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Supramolecular aggregates of oligosaccharides with co-solvents in ternary systems for the solubilizing approach of triamcinolone

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ABSTRACT

A second compound is generally associated with oligosaccharides as a strategy to maximize the solubilizing effect for nonpolar compounds. This study elucidated the role and the mechanism whereby liquid compounds interact in these supramolecular aggregates in the solubilization of triamcinolone. Three different oligosaccharides (beta-cyclodextrin, 2-hydroxipropil-beta-cyclodextrin, and randomly methylated beta-cyclodextrin) and two potent co-solvents (triethanolamine and *N*-methyl pyrrolidone) were carefully evaluated by using three distinct experimental approaches. Incredibly stable complexes were formed with cyclodextrins (CDs). The structure of the complexes was elucidated by magnetic resonance spectra 2D-ROESY. The interactions of the protons of ring "A" of the drug with H^3 and H^5 protons of the CD cavity observed in the binary complexes remained in both ternary complexes. Unlike the observed ternary associations with triethanolamine, *N*-methyl pyrrolidone competed with the triamcinolone CD cavity and considerably decreased the stability of the complex and the solubility of the drug. The molecular dynamics (MD) and quantum mechanics:molecular mechanics (QM:MM) calculations supported that triethanolamine stabilized the drug-CD interactions for the conformer identified in the 2D-ROESY experiments, improving the quality and uniformity of the formed complex. The role played by the co-solvent in the ternary complexes depends on its specific ability to interact with the CD cavity in the presence of the drug, which can be predicted in theoretical studies to select the best candidate.

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1. Introduction

Triamcinolone (TRI) is an anti-inflammatory corticosteroid with broadly proven glucocorticoid activity. It has the drawback of low water solubility, approximately 120.93 μg/mL for triamcinolone and 26 μg/mL for triamcinolone acetonide, determined in phosphate buffered saline (Thakur, Kadam, & Kompella, 2011). This low drug solubility has limited its application and has led to its use in formulations that need invasive procedures such as intraocular, intra-articular, or intramuscular suspensions (Chen, Li, Liu, Han, & Cheng, 2015; Konai, Vilar Furtado, Dos Santos, & Natour, 2009). Besides the discomfort, these parenteral administrations can cause some side effects, such as joint sepsis (Marsland, Mumith, & Barlow, 2014), vitreous hemorrhage, or intraocular infections (Chen et al., 2015).

For TRI solubilization, several agents can be indicated to have the potential to increase its apparent aqueous solubility. A co-solvent is one of the most used agents and, as examples, polyethylene glycols and alcohols can be cited (Matsuda et al., 2011). The *N*-

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methyl pyrrolidinone (NMP) is also a co-solvent and has been investigated in the solubilization of several compounds. In a recent study, the solubility of a similar corticosteroid drug, testosterone, was enhanced about 14-fold by using NMP (Sanghvi, Narazaki, Machatha, & Yalkowsky, 2008). Triethanolamine was used as an antichagasic benznidazole solubilizer (de Melo et al., 2013) and the authors also identified the co-solvent mechanism, showing the potential co-solvent activity for further studies with nonpolar drugs.

Uncountable formulations have also applied oligosaccharides to increase the aqueous solubility or physical-chemical stability of many therapeutic agents including corticosteroids (Bary, 2000; Vianna et al., 1998). The molecule of cyclodextrins (CD) allows the formation of water-soluble complexes with several solutes by weak interactions (hydrogen bond or Van der Waals interactions). Its cyclic structure with a nonpolar cavity (carbon and ether chains) and a polar exterior (hydroxyl groups) usually allows the nonpolar solute to be included in the CD cavity (inclusion complex), a less energetic environment for the molecule in an aqueous media (Kurkov & Loftsson, 2013).

Chemically-modified α, β, and γ-CDs from their natural form (obtained from *Bacillus macerans* bacteria) have been developed, and they bring even more solubilizing power (Kurkov & Loftsson, 2013). In fact, the importance of CDs has created continuous interest in improving their effectiveness, which can be seen with the CD derivatives and the multicomponent complex concept. A drug:CD complex that can be optimized with the addition of a second solubilizing compound can be called a multicomponent or ternary complex. In these cases, a synergic effect is desired to use low concentrations of the non-drug compounds. However, the addition of a third compound modifies the forming and breaking balance of the drug:CD complex. Liquid compounds can stabilize the drug-CD interaction, compete with the drug by the CD cavity or simply act by cosolvency without any synergism with the CD, as previously demonstrated by our group (de Melo et al., 2013). The use of triethanolamine (TEA), for example, is extensively reported with ternary complexes with CDs and drugs (Palma et al., 2009; Sami, Philip, & Pathak, 2010) and additionally allowed the enhancement of drug bioavailability by the solubilizing effect of inclusion complexes.

The aim of this study was to elucidate the role and the mechanism whereby liquid compounds interact in inclusion complexes with CDs for solubilization of a widely used anti-inflammatory corticosteroid drug. A new raw material containing a binary or ternary complex, able to provide water-soluble TRI, allows its incorporation in pharmaceuticals such as eye drops, lotions, solid and liquid oral dosage forms, as well as drug delivery systems. In this study, we investigated the TRI solubilization by using several strategies, including three different and most commonly used CDs (beta-cyclodextrin [βCD], 2-hydropropil beta-cyclodextrin [HPβCD], and randomly methylated beta-cyclodextrin [RMβCD]; and two co-solvents (triethanolamine and *N*-methyl pyrrolidinone). The phenomena involved in a ternary association approach applied to study CDs:co-solvent interactions in the supramolecular aggregates were carefully explained by using experimental spectroscopy studies and distinct computational simulations.

2. Experimental

2.1. Materials

Triamcinolone, 11beta,16alpha,17alpha,21-Tetrahydroxy-9alpha-fluoro-1,4-pregnadiene-3,20-dione, was purchased from DEG Roche (Brazil); beta-cyclodextrin, 2,3,6-hydroxipropil-beta-cyclodextrin (DS = 5.6/MS = 0.8) and 2,3,6-randomly-methylated-beta-cyclodextrin (DS = 11.9/MS = 1.7) were purchased from Trappsol (USA); and Triethanolamine and *N*-methyl pyrrolidinone were purchased from Synth (Brazil). All other reagents were analytical grade. The purified water (1.3 μS) was prepared from a reverse osmosis purification apparatus, OS50 LX model, Gehaka (Brazil).

2.2. Phase solubility diagrams: co-solvent effect

The solubility diagrams were made to obtain the saturated solutions of TRI in the presence of co-solvents. The experiments were conducted according to the method established by Higuchi and Connors (Reilley, 1965), in which an excess of TRI was added to flasks containing solutions of TEA (neutralized with acetic acid) (0; 0.191; 0.386; 0.597; 0.816; 1.052; 1.329 M) or NMP (0; 0.251; 0.502; 0.753; 1.00; 1.507; 2.010 M). All flasks were hermetically closed and placed in a thermostatic bath at 25.0 ± 0.5 °C for 72 h, during which the flasks were shaken for 15 min in an ultrasonic bath every 12 h. Then, the different solutions were filtered through 0.45 μm membranes of cellulose acetate (Sartorius® Biolab Products, Germany). The pH of each solution was measured and the TRI concentration analytically determined using the UV–vis spectrophotometric assay with a previously validated method (Aquino et al., 2011). The experimental results were expressed as the mean values of three replicates (n = 3).

