the guest and the CD, respectively. E_{gas} is the standard force field energy, that consists of strain energies (E_{bond}, E_{angle} and $E_{torsion}$). The solvation free energy (G_{solve}) is further divided into a polar component (GPB) and a nonpolar one (GSASA). The polar component was calculated by using the PBSA program in AMBER 9.0 [14]. In this work the dielectric constant was set to 1 inside the solute, and to 80 in the solvent. The nonpolar component was determined by $\Delta G_{nonpol} = \gamma SASA + \beta$, in which SASA is the solvent-accessible surface area determined with MOLSURF [22]. In our calculations, the values for γ and β were set to 0.0072 kcal/mol Å^2 and 0 kcal/mol, respectively. The contributions of entropy (TS) to binding free energies via normal mode analyses are not evaluated, since they usually have large error bars and require long simulation times. In order to compare the binding free energies of the complexes, the 30% of the minimum calculated binding free energies for all the trajectories of MD simulation were considered, and the means ± SD were calculated.

3.2. Statistical analysis

The results are expressed as mean ± SD of n separate experiments. The stability constant values obtained by phase solubility studies were compared by one-way ANOVA, followed by Tukey multiple comparison test. Values of $P < 0.05$ were considered ignificant. Calculations were performed using the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA; www.graphpad.com).

4. Results and discussion

4.1. Phase solubility studies

The results of phase solubility studies showed that, in all cases, the BVP HCl solubility linearly increased as a function of the cyclodextrin concentration, showing an A_L type diagram according to the classification of Higuchi and Connors (Figure 1) [11]. This suggested the formation of soluble inclusion complexes with 1:1 drug/CD molar ratio, irrespective of the type of cyclodextrin examined. The stability constants calculated for parent α-, β- and γ-CD were 19.62 ± 2.55, 21.02 ± 5.14 and 20.63 ± 6.46 M^{-1}, respectively. The absence of a significant difference among these values ($P > 0.05$), suggested that the CD cavity size does not seem to be an important factor in determining the CD effectiveness as carrier for BVP HCl. Moreover, the low value of stability constants indicated poor suitability of parent CDs as complexing agents for the drug. In case of β-CD derivatives, K_s values were growing in order HP-β-CD ≤ RAMEB < EPI-β-CD < SBE-β-CD (35.06 ± 5.43, 40.12 ± 5.35, 82.68 ± 11.61 and 114.38 ± 9.74 M^{-1}, respectively), clearly indicating that affinity of BVP HCl for complexation with cyclodextrins was positively influenced by the presence of substituents on the β-CD core ($P < 0.01$).

As for the BVP HCl complexes with γ-CD and HP-γ-CD, the higher K_s value observed with the hydroxypropylated derivative (K_s = 43.91 ± 3.84 M^{-1}) compared to the parent γ-CD ($P < 0.05$) confirmed the favourable influence of the presence of substituents on the cyclodextrin complexation power.

4.2. One-dimensional ^{1}H NMR studies

The increased drug solubility in the presence of cyclodextrin demonstrated by phase solubility analysis cannot be considered as an ultimate proof of the actual inclusion complex formation, therefore ^{1}H NMR studies were performed. This technique provides the clear answer about the type of complex formed (inclusion complex or adsorbate) and it is capable to differentiate the part of the guest molecule involved in the interaction with the cyclodextrin cavity [23]. The binary systems of BVP HCl with EPI-β-CD and SBE-β-CD were selected for the NMR studies, since phase solubility studies demonstrated the highest affinity of the drug for interaction with these cyclodextrins. The binary system with β-CD was taken as a reference. Since phase solubility studies have indicated the formation of complexes in equimolar drug:cyclodextrin ratio, binary systems for ^{1}H NMR studies were prepared in such ratio. The assignments of ^{1}H chemical shifts of BVP HCl, β-CD, EPI-β-CD and SBE-β-CD were done according to the related literature [24–27]. In ^{1}H NMR spectra of all binary systems investigated, the signals of BVP HCl were well separated from those of the cyclodextrins tested. No new peaks were observed when the complexes were formed indicating that the guest molecule was in rapid exchange between the free and the complexed states [28]. The proton labels for BVP HCl and cyclodextrins studied are presented in Figure 2.

β-CD exhibited a well resolved ^{1}H NMR spectrum, and its assignments are presented in Table 1. The internal β-CD protons (H3 and H5) experienced a shielding effect resulting in their upfield shift ($\Delta\delta < 0$), which clearly suggested the formation of an inclusion complex between BVP HCl and β-CD [29]. In particular, the shielding effect can be attributed to the diamagnetic