The drug solubilization was expressed by a mathematical treatment (log-linear model) to demonstrate the co-solvent effect of TEA and NMP in contrast with other solubilizing mechanisms as a complex formation (details in Section 3.1). Eq. (1) expresses this model:

$$\log S_{mix} = \log S_w + \sigma f \quad (1)$$

Where S_{mix} and S_w represent the drug solubility in water/co-solvent mixture and pure water, respectively. The σ and f represent the co-solvent power of the studied solute and the percentage fraction of co-solvent in aqueous mixture (v/v), respectively.

2.3. Phase solubility diagrams: binary association of TRI with βCD, HPβCD, and RMβCD

An excess of TRI was added to flasks containing 3 mL of different CDs at concentration ranges of βCD (0; 2.5; 5; 7.5; 10; 12.5; 15 mM), HPβCD, or RMβCD (0; 0.020; 0.040; 0.070; 0.090; 0.116; 0.140 M). After 72 h of incubation, the samples were filtered and the drug content was measured (with three replicates for all analyses).

The apparent stability constant (K_s) was estimated from the plot of drug solubilization versus CD concentration. A linear relationship with a slope less than one indicates a complex with 1:1 drug:CD stoichiometry. Thus, with a linear plot, the K_s can be calculated by Eq. (2) with a 1:1 complex stoichiometry (Brewster & Loftsson, 2007; Cevher et al., 2014).

$$K_s = slope/S_0(1 - slope) \quad (2)$$

Where K_s represents the stability constant of the complex, S_0 is the solubility of the drug in water without CD presence, and the slope represents the angular coefficient of linear regression.

2.4. Phase solubility diagrams: effect of co-solvents on TRI:CD complexation

The TRI solubility was measured as a function of 3 mL of CD solutions at concentration ranges of βCD (0; 2.5; 5; 7.5; 10; 12.5; 15 mM), or HPβCD, or RMβCD (0; 0.020; 0.040; 0.070; 0.090; 0.116; 0.140 M). The CD samples were associated with fixed concentra-

tions of co-solvents, such as TEA (0.820 M) neutralized with acetic acid (0.800 M) or NMP (1.000 M).

2.5. Job's plot method

The complex stoichiometry between TRI and the studied CDs was assessed by Job's method of continuous variation (Job, 1928). Solutions of TRI, βCD, HPβCD, and RMβCD were prepared at 0.05 mM, and the UV/Vis spectra were obtained from different solutions containing the same total molar content (TRI + each CD), but varying the ratio between them. The difference in absorbance at 242 nm between the solutions with and without CDs was plotted (Δabs X [TRI]) in function of *R* (*R* = [TRI]/[TRI] + [CDs]). Each absorbance spectra was expressed as the mean of three replicates. The same assay was performed for the ternary complexes, in which the CD solutions were prepared and mixed with TEA (0.820 M), neutralized with acetic acid (0.800 M), or mixed with NMP (1.000 M).

2.6. ^{1}H NMR and 2D-ROESY spectra

The ^{1}H NMR and 2D-ROESY spectra were recorded for the solubilizing compounds, TRI and their respective binary and ternary complexes. The pure compounds (βCD, HPβCD, RMβCD, TEA, and NMP) and their associations were weighed and diluted in 0.6 mL of deuterium oxide (D_2O) to obtain a final concentration of 1.66 mg/mL. TRI solution was prepared by weighing 1 mg of the drug and suspending in 1 mL of D_2O in ultrasonic bath for 2 h. The non-soluble drug was then removed by filtering with 0.45 μm membrane of cellulose acetate. The associations were prepared to obtain a drug:CD proportion of 1:1 molar. For ternary complexes, the solutions were prepared to contain 0.13 mg/mL of the co-solvents TEA and NMP, using the equimolar content of acetic acid to neutralize TEA. Each solution was transferred to 5 mm flasks and hermetically sealed. The ^{1}H NMR and 2D-ROESY sample spectra were obtained on a Bruker Advance spectrometer of 400.16 MHz with a broad-band inverse probe (BBI) and a variable temperature unit (VTU), at 298 K temperature. The experimental comparisons were made using the mixing time of 350 ms. The chemical shifts (δ) were expressed as ppm and the residual signal of the solvent was used as an internal reference. The δ values of TRI and CDs were identified in free or non-complexed and complexed samples. The δ changes induced by complex formation were calculated by Eq. (3):

$$\Delta\delta = \delta\text{free} - \delta\text{complex} \quad (3)$$

2.7. Molecular dynamics simulation

The interactions between triamcinolone with βCD and co-solvents (TEA and NMP) in binary and ternary complexes were simulated and evaluated by molecular dynamic simulations (MD) in GROMACS 4.5 (Pronk et al., 2013). All simulations were performed at a constant temperature of 298 K and pressure at 1 bar by rescaling velocities and using an isotropic pressure bath with the Parrinello–Rahman barostat in a periodic truncated triclinic box. The explicit TYP3 P water model was used to simulate an aqueous environment. Simulations that contained TEA and NMP were made with the same molar concentration of the experimental data including acetate for systems that contained TEA. The TRI molecular model was positioned outside the CD binding cavity and permitted to meet freely and bind to the CDs. Prior to MD simulations, the geometries were optimized with energy minimization using the steepest descent algorithm, followed by conjugated gradient minimization. The molecular topologies were created using Automated Topology Builder (ATB) and Repository version 2.0 (Koziara, Stroet, Malde, & Mark, 2014), employing the GROMOS 54a7 force field (Schmid et al., 2011). Covalent bonds with hydrogen atoms were constrained using the LINCS algorithm. A 100-ps protein position-restrained molecular dynamics simulation was performed to gently relax the water molecules. Unrestrained NVT and NPT molecular dynamics simulations were performed for at least 5 ns to assess the stabilization of the box density. A productive simulation was then performed for 200-ns or until the stabilization of the interaction energies.

The MD simulation results were evaluated by cluster conformation into the most relevant during the simulation analysis according to the root mean square deviation method of the atom positions. This task was performed by the use of the UCSF Chimera MD analysis module (Huang, Meng, Morris, Pettersen, & Ferrin, 2014).

2.8. Ab initio and QM:MM interaction energies calculation

The most relevant clusters from the molecular dynamics calculations were selected for energy minimizations by the quantum mechanics:molecular mechanics technique. QM:MM optimization was performed within the multilayered ONIOM (our own N-layer integrated molecular orbital and molecular mechanics) framework (Dapprich, Komáromi, Byun, Morokuma, & Frisch, 1999) implemented into the program package Gaussian09.

Developed by Morokuma and co-workers, ONIOM is a powerful and systematic method that divides a large system into two or three zones (layers) and uses an extrapolation to facilitate accurate *ab initio* calculations of total energy of large chemical complexes (Dapprich et al., 1999) by Eq. (4):

$$E^{ONIOM} = E^{REAL}{}_{LOW} + E^{MODEL}{}_{HIGHT} - E^{MODEL}{}_{LOW} \quad (4)$$

where "high" ("low") represents the QM (MM) method and "model" ("real") refers to the chemically most important region (full system). The core system is treated at the QM level, while the real (entire) system is treated with the MM method.

This method allows for different levels of theory to be applied to different parts of a bimolecular system. In this approach, each complex was divided into two layers. The 54 ligand atoms of TRI with a net neutral charge were selected for the high layer, called the QM zone, which is mechanically treated quantum with the popular B3LYP (Becke, three-parameter, Lee-Yang-Parr) exchange-correlation functional and the 6-311G(d,p) triple split valence basis-set. The CDs, water, and co-solvent molecules were delimited for the MM zone by a 6.0 Å radius sphere with its origin in the triamcinolone centroid and all atom types were assigned by atomic number, hybridization, and formal connectivity using the Universal Force Field.

2.9. Statistics

The phase solubility results (stability constant and increased solubility shown in Table 2) were used to compare different systems. An ANOVA test was selected and when the F value was significantly different, the multiple comparison Tukey's test was applied. A $p < 0.05$ was established to reject the null hypothesis.

3. Results and discussion

3.1. Phase solubility studies

The co-solvency of TEA and NMP for the TRI drug was investigated according to the log-linear model. This model predicts an exponential enhancement of drug solubility as a function of co-solvent concentration. On a logarithm scale this relationship becomes linear, according to Eq. 1. Thus, a linear increment of drug soluble fraction occurs when the co-solvency mechanism is involved. In Fig. 1a the log-linear model for TRI solubility versus the

Table 1
The solubility parameters assessed in the log-linear model versus the physicochemical properties of the co-solvents.

	Increased solubility (fold)	Maximum solubility (mg/mL)	σ ($_{co\text{-}solvent}$)	$LogP_{ow}$	HBD
TEA	1.42 ± 0.1	0.144 ± 0.002	0.76	−2.3 (Verschueren, 1996)	30.13
NMP	6.72 ± 0.12	0.85 ± 0.02	3.97	1.21 (Sangster, 1989)	0

σ = Co-solvent power; Pow = octanol/water partition coefficient; HBD = Hydrogen bond density.

Table 2
The solubility parameters assessed in the phase diagrams for binary and ternary systems.

Complexes	K_s (M^{-1})	Increased solubility (fold)	Maximum solubility (mg/mL)	r^2	*Slope*
TRI:βCD	2036 ± 161[a]	19.0 ± 2.0[a]	2.39 ± 0.17	0.99	0.393 ± 0.018
TRI:βCD:TEA	1694 ± 47[b]	18.0 ± 1.0[a]	2.02 ± 0.03	0.99	0.325 ± 0.006
TRI:βCD:NMP	293 ± 13[c]	7.0 ± 0.1.[b]	0.86 ± 0.04	0.99	0.085 ± 0.003
TRI:HPβCD	1340 ± 33[d]	137.0 ± 1.0[c]	15.35 ± 0.10	0.99	0.276 ± 0.005
TRI:HPβCD:TEA	1001 ± 11[e]	110.0 ± 2.0[d]	12.34 ± 0.17	0.99	0.221 ± 0.002
TRI:RMβCD	1705 ± 36[b]	161.0 ± 5.0[e]	18.15 ± 0.41	0.99	0.327 ± 0.003
TRI:RMβCD:TEA	1564 ± 14[b]	152.0 ± 2.0[f]	17.07 ± 0.17	0.99	0.308 ± 0.002

KS = Stability constant.
a–f = Letters used to express ANOVA statistic test (systems with the same letters express values statistically equal).

concentration of the distinct co-solvents is represented, in which a linear mathematical adjustment ($r^2 > 0.97$) occurred for both TEA and NMP, demonstrating the co-solvent character of the selected substances (Li & Yalkowsky, 1994). The experimental data are fully presented in the Supplementary Table S1 in the online version at DOI: 10.1016/j.carbpol.2016.06.044.

The log-linear model considers only solute-water and solute-co-solvent interactions. Thus, deviations from linearity could be noticed when the solubilizing compounds modify these interactions, for example, when the solubilization ionizes the drug or forms a complex, modifying its initial structure and interaction mechanisms with the aqueous medium (Sanghvi et al., 2008). For example, in a previous study, it was demonstrated that the solubilizing effect of NMP for estrone and griseofulvine drugs showed a positive deviation of linearity from the log-linear model, as a consequence of the dual mechanism whereby NMP modified drug solubility (co-solvency and complexing) (Sanghvi et al., 2008). In this present study, deviations of linearity were not identified, corroborating the idea that the solubilizing effect of the TEA and NMP for the TRI drug occurred through the co-solvency mechanism.

As can be observed in Table 1, the increase of drug solubility was more expressive using NMP compared to TEA, which demonstrates that NMP was more efficient as a co-solvent. The co-solvent power (σ) value of the two substances also indicates this result.

The different polarities of the two liquid substances may explain why they also had different values of σ. In an enlightening study about this issue, the authors showed that the σ value of a co-solvent is higher as its polarity decreases (Rubino & Yalkowsky, 1987). Some parameters such as $LogP_{ow}$ (logarithm of solute partition between octanol and water phases) and the hydrogen bond formation ability can be used to represent the polarity of co-solvents. The $LogP_{ow}$ is a direct measurement of the solute affinity in the water or non-polar phase. The hydrogen bond formation ability in turn can be correlated with the interaction of the solute with the water, since the chemical groups responsible for hydrogen bonds contribute to the polarity of a specific molecule. This property can be mathematically assessed by calculating the density of proton-donating groups (HBD) or acceptor groups (HBA) (Rubino & Yalkowsky, 1987). Therefore, to explain these correlations we have calculated the co-solvent power (σ), the $LogP_{ow}$, and HBD values, as shown in Table 1. The HBD was calculated as follows: HBD = (No. of proton donor groups) × ($\rho_{co\text{-}solvent}$)/(molecular weight of co-solvent) × 1000.

The results shown above in Table 1 also demonstrated that the σ values were inversely correlated with co-solvent polarity (direct and inverse correlations with $LogP_{ow}$ and HBD, respectively). These correlations are expected, since co-solvents act in an intercalating manner between water molecules, decreasing hydrogen bond density and reducing the polarity of the aqueous environment, which thus enhances the non-polar solute dispersion (He, Li, & Yalkowsky, 2003).

Solubility diagrams were assessed in binary associations between TRI and distinct CDs (βCD, HPβCD and RMβCD), and in the ternary approach in the presence of NMP or TEA, in a continuous effort to reach better drug solubilization. The solubility diagrams of binary associations are shown in Fig. 1b, and TRI solubility is clearly enhanced when the CD concentration was increased. This relationship followed an A_L profile (when drug saturation and CD concentration rise together in a linear mode), with a slope up to 0.39, which indicates a 1:1 drug:CD complex stoichiometry (Brewster & Loftsson, 2007).

The stoichiometry of the complexes was confirmed by Job's plot method, as shown in Fig. 1c. The maximum absorbance variation (Δabs × TRI concentration) was observed at a TRI:CD ratio of 0.5, which is associated with the formation of soluble complexes with a 1:1 stoichiometry (Buranaboripan, Lang, Motomura, & Sakairi, 2014). The same assay carried out for ternary associations led to identical results, as shown in Supplementary Table S2 in the online version at DOI: 10.1016/j.carbpol.2016.06.044. The spectral changes that allowed the Job's plot findings can be correlated to the insertion of chromophore chemical groups of TRI in the CD cavity.

Regarding solubility diagrams, the complexes with derivative CDs (RMβCD and HPβCD) were able to solubilize more drug than the βCD complexes (binary–binary and ternary–ternary comparisons) through the increment of solubility parameters, as can be observed in Table 2, and further in Supplementary Table S3 in the online version at DOI: 10.1016/j.carbpol.2016.06.044.