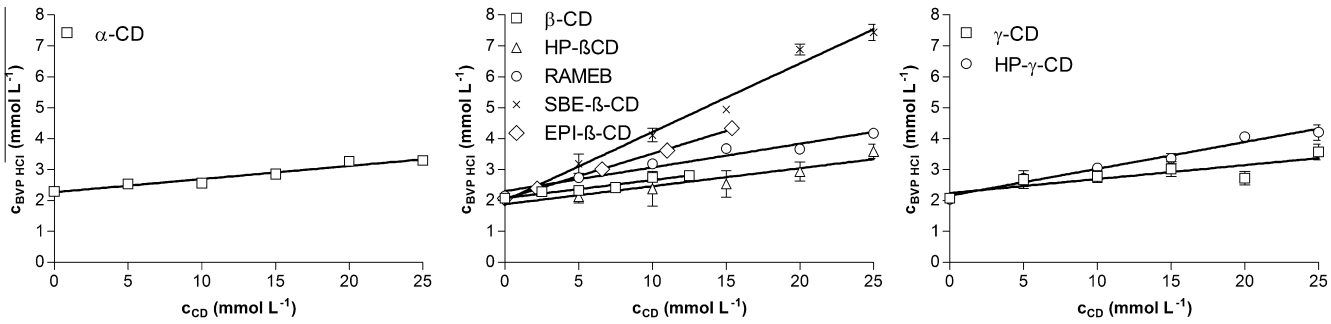

Figure 1. Phase solubility diagrams of BVP HCl with the different cyclodextrins tested in 0.1 M phosphate buffer solution (pH 8.32) at 25 °C (mean ± SD).

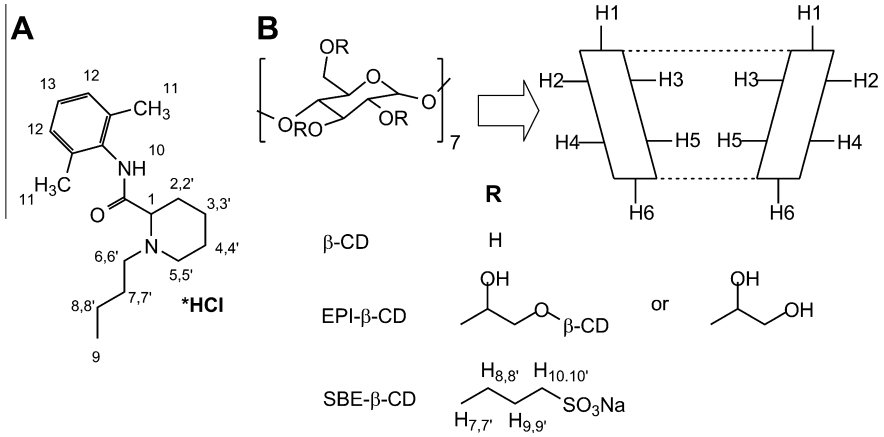

Figure 2. Structures and proton naming of drug (A) and cyclodextrins (B) investigated by the ^{1}H NMR study.

Table 1
^{1}H NMR chemical shifts of β-CD in free and complexed state determined in D_2O at 300 K.

Proton	δ_{free} (ppm)	$\delta_{complex}$ (ppm)	$\Delta\delta$[a] (ppm)
H1	4.999	4.997	−0.002
H2	3.591	3.592	0.001
H3	3.887	3.860	−0.027
H4	3.582	3.583	0.001
H5	3.777	3.765	−0.011
H6	3.802	3.802	0.000

[a] $\Delta\delta = \delta_{complex} - \delta_{free}$.

anisotropy effect due to inclusion of a group rich in π-electrons [30]. The only large group rich in π-electrons in the structure of BVP HCl is the methylated phenyl ring. Thus, the observed results are an indication that this drug moiety was included into the central cavity of the β-CD. Furthermore, because of the higher shielding effect on H3 proton with respect to H5 proton ($\Delta\delta$H3 > $\Delta\delta$H5), it could be hypothesized that complexation of BVP HCl with β-CD occurred by inclusion of the methylated phenyl ring of the drug molecule into β-CD cavity through the larger rim of the torus.

Of the external protons of β-CD, only H1 was slightly affected by the presence of BVP HCl, while signals corresponding to other external protons showed no or negligible change of their resonance (Table 1). Since the drug/β-CD interaction affected the resonance not only of the internal, but also of some external cyclodextrin protons (H1), it may be concluded that inclusion complex formation occurred by only partial insertion of the drug into the central cavity of β-CD, while the portion remaining outside can interact with the external protons of β-CD.

^{1}H NMR signals of some BVP HCl protons, listed in Table 2, experienced a downfield shift, which is a further indication of the inclusion complex formation.

A detailed inspection of the chemical shifts changes of the BVP HCl protons confirmed the previous assumption about the drug/cyclodextrin binding mode. The most influenced BVP HCl signals were those of H11 protons followed by those of H12 and H13 protons (Table 2). Probably, this was caused by inclusion of methylated phenyl ring into the CD cavity. The downfield shift of ^{1}H NMR signal corresponding to H9 protons of BVP HCl was less pronounced, suggesting that the butyl chain of the drug remained outside of the cyclodextrin cavity and probably was involved in interactions with external (H1) protons of β-CD.