The RMβCD and HPβCD are much more soluble than βCD, which permitted the largest amount of complexed drug and consequently the increment of solubility. The TRI:RMβCD system solubilized the drug about eight-fold more than TRI:βCD. A curious fact is that the maximum studied concentration of derivative CDs was ten-fold higher than natural βCD. However, the increment of solubility did not follow this ratio. A more intense response in TRI solubility

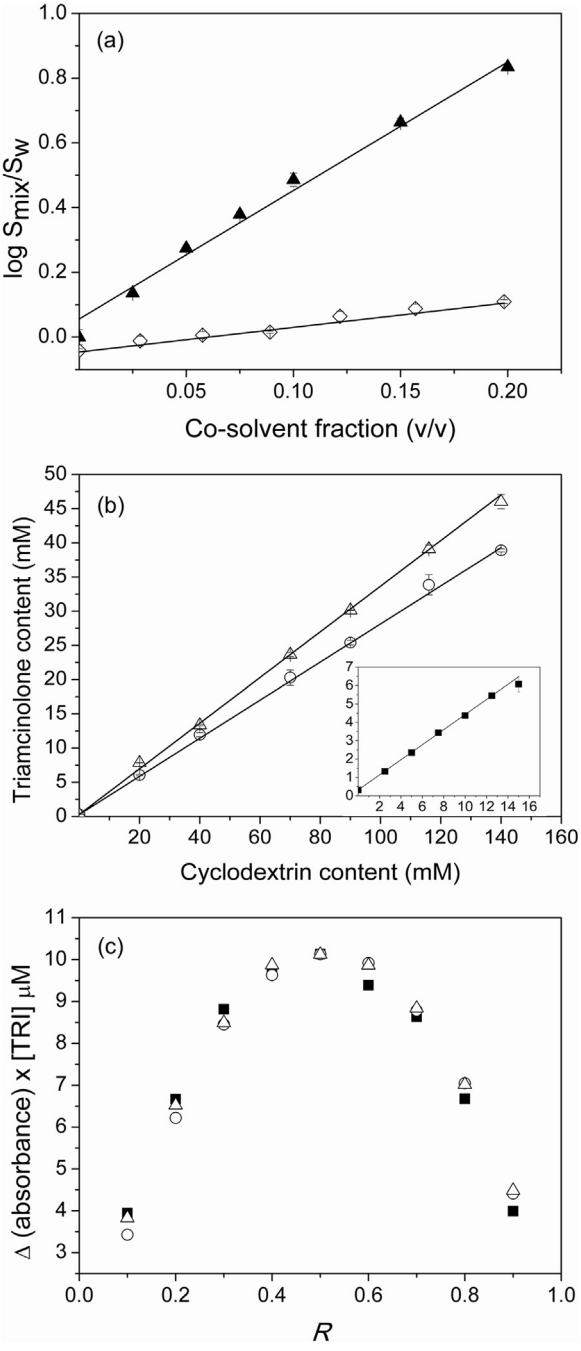

Fig. 1. (a) Solubility diagrams fitted by log-linear model of TRI content (axis y) as a function of co-solvents TEA (◊) and NMP (▲). (b) Solubility diagrams of TRI content as a function of βCD (■ insert), HPβCD (○), and RMβCD (Δ). (c) Job's plot of UV/Vis absorbance variation as a function of different ratios of TRI:βCD (■),TRI:HPβCD (○), and TRI:RMβCD (Δ) associations.

was observed with the βCD association, followed by RMβCD and HPβCD, respectively. It can also be seen in Table 2 that the βCD systems had a higher stability constant than the derivative ones. It seems that modified chemical groups in CDs changed the complex mode and the affinity of the drug:CD complex. Similar results were observed for different drugs in previous studies, in which, depending on the guest molecule, the K_s was higher or lower with native CDs than their derivatives, where the stabilizing effect of hydroxypropyl or methyl substituents only act in some cases. For example, in a study of the inclusion complex between gibberellic acid, βCD, and HPβCD, the stability constant for the derivative CD was higher than that of the natural βCD (Yang et al., 2012). However, the opposite effect can also be present, as shown in a distinct study with clonazepam and several CD complexes (Mennini, Bragagni, Maestrelli, & Mura, 2014). The authors found a major K_s with HPβCD and methyl-βCD rather than βCD.

Different ligands in the drug may also change the interactions and consequently macroscopic properties, like solubility. Previous studies with triamcinolone acetonide (TA) demonstrated a different way with HPβCD, in which the solubility diagram (type B_S profile) was markedly different (Miro et al., 2012). Thus, the experimental data presented in Fig. 1, and Table 2 supported the importance of free hydroxyls groups in ring D of TRI (Fig. 2) for drug-CD interactions. This fact was further investigated in the molecular modeling simulations and QM/MM experiments.

We conclude that the kind of CD should be chosen according to the desired TRI concentration. For example, if the solubilizing capacity of βCD is sufficient, its application allows the use of less CD content than HPβCD or RMβCD. Thus, the secondary properties should also be evaluated, such as drug stability, absorption, or interaction with specific tissues.

Despite NMP having shown an σ value larger than TEA (Table 1), the experiments with the ternary approach revealed that the first substance considerably decreased drug complexation with βCD. The K_s of the TRI:βCD:NMP system decreased about three times compared to the βCD binary complex. This is a peculiar effect since this co-solvent can enhance the TRI solubility approximately seven times. However, the importance of the solvent medium for complex stability should be considered. The theory of the solvophobic effect explains that during the dissolution, the solute has to create a cavity in the solvent net-structure, and the energy cost of the dissolution is equal to the product of surface area that was dislocated and the solvent surface tension. The energy cost can be covered by the solute solvation, which is minimal for hydrophobic solutes. An inclusion complex represents a decrease in the solute surface area and is energy-driven based on this theory (Connors, Mulski, & Paulson, 1992). So, the addition of a solvent that reduces surface tension, as occurs with some co-solvents and with NMP (Sanghvi et al., 2008), can increase the solute dispersion and reduce the driven force to form the complex. Another approach to explain the solvent effect is to consider the solvent-solute interactions. When a change in the solvent medium makes the solute-solvent interactions stronger, through hydrogen-bonds or Van der Waals, for example, the solute will consequently interact more weakly with the CD (de Garcia Venturini, Andreaus, Machado, & Machado, 2005).

Conversely, as occurred with NMP, the ternary complex TRI:βCD:TEA leads to an equivalent drug solubilization compared to the TRI:βCD complex. The slight decrease in the stability constant shows that different parameters can lead to different results and an overview of them is important. Considering the intuitive effect of NMP on TRI:βCD complex solubility, ternary association using derivative CDs was made only with TEA. The TEA association with derivative CDs decreased drug solubilization compared to the binary complexes (Table 2). It seems that TEA can be detrimental to the formation of the inclusion complex, as previously discussed for the TRI:βCD:NMP system.

Despite the negative effect of the co-solvents on TRI solubility when added to some TRI:CD systems, the drug solubility remained higher than needed for therapeutics. The clinically used concentration of TRI in intraocular injection is up to about 8 mg (Lam et al., 2007) and 20 mg for injection in intraarticular and soft tissues (Gaffney, Ledingham, & Perry, 1995), both used as depot suspensions. The complexes evaluated in this paper present a potential alternative for these depot suspensions, allowing formulations to administrate the drug in the eye by water soluble non-invasive eye drop or improving the oral administration effect due to the higher solubility. The TRI complexes of this work could entirely solubi-

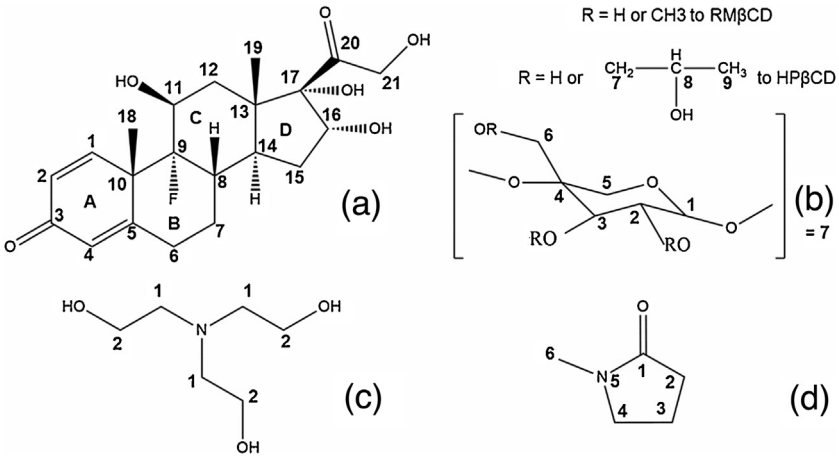

Fig. 2. Schematic representation of the chemical structures of (a) triamcinolone, (b) βCDs, (c) triethanolamine, and (d) *N*-methyl-pyrrolidinone.

lize the depot doses with less than 1 or 2 mL, depending on the complexes used, as shown in Table 1, which includes the ternary systems.

The presence of NMP and TEA can evaluate more drug in a non-complexed state in the target environment, since the K_s values of some ternary complexes are lower than their respective binaries. Similar results were identified in a study with acetazolamide, which demonstrated that the TEA ternary complex with HPβCD increased therapeutic activity of drug compared to a binary system. This effect was attributed to the decreased complex stability and, consequently, more drug availability for absorption (Palma et al., 2009).

The presence of NMP and TEA in the ternary complexes can also enhance drug permeability, since both co-solvents were previously studied for this purpose involving NMP (Pappinen et al., 2007; Ren et al., 2008; Yerramsetty, Neely, Madihally, & Gasem, 2010) and TEA (Cheong & Choi, 2002; Feng et al., 2008). In this study, we recognize that the ternary complexes could have different properties related to binaries, with some potential enhancement of TRI absorption or due to the calculated different stability constants that modify drug release from the CD complex.

3.2. ^{1}H NMR spectroscopy and 2D-ROESY spectra

The ^{1}H NMR experiments were carried out to elucidate the interactions between TRI drug with CDs (βCD, HPβCD, and RMβCD) and third compounds (TEA and NMP) by using the approach of binary and ternary complexes. The individual protons were assigned according to their position in the chemical structure of the compounds as shown in Fig. 2.

The spectra of the compounds were recorded and the chemical shifts (δ) of TRI and CDs are represented in Table 3. Some proton signals of the drug appeared to overlap and could not be identified. The chemical shifts obtained from solutions of a single solute are expressed as δfree and the chemical shifts of the complexes are expressed as δcomplex, while the spectra modifications induced by the complexes are expressed as Δδ.

The drug association with CDs induced changes in the chemical shifts of TRI protons, mainly those located on ring "A" of the molecule. The three binary complexes presented noticeable spectral modifications induced by the complexes (Δδ) in the signals of $H^{2'}$ and $H^{18'}$. The TRI:HPβCD and TRI:RMβCD associations

Table 3
Proton signalization of TRI and CDs by ^{1}H NMR as free-state, binary, and ternary complexes. The proton numbering follows the carbon numbering, with H^1 being H-linked to C^1.

Studied Protons	TRI Free (ppm)	βCD Free (ppm)	βCD Bin. (Δδ)	βCD TEA-Tern. (Δδ)	βCD NMP-Tern. (Δδ)	HPβCD Free (ppm)	HPβCD Bin. (Δδ)	HPβCD TEA-Tern. (Δδ)	RMβCD Free (ppm)	RMβCD Bin.(Δδ)	RMβCD TEA-Tern. (Δδ)
CDs											
H^1		5.09	−0.01	−0.01	−0.02	5.09	−0.01	−0.01	5.08	−0.01	−0.01
H^2		3.68	0.00	0.00	−0.01	3.64	0.00	−0.01	3.68	0.00	0.00
H^3		3.98	−0.08	−0.09	−0.09	3.98	−0.09	−0.09	3.88	0.01	0.02
H^4		3.60	0.01	0.01	0.00	3.52	−0.04	−0.04	3.68	0.00	0.00
H^5		3.89	−0.14	−0.12	−0.14	3.72	0.03	–	3.98	−0.09	−0.09
H^6		3.90	0.00	0.00	0.00	3.89	−0.01	−0.02	3.68	0.00	0.00
H^7						4.04	0.00	−0.01			
H^8						4.04	0.00	−0.01			
H^9						1.17	0.00	−0.01			
H (2methyl)									3.58	0.01	0.02
H (6methyl)									3.41	0.00	0.01
TRI											
$H^{1'}$	7.55		−0.01	−0.01	−0.02		−0.01	−0.02		−0.04	−0.03
$H^{2'}$	6.45		−0.07	−0.08	−0.08		−0.07	−0.07		−0.11	−0.09
$H^{4'}$	6.26		−0.01	−0.01	−0.02		−0.03	−0.04		−0.04	−0.03
$H^{18'}$	1.58		0.10	0.10	0.09		0.07	0.06		0.09	0.08
$H^{19'}$	0.97		0.04	0.04	0.03		0.01	0.01		0.01	0.02
$H^{21'}$	4.43		0.02	0.02	0.01		−0.01	−0.01		0.00	0.01

δ free = Single solute signal, δ complex = complex signal, Δδ = δ free − δ complex.

also showed expressive changes in $H^{4'}$, and changes in $H^{1'}$ for TRI:RMβCD. The involvement of ring "A" in the complex formation is quite expected, since the formation of inclusion complexes generally involves non-polar portions of the guest to be included in the CD cavity. This trend is commonly shown in the literature for saturated or aromatic carbon rings, which are mostly complexed with CDs (Barbosa et al., 2014; Shanmuga Priya, Sivakamavalli, Vaseeharan, & Stalin, 2013). Relative to ternary complexes, there were no notable changes in δ values between binaries and ternaries.