^{1}H NMR spectra of EPI-β-CD and SBE-β-CD were not well resolved. In case of EPI-β-CD, lack of symmetry, due to the completely random polymeric structure of this cyclodextrin derivative, resulted in a ^{1}H NMR spectrum that appeared as several strong and unresolved broad peaks. The SBE-β-CD is a rather complex mixture of different stereoisomers. As a consequence, only some of ^{1}H NMR signals in the spectra of these cyclodextrin derivatives could be unambiguously identified, while others are uncertain and cannot be used for interpretation of the drug inclusion mode. Therefore, information about the inclusion complex formation between both these cyclodextrin derivatives and BVP HCl was deduced solely on the basis of chemical shifts changes of BVP HCl protons. The signals of drug protons most affected by the presence of EPI-β-CD and SBE-β-CD and their assignments are listed in Table 2.

As can be seen from the presented data, the magnitude of the drug chemical shift changes followed the line β-CD < EPI-β-CD < SBE-β-CD. The increasing chemical shift perturbation can be attributed to a higher fraction of bound drug molecules, which is consistent with the increasing stability constant values of the complexes with these cyclodextrins, obtained by phase solubility studies.

Although the obtained data did not allow exact determination of the drug/cyclodextrin binding mode, because of uncertain position of signals corresponding to the internal protons of these cyclodextrins, it seems probable that complexation occurred by inclusion of the drug into cyclodextrin cavity through the wider rim of the torus. The presence of substituents on the cyclodextrin ring resulted in interactions between them and the butyl chain of the drug molecule that additionally contributed to increase the stability of the inclusion complex formed. This may be deduced from

Table 2
The most pronounced ^{1}H NMR chemical shifts of BVP HCl in free state and in presence of cyclodextrins determined in D_2O at 300 K.

Proton	β-CD			EPI-β-CD			SBE-β-CD		
	δ_{free} (ppm)	$\delta_{complex}$ (ppm)	$\Delta\delta$[a] (ppm)	δ_{free} (ppm)	$\delta_{complex}$ (ppm)	$\Delta\delta$ (ppm)[a]	δ_{free} (ppm)	$\delta_{complex}$ (ppm)	$\Delta\delta$ (ppm)[a]
H9	0.791	0.795	0.004	0.791	0.797	0.006	0.791	0.819	0.028
H11	2.066	2.079	0.013	2.066	2.079	0.013	2.066	2.134	0.068
H12 and H13	7.088	7.094	0.006	7.088	7.097	0.009	7.088	7.113	0.025

[a] $\Delta\delta = \delta_{complex} - \delta_{free}$.

the more pronounced change in chemical shift values of drug H9 protons in presence of EPI-β-CD and SBE-β-CD, compared to β-CD (Table 2).

Moreover, the upfield shift of ^{1}H HMR signals corresponding to the sulphobutylether chains of SBE-β-CD confirmed their interaction with the drug molecule. The signal of $-CH_2-$ group attached to $—SO_3$ group (H10,10′, $\delta = 2.834$ ppm) showed the most pronounced upfield shift in the drug presence ($\Delta\delta = -0.010$ ppm), while the signal corresponding to the middle $—CH_2CH_2—$ protons (H9,9′ and H8,8′, $\delta = 1.661$ ppm) was less affected ($\Delta\delta = -0.006$ ppm). The interaction of BVP HCl with the sulphobutylether chains of SBE-β-CD is mainly attributable to electrostatic attraction between the oppositely charged molecules. This interaction additionally contributes to enhance the complex stability [25], leading to the highest increase of the drug aqueous solubility (Figure 1).

4.3. Two dimensional ^{1}H NMR studies (ROESY)

Two dimensional NMR is a powerful technique for investigation of inter- and intra-molecular interactions. The presence of nOe cross-peaks between protons of two different species in 2D ROESY spectrum is an indication that they are in spatial contact through space within 3–5 Å. Based on the results obtained from 2D ROESY spectra, the spatial conformations of inclusion complexes may be determined. Thus, to obtain more insight about the binding mode between BVP HCl and cyclodextrins tested and to confirm the proposed structure of inclusion complexes formed, 2D ROESY NMR experiments were performed.

In 2D ROESY spectrum of BVP HCl/β-CD binary system, two groups of intermolecular nOe cross-peaks were observed (Figure 3A and B): the first belongs to the interaction between H11 protons of BVP HCl and β-CD protons, the other to the interactions between H12 and 13 and β-CD protons. In both cases, interaction with internal and external β-CD protons was observed. One has to be aware that an inclusion complex is characterized by existence of a dynamic equilibrium between the complexed and free drug forms. This equilibrium is extremely fast, even on a NMR-timescale. Thus, it is possible to detect nOe cross-peaks that are the consequence of inclusion complex formation, as well as those that corresponded to non-inclusion interactions between free drug and cyclodextrin molecule in the solution. Moreover, cross-peaks observed between BVP HCl protons and internal β-CD protons (H3 and H5) were always more intense than those of external β-CD protons (H2 and H4), which may be taken as an indication that the inclusion complex formation was the prevalent interaction between the components. These results also confirmed the assumed structure of the complex formed. The nOe cross-peak corresponding to the interaction of the drug H9 protons and β-CD protons was not observed, indicating that these protons are in greater distance, probably as a consequence of the geometry of the inclusion complex formed.