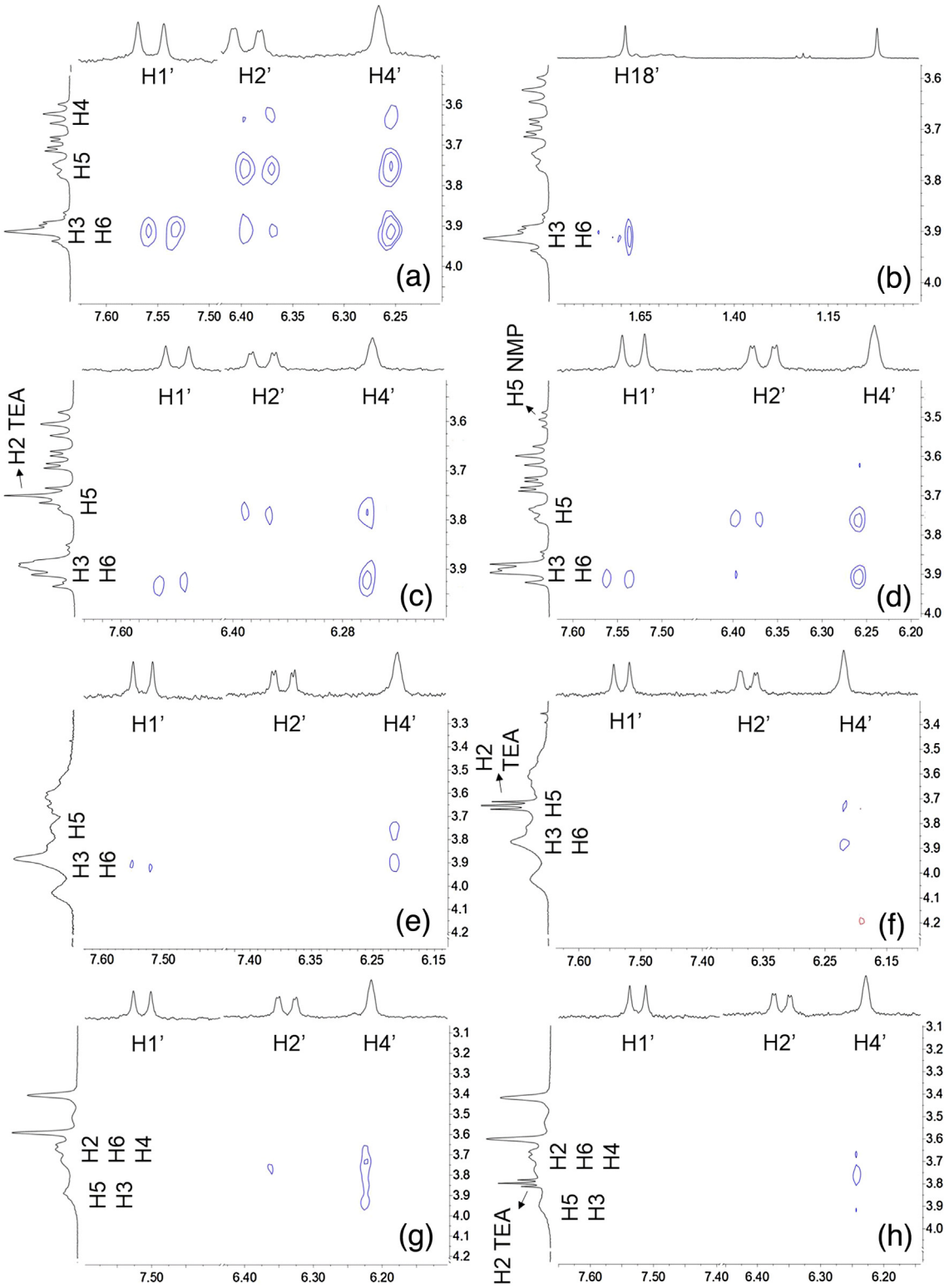

Fig. 3. 2D-ROESY spectra for binary and ternary complexes. TRI protons are represented on the horizontal axis, with third compounds and CDs on the vertical (axis as ppm). The crossing signal represents the NOE effect with a blue-red scale for high-low intensity, respectively. (a) TRI:βCD, (b) TRI:βCD, (c) TRI:βCD:TEA, (d) TRI:βCD:NMP, (e) TRI:HPβCD, (f) TRI:HPβCD:TEA, (g) TRI:RMβCD, (h) TRI:RMβCD:TEA.

On the internal surface of the CD cavity, the H^3 and H^5 protons of the oligosaccharide can be found, while on the external surface are the H^1, H^2, H^4, and H^6 protons (Mura, 2014; Zoppi, Quevedo, & Longhi, 2008). In this study, the ^{1}H NMR spectra showed some differences for CD protons when comparing the single solute samples and the complexes. In the TRI:βCD complex, the H^3 and H^5 βCD protons were the only ones that experienced notable changes in δ values, with a shielding effect. For the TRI:RMβCD complex, the H^5 chemical shift appeared modified, instead of the other protons which had barely changed. The TRI:HPβCD complex showed modifications in H^3, H^5, and H^4, corroborating the differences evidenced in the solubility diagrams by distinct stability constants (K_s) identified for the three different complexes. In general, CD internal protons H^3 and H^5 appeared modified in complex spectra. The changes in spectra involving H^3 and H^5 signals indicate the formation of an inclusion complex, as shown in the literature for other complexes (Chao, Wang, Zhao, Zhang, & Zhang, 2012; Pedotti et al., 2015).

Shielding and de-shielding effects varied among the three types of complexes. An insertion of the TRI ring "A" in the CD cavity could explain these effects. In the literature, it is demonstrated that the spectra of organic molecules can be influenced by interactions with other compounds. The changes can be caused mainly by interactions with electropositive or electronegative atoms (Wishart, Sykes, & Richards, 1991). Therefore, differences of TRI arrangement in the CD cavity can be considered among the three types of binary systems due to the slight differences among the Δδ products. Concerning ternary complexes, spectral modifications were not evidenced between binaries and ternaries.

The ^{1}H NMR spectra of a substance can be influenced by the chemical environment, such as solvent modifications (Usula et al., 2014). Therefore, 2D-ROESY spectra were conducted for the same systems (binaries and ternaries) in order to observe space proximity between two protons through the NOE effect and to confirm the complex occurrence (Fig. 3). The δ signals of TRI protons can be seen on the horizontal axis, and CDs and third components on the vertical axis. Crossing signals represent the NOE effect. In general, it was possible to note interactions between protons of the TRI ring "A" and CDs (on binary and ternary systems), especially the internal H^3 and H^5 of the oligosaccharides. This result reinforces the idea that ring "A" of TRI can be included in the cavity of the three CDs, forming a complex even with the presence of the third compound. The TRI $H^{18'}$ methyl group also showed interaction with the βCD cavity. In general, other CD protons besides H^3 and H^5 showed a NOE effect with TRI protons, due to the dynamic phenomenon involving CD complexes.

The proton $H^{4'}$ of TRI appeared interacting with several protons of the three CDs, including the ones located in the cavity. Mainly in the case of HPβCD it would be expected that the inserted hydroxypropyl chains could offer some stabilization effect for the complex. To get light on the structure of TRI:HPβCD complex, 2D ROESY spectra were recorded at different mixing times of 500 and 750 ms (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.carbpol.2016.06.044). However, any NOE signal referring to the interactions between hydroxypropyl chains of HPβCD and TRI was observed for different mixture times. Thus, the hydroxypropyl seems decreased the stabilization of TRI- HPβCD complex, as previously discussed in the phase diagrams and further supported by molecular modeling simulations.

The ternary systems showed similar NOE signals between TRI and CDs compared with the binaries, indicative of the inclusion complex. There did not appear to be interaction signals between CDs and the co-solvents, or between the co-solvents and the drug. In addition, any different NOE effect was observed for the 2D ROESY spectra of TRI:HPβCD:TEA system recorded at different mixing times (Supplementary Fig. S2 in the online version at DOI: 10.1016/j.carbpol.2016.06.044).

Table 4
Energetic values for TRI interactions with their surroundings.

Systems	Energy (kcal/mol)	
	MD interactions (phase 1)	QM/MM interactions (phase 2)
TRI:βCD	−43.201	−119.9
TRI:βCD:NMP	−41.420	−82.561
TRI:βCD:TEA	−43.553	−124.32

These experimental data permitted us to postulate that the chemical shift differences between pure compounds and complexes occurred due to the inclusion complex formation, which was confirmed by 2D-ROESY assay, and that the TRI ring "A" inclusion in the CD cavity is a suitable and stable form of an inclusion complex.

3.3. Molecular dynamics simulations

Simulations with the molecular dynamics (MD) technique were used to predict interactions between the compounds in the binary and ternary associations. The software UCSF chimera was used to cluster the most relevant conformations from the MD frames. Simulations containing RMβCD and HPβCD were not performed due to the uncertain structure derived from the randomized substitution. The formation of complexes between the drug and the CDs was observed. The clusters obtained for the binary complexes with βCD are presented in Fig. 4a and b. It can be noticed that TRI ring "A" easily entered through the narrower edge of the βCD cavity since this was the most frequent complex during the simulated time. The TRI ring "A" entering through the wider edge of βCD is much less frequent during the simulation due to its instability.

Fig. 4 also shows the most frequent clusters for the ternary complexes. The narrower edge of CDs was the most preferable way of entrance for TRI ring "A". The co-solvents are shown dispersed in the media. What did not appear were conformers of important frequency that represent a complex between TEA or NMP and the CDs, which leads us to discredit a competitive effect between co-solvents and the drug by CD cavity. The competitive effect between TEA and the benznidazole drug by βCD cavity was previously demonstrated (de Melo et al., 2013), showing that the complex phenomenon must take into account all compounds involved. Therefore, the detrimental effect of the co-solvents on complex formation with TRI must be due to the reduction of the driving force for the drug inclusion, as previously discussed in this study. In addition, it was observed that the ternary systems presented a lower number of possible conformations when compared to the binary complexes. In fact, there is only one important representative conformation for every ternary complex. This fact suggests that the third compound contributes to stabilizing the most frequent conformer, corroborating experiments of Jobís plot and ^{1}H NMR spectroscopy, which have demonstrated that the third compound did not impair drug complexation.

3.4. Ab initio and QM/MM interaction energy calculation

Following the MD simulations, the most frequent inclusion complexes (binary and ternary) were submitted to an accurate quantum mechanics: molecular mechanics (QM:MM) protocol to find the respective optimum (stable) conformational geometries. The complex energetic values determined by MD and QM:MM simulations are presented in Table 4.

The energy values calculated from MD simulations were not completely consistent with the QM:MM results. Considering the more demanding energy calculation of the QM:MM technique and that MD presented an average energy during all the simulation

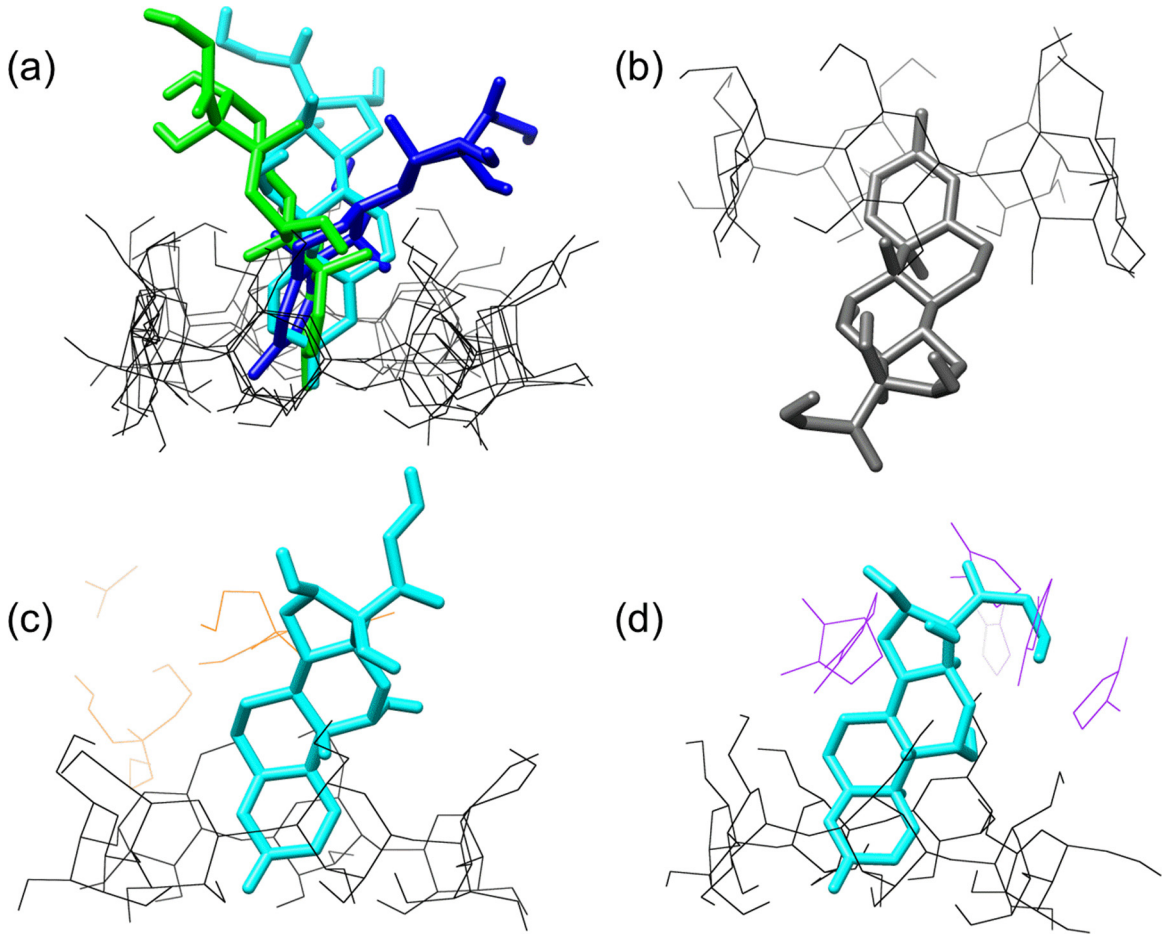

Fig. 4. Most frequent clusters (overlapped) for the complexes TRI:βCD (a and b), TRI:βCD:TEA (c), and TRI:βCD:NMP (d). The TRI placement in the complex is represented by different colors according to its frequency: light blue> dark blue> green > gray. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

time, even before the complex formation, these differences were already expected. However, the trends in the energy changes due to the added co-solvents were the same for the two techniques.

The QM:MM theoretical energies are in agreement with the experimental results. The experimental stability constants presented the following decreasing order: TRI:βCD > TRI:βCD:TEA > TRI:βCD:NMP. The theoretical QM:MM energies showed that TRI:βCD and TRI:βCD:TEA were the most favorable for enthalpy complexes, with −119.90 and −124.32 kcal/mol, respectively. Differences between the theoretical energy of binary and TEA ternary complexes were too small to be considered, a different conclusion to when the experimental K_s value was used for this comparison. However, the QM:MM energy difference of the TRI:βCD binary complex and the TRI:βCD:NMP complex was conclusive, showing the lower stability of this ternary complex, corroborating the experimental results.

Fig. 5 shows the intermolecular H-bonds between TRI and the βCD. Considering that the interactions between TRI and the CDs are non-covalent, it is important to remember that the structures represented in this study were geometrically optimized, but interaction changes, breaking, and associations of the complexes occurred constantly.