The 2D ROESY spectrum of BVP/EPI-β-CD binary system showed a strong intramolecular nOe cross-peak that may be attributed to the interaction of H1 proton with other protons of CD core (Figure 3C). The same interaction was not observed in 2D ROESY spectrum of parent β-CD. This result suggested that chemical modification of β-CD has resulted in a distortion of the cone structure. As a consequence, the binding mode between BVP HCl and EPI-β-CD has been changed. Cross-peaks corresponding to the interaction of cyclodextrin protons with protons H11 and H12,13 of the drug molecule were observed ($\delta = 1.179$–1.297 ppm and 3.031–3.3133 ppm, Figure 3D), but their intensity was reduced in comparison with those of β-CD. Also, strong nOe cross-peaks corresponding to the interaction between protons of piperidine ring of BVP HCl and EPI-β-CD protons were observed (Figure 3E). This interaction was absent in ROESY spectra of the sample with β-CD. Probably, the distortion of the CD cone caused by chemical modification in case of EPI-β-CD allowed deeper penetration of the drug into the central cavity of this cyclodextrin derivate. This directly contributed to the formation of a more stabile inclusion complex, as observed by phase solubility studies.

The nOe cross-peaks observed in 2D ROESY spectra of BVP/SBE-β-CD binary system belonged to SBE-β-CD intramolecular interactions (Figure 3F and G) and to interaction between cyclodextrin and BVP HCl (Figure 3G and H) The obtained results were a basis for elucidation of the mechanism that contribute to the stability of the BVP HCl/SBE-β-CD inclusion complex.

As in case of EPI-β-CD, chemical modification of SBE-β-CD resulted in a distortion of the cone structure with respect to native β-CD, allowing intramolecular interactions between H1 and other protons of the cone (Figure 3F). Other intramolecular nOe cross peaks were attributed to the interaction between protons of sulphobutylether sidearms and protons of the cone (Figure 3G). In fact, sulphobutylether sidearms are capable of free rotation that allowed their interaction with some protons of the cone, resulting in strong nOe intramolecular cross-peaks. The interaction seems to be more intense for middle $—CH_2—CH_2—$ protons of sulphobutylether sidearms of SBE-β-CD than for $—CH_2—$ group directly bonded to the $—SO_3$ group. The observed structural change of SBE-β-CD with respect to parent β-CD seems to be more favourable for the complexation of BVP HCl. Pronounced intermolecular nOe cross-peaks indicated a strong interaction between cyclodextrin cone protons and H11 and H12,13 protons of drug (Figure 3G and H). This confirmed that a complex between BVP HCl and SBE-β-CD has been formed by inclusion of the methylated phenyl group of BVP HCl into the central cyclodextrin cavity. The extension of its lipophilic cavity caused by the presence of sulphobutylether sidearms on the β-CD core contributed to more intense interactions between the drug and SBE-β-CD, resulting in formation of the most stable inclusion complex, as observed by phase solubility studies.

4.4. Molecular modeling studies

2D-ROESY spectra demonstrated several intermolecular cross-peaks related to the nOe interactions between the internal protons of the cyclodextrins (H3 and H5) and the aromatic and methyl protons of BVP HCl. Since the nOe cross-peak's intensity values are inversely proportional to the sixth power of the distance between the protons ($nOe \propto 1/r^6$), they can be correlated with the lowest measurable distance between interacting protons. By using a known distance as a reference (in this case that between the aliphatic protons H-8,8′ and H6,6′ of BVP HCl), it is possible to calculate the distances between interacting protons from the corresponding cross-peaks intensity values according to the following equation:

$$r_{i,j} = \sqrt[6]{\frac{\eta_{\mathrm{ref}}}{\eta_{i,j}} \times r_{ref}^6} \tag{6}$$

where $\eta_{i,j}$ and $r_{i,j}$ represent the nOe cross-peak intensity and the inter-proton distance of two interacting protons i and j, respectively, while η_{ref} and r_{ref} are the corresponding values of the reference protons. The mean values of the calculated distances for the BVP HCl complexes with β-CD and SBE-β-CD are presented in Table 3. These data were taken as a basis for the molecular dynamic calculations, in order to hypothesize the most probable conformations of the inclusion complexes formed.

Since BVP molecule has a chiral centre, 4 possible inclusion modes have been considered in case of β-CD complex. In particular, for each enantiomer, S and R, two different orientation modes into the β-CD cavity have been considered, i.e. one with the aromatic portion inserted from the secondary rim (A type complex) and another from the primary rim (B type complex) of the cone.

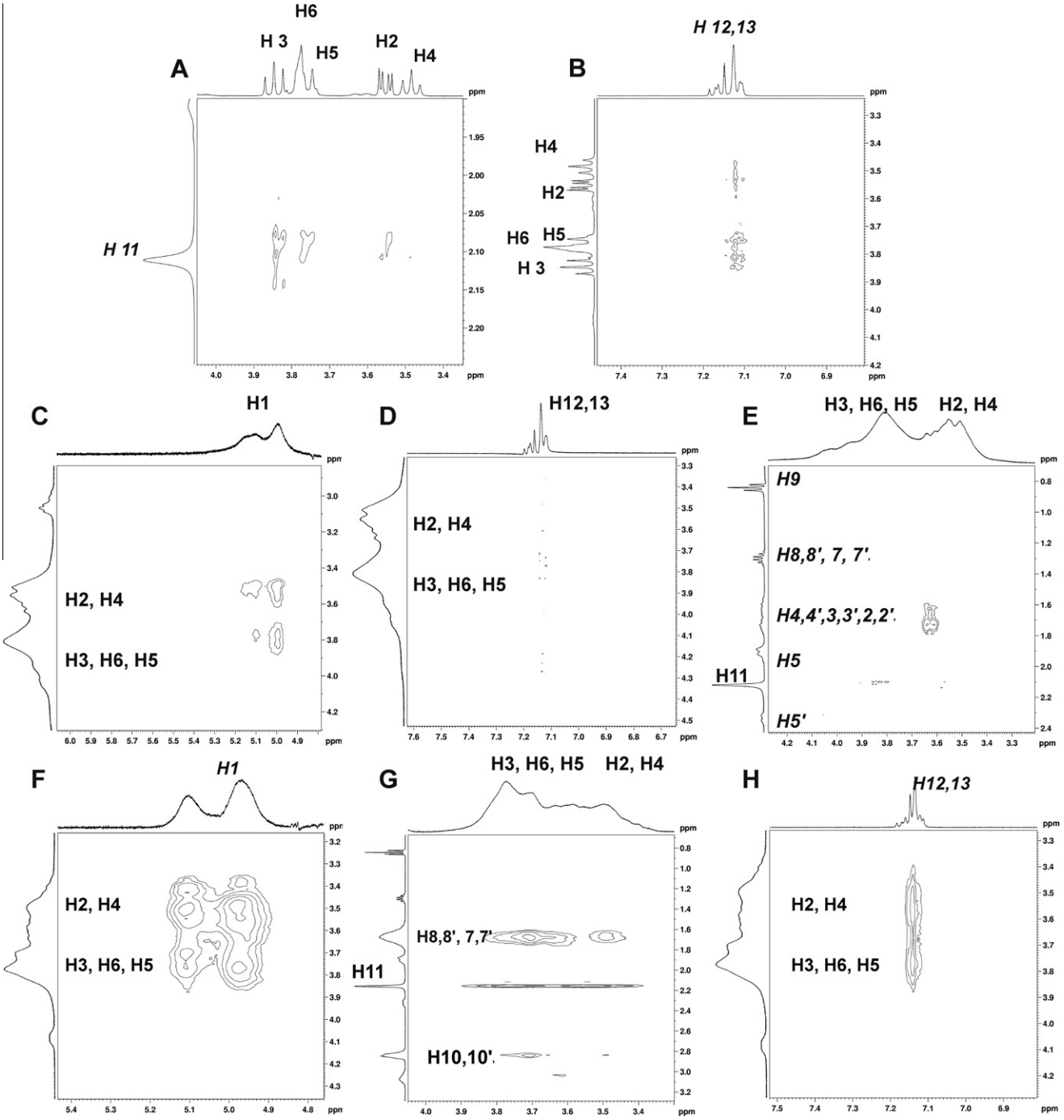

Figure 3. Partial contour plots of 2D ROESY spectrum of BVP HCl in presence of β-CD (A and B), EPI-β-CD (C, D, and E) and SBE-β-CD (F, G, and H) in D_2O at 300 K. The signals corresponding to the BVP HCl are presented as italic.

Table 3
The nOe cross-peak intensity values and calculated distances for interacting protons of BVP HCl and cyclodextrin tested.

Protons	Cross-peak intensity		Distance (Å)	
	β-CD/BVP	SBE-β-CD/BVP	β-CD/BVP	SBE-β-CD/BVP
H11–H3 CD	472320	1090240	2.5 ± 0.2	2.2 ± 0.2
H11–H5 CD	462272	1093248	2.5 ± 0.2	2.2 ± 0.2
H12/13–H3 CD	462208	1070976	2.5 ± 0.2	2.2 ± 0.2
H12/13–H5CD	476160	1095808	2.5 ± 0.2	2.2 ± 0.2
H8–H6 (ref)	507456	469024	2.5 ± 0.2	2.5 ± 0.2

MD studies allowed determination of the most probable conformations for each complex type, by comparison of the distances calculated with those experimentally measured. The distance error (e_{ij}) was defined as the difference between the proton distance calculated from MD (d_{ij}) and that measured from nOe experiments (r_{ij}). The number of conformations represented as a percentage of all conformations generated during MD studies, for both complex types between β-CD and the BVP HCl enantiomers *versus* the maximum distance error in Å (for an error range of 0.2 Å), are presented in Figure 4.