Regarding the TRI complexes with βCD, the binary TRI:βCD presents two important H-bonds between primary hydroxyl groups (narrow cavity hydroxyls) of the βCD and TRI, supporting the greater stability constant calculated in the experimental phase solubility diagrams compared with that HPβCD complex, which some primary hydroxyl groups are substituted. In this system, the TRI can penetrate deeply into the cavity of the molecule, and another H-Bond is present between the TRI aromatic-like ketone with the secondary hydroxyl of the βCD. Similarly, there exists in the TRI:βCD:TEA system stabilizing H-bonds between the drug and hydroxyls of the βCD (primary and secondary). In this ternary complex, different from that which occurs with the binary, TRI also interacts with the CD by etheral oxygens of the glucose residues at the central cavity, corroborating the stabilization effect for this conformer detected in the MD simulations. In the TRI:βCD:NMP system, an H-bond occurs between the deeply penetrated TRI aromatic-like ketone and the secondary hydroxyl of βCD. The other H-bond represents the only one where the fluorine atom acts as the H-bond acceptor, and this interaction is not quite strong enough to generate complex stability. Besides being extremely rare in crystallographic structures, the contact between fluorine and the acidic hydrogens is less than half the strength (2.38 kcal/mol) of a conventional H-bond (Howard, Hoy, O'Hagan, & Smith, 1996). Due to the high electronegativity of fluorine and its strong electrostatic nature, it holds the lone pair of electrons (poor polarisability) and, unlike oxygen or nitrogen, renders to the organic fluorine a poor donor and hydrogen bonding acceptor character (O'Hagan, 2008).

As previously discussed, the NMP presence as the TRI:βCD:NMP third compound represents a decrease in the surface tension due to its co-solvent effect and, consequently, the complex stability. This could lead to a downturn in the complex occurrence, specifically changing its interaction type to a more energetic one, as

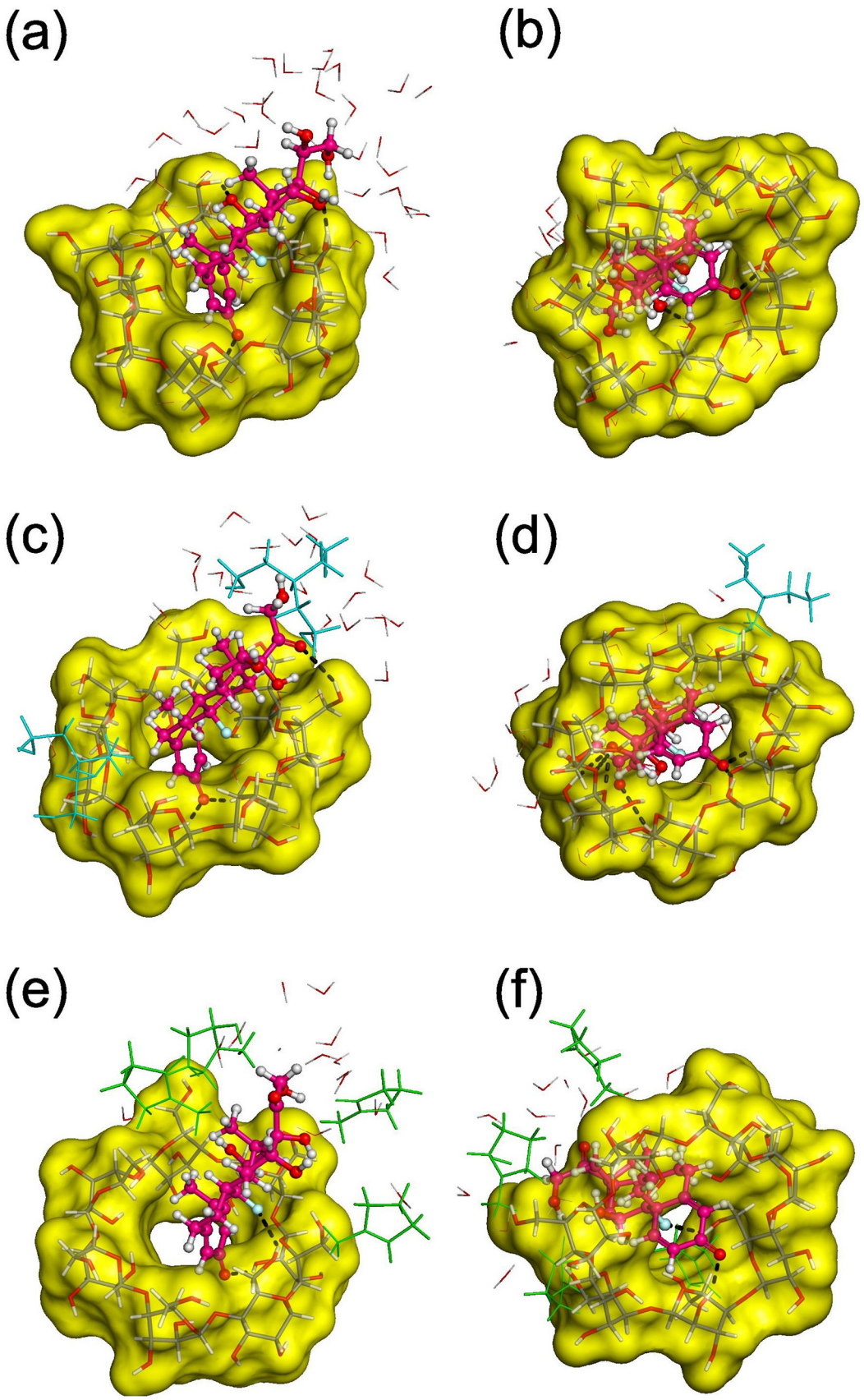

Fig. 5. TRI complexes with βCD optimized by QM:MM simulations. The intermolecular H-bonds are represented as dashed lines. (a) and (b) represent TRI:βCD complex (narrow edge and wide edge view); (c) and (d), TRI:βCD:TEA; and (e) and (f), TRI:βCD:NMP.

we demonstrated with the much lower total QM:MM interaction energy (Table 4) and experimental stability values (Table 2) of this system compared to the TRI:βCD binary complex.

4. Conclusions

The latest state-of-art was used to study triamcinolone/cyclodextrin/co-solvent associations. The detailed study demonstrated the interactions between the compounds in binary and ternary complexes by ^{1}H NMR and molecular modeling, contributing to the understanding of their behavior and correlating its properties with the practical application. The complexation phenomena and the structure of the formed conformers were confirmed by ^{1}H NMR and 2D-ROESY, and supported by different computational simulations. The two selected co-solvents have different effects in the ternary associations. The NMP considerably decreased the soluble fraction of drug and stability of formed complexes due to the competition with TRI by CD cavity. The TEA did not present a synergic effect with the CD in the drug solubilization, but stabilized the main conformer of the formed ternary complex, improving the uniformity and the quality of complexation. Considering the similar drug solubility of the binary and ternary complex with TEA, the latter would be the reasonable choice in practical application since previous studies demonstrated that these co-solvents improved drug absorption and bioavailability when used in ternary complexes (Feng et al., 2008; Palma et al., 2009; Yerramsetty et al., 2010). Finally, the large soluble fraction of drug identified for the binary and ternary complexes with TEA was superior to all therapeutic doses commercially available in different dosage forms, mainly in depot suspensions, needed in invasive injections in the eye, joint, or soft tissues (Gaffney et al., 1995; Lam et al., 2007). Therefore, this study attended to its purpose with a promising and new pharmaceutical raw material for aqueous solution and improved TRI dissolution in dispersions or solid dosage forms.

Conflicts of interest

The authors declare no conflict of interest.

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