Molecular modelling of BVP HCl complexes with SBE-β-CD was further complicated by the random substitution of this cyclodex-

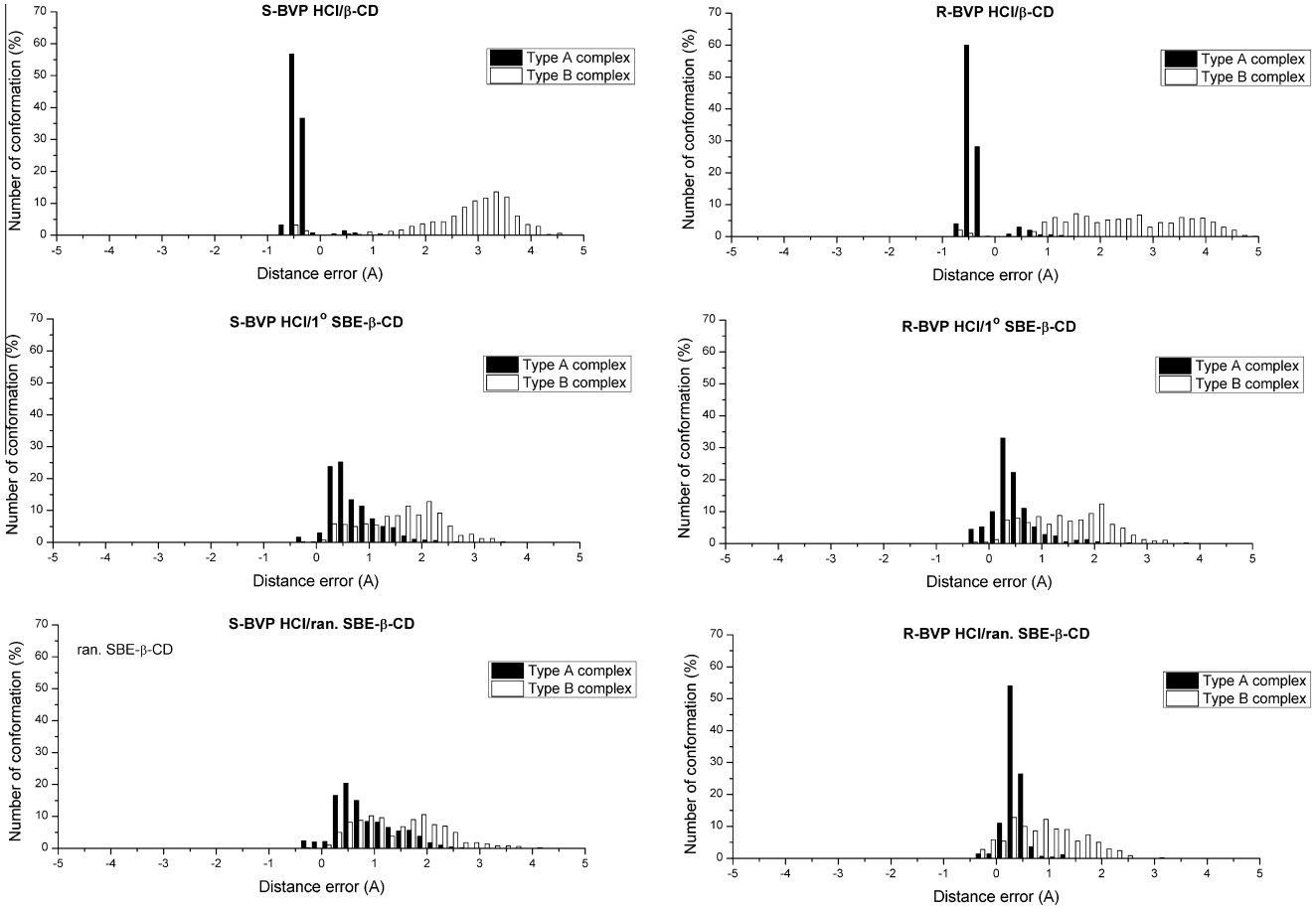

Figure 4. Conformational population distributions *versus* the maximum distance error in Å (for an error range of 0.2 Å), for the R- and S-BVP complexes with β-CD and SBE-β-CD with all the primary OH substituted (1° SBE-β-CD) or randomly substituted (ran. SBE-β-CD). The black and gray bars represent the distribution for the A and B type insertion modes of drug into the CD cavity, respectively.

trin derivate (D.S. 6.4). Two different patterns of substituent distribution have been hypothesized while building the SBE-β-CD structure: the first one by replacing all the primary OH groups and the second one by randomly replacing 4 primary and 3 secondary OH groups. Due to the presence of R and S enantiomers of BVP HCl and the two possible modes of inclusion inside the CD cavity, 8 different complexes were then designed and subjected to MD simulation. The conformational population distribution obtained by MD studies for each complex type between SBE-β-CD and the BVP HCl enantiomers *versus* the maximum distance error in Å (for an error range of 0.2 Å), are also presented in Figure 4.

As it could be seen from the presented data, in the case of β-CD, the very similar results obtained for R and S drug enantiomers indicated the absence of chiral selectivity in the complexation process. Moreover, the probability of the A type complex formation was always clearly higher compared to the B type. This indicated that the inclusion of the methylated phenyl ring of BVP HCl into β-CD molecule occurs via the wider (secondary) rim of the cone, as shown in Figure 5, thus confirming the conclusion made on the basis of one-dimensional ^{1}H NMR studies.

The data obtained in the case of complexes with SBE-β-CD indicated that inclusion of the methylated phenyl ring of BVP HCl via the wider (secondary) rim of the cone is still the dominant binding mode (Figure 5). However, there is an increased probability of formation of the B-type of the complex, i.e. inclusion of the drug via the primary rime of the cone. The difference in the drug inclusion mode observed between β-CD and SBE-β-CD may be related to cone distortion upon chemical modification, as suggested earlier on the basis of NMR results. Furthermore, the substitution pattern of the cyclodextrin molecule had a great impact on the complex formation probability. It was always higher in case of randomly substituted SBE-β-CD, which can be considered as a more reliable model with respect to that substituted only on the primary rim. In fact, as it could be seen in Figure 5, the obtained structure in case of randomly substituted SBE-β-CD was more consistent with NMR data, which suggested the interaction of the BVP HCl butyl chain and the cyclodextrin sulphobutylether sidearms. Additionally, differently from the case of β-CD, there was always a significantly higher probability of inclusion complex formation with R-enantiomer of the drug, irrespective on the substitution pattern of the SBE-β-CD (Figure 4). These data may be considered as an indication of some stereoselective binding between R-BVP HCl and SBE-β-CD, which is in agreement with results of Salama [31], who demonstrated that β-CD derivatives are useful chiral selectors which enabled stereoselective TLC analysis of BVP HCl. This corroborated the validity of our approach, which, for the first time, may allow exact quantification of the most probable conformations for each complex studied.

MD simulations allowed calculation of the binding free energy of the drug complexes with βCD and SBEβCD, this last in the random distribution, which can be considered as the most reliable model. The binding free energy values, obtained for complexes of R and S enantiomer of BVP HCl with βCD were -20.0 ± 0.8 and -20.3 ± 1.0 kcal/mol, respectively, while in case of complexes with SBEβCD values of -22.8 ± 1.1 and -20.7 ± 1.3 kcal/mol were obtained. These results confirmed both the trend obtained from

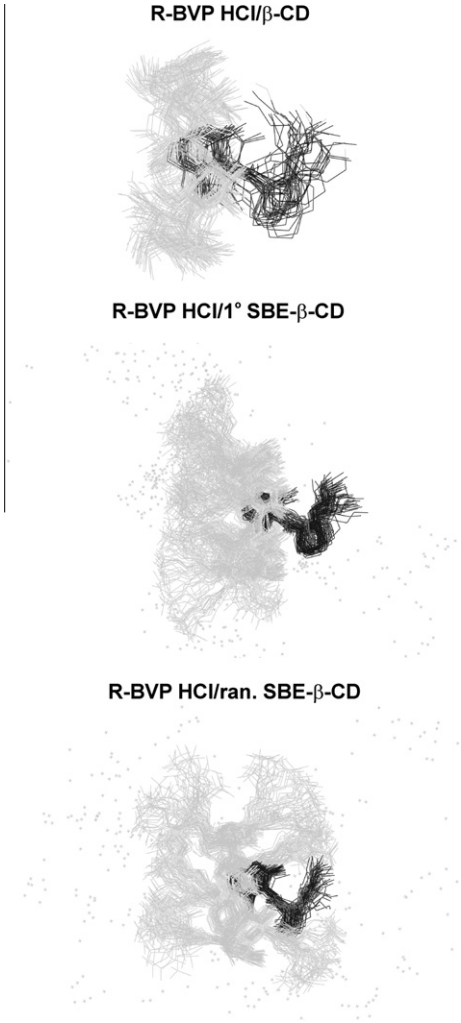

Figure 5. A group of conformations, within a distance error of 0.3 Å obtained by MD simulation studies on the basis of ROESY data, of the inclusion complex between R-BVP HCl (black) and β-CD, SBE-β-CD with all the primary OH substituted (1° SBE-β-CD) or randomly substituted (ran. SBE-β-CD). The gray dots represent the Na^+ counter ions.

phase solubility studies, supporting the higher stability of the BVP HCl complex with SBEβCD than with the parent βCD, and the preferential interaction of SBEβCD with the R chiral form of the drug.

5. Conclusion

Among the different tested cyclodextrins, EPI-β-CD and SBE-β-CD were found to be the best complexing and solubilizing agents for BVP HCl. Molecular modelling studies, carried out on the base of ^{1}H NMR and ROESY data, allowed the construction of reliable three-dimensional models of the complexes formed, and provided further insight about the drug inclusion modes into the cyclodextrin cavity. The binding free energy, the most probable conformations and their relative population have been determined for each complex. The inclusion of methylated phenyl ring of BVP HCl was the dominant binding mode, while the presence and type of substituents on β-CD core had an important impact in determining the stability and aqueous solubility of complexes formed. Furthermore, some stereoselective binding of SBE-β-CD towards BVP HCl was also evidenced.

6. Supporting information

The radial distribution functions (RDFs) for different BVP HCL/CD proton pairs as well as distribution of conformational population, with the nearest distances from guest – host protons measured during the MD simulation, respect to the distance calculated from the NOE cross-peak in the ROESY experiments, for the same pairs of protons, are presented in Supporting Information section.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cplett.2010.10.046.

References

[1] S. Leone, S. Di Cianni, A. Casati, G. Fanelli, Acta Biomed. 79 (2008) 92.
[2] R. Gristwood, Drug Safety 25 (2002) 153.
[3] F. Hirayama, K. Uekama, Adv. Drug Del. Rev. 36 (1999) 125.
[4] J.C. Fréville, G. Dollo, P. Le Corre, F. Chevanne, R. Le Verge, Pharm. Res. 13 (1996) 1576.
[5] G. Dollo, P. Le Corre, J.C. Freville, F. Chevanne, R. Le Verge, Ann. Pharm. Fr. 58 (2000) 425.
[6] D.R. de Araujo, S.S. Tsuneda, C.M.S. Cereda, F.G.F. Del Carvalho, P.S.C. Preté, S.A. Fernandes, F. Yokaichiya, M.K.K.D. Franco, I. Mazzaro, L.F. Fraceto, A.F.A. de Braga, E. de Paula, Eur. J. Pharm. Sci. 33 (2008) 60.
[7] P. Mura, S. Furlanetto, M. Cirri, F. Maestrelli, G. Corti, S. Pinzauti, J. Pharm. Biomed. Anal. 37 (2005) 987.
[8] M. Cirri, F. Maestrelli, G. Corti, S. Furlanetto, P. Mura, J. Pharm. Biomed. Anal. 42 (2006) 126.
[9] F. Maestrelli, M. Cecchi, M. Cirri, G. Capasso, N. Mennini, P. Mura, J. Incl. Phenom. Macrocycl. Chem. 63 (2009) 17.
[10] P. Cairo, F. Ortusi, S. Alcaro, E. Fontananova, E. Tocci, E. Drioli, Chem. Phys. Lett. 454 (2008) 374.
[11] T. Higuchi, K. Connors, Adv. Anal. Chem. Instrum. 7 (1965) 117.
[12] G. Zheng, W.S. Price, Prog. Nucl. Magn. Reson. Spectrosc. 56 (2010) 267.
[13] L.B. Luo, Y. Chen, H.L. Chen, Z.Y. Zhang, Z.Y. Zhou, T.C.W. Mak, Inorg. Chem. 37 (1998) 6147.
[14] D.A. Case et al., University of California, San Francisco, 2006.
[15] Y. Duan, C. Wu, S. Chowdhury, M.C. Lee, G. Xiong, W. Zhang, R. Yang, P. Cieplak, R. Luo, T. Lee, J. Comput. Chem. 24 (2003) 1999.
[16] J. Wang, R.M. Wolf, J.W. Caldwell, P.A. Kollamn, D.A. Case, J. Comput. Chem. 25 (2004) 1157.
[17] A. Jakalian, B.L. Bush, D.B. Jack, C.I. Bayly, J. Comput. Chem. 21 (2000) 132.
[18] A. Jakalian, D.B. Jack, C.I. Bayly, J. Comput. Chem. 23 (2002) 1623.
[19] W.L. Jorgensen, J. Chandrasekhar, J. Madura, M.L. Klein, J. Chem. Phys. 79 (1983) 926.
[20] J.P. Ryckaert, G. Ciccotti, H.J.C. Berendsen, J. Comput. Phys. 23 (1977) 327.
[21] S. Miyamoto, P.A. Kollman, J. Comput. Chem. 13 (1992) 952.
[22] M.L. Connolly, J. Appl. Cryst. 16 (1983) 548.
[23] L. Ribeiro, R.A. Carvalho, D.C. Ferreira, F.J.B. Veiga, Eur. J. Pharm. Sci. 24 (2005) 1.
[24] L.F. Fraceto, A. Sposni, S. Schreier, E. de Paula, Biophys. Chem. 115 (2005) 11.
[25] S. Astilean, C. Ionescu, G.H. Cristea, S.I. Farcas, I. Bratu, R. Vitoc, Inc. Biospect. 3 (1997) 233.
[26] J. Li, H. Xiao, J. Li, Y.P. Zhong, Int. J. Pharm. 278 (2004) 329.
[27] Q. Qu, E. Tucker, S.D. Christian, J. Incl. Phen. Macr. Chem. 43 (2002) 213.
[28] Y. Zheng, I.S. Haworth, Z. Zuo, M.S.S. Chow, A.H.L. Chow, J. Pharm. Sci. 94 (2005) 1089.
[29] H.J. Schneider, F. Hacket, V. Rüdinger, H. Ikeda, Chem. Rev. 98 (1998) 1755.
[30] F. Djedaine, S. Lin, B. Perly, D. Wouessidjewe, J. Pharm. Sci. 79 (1990) 643.
[31] N.N.E.D.A. Salama, J. Planar. Chromatogr. 21 (2008) 41.