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ABSTRACT

This work provides a new insight on fundamental principles of the interaction mechanism between two forms of tetracaine – a potent local anesthetic – both in neutral (TC) and ionized (TC^+) states, with beta-(β-CD) and hydroxypropyl-beta-cyclodextrin (HP-β-CD), and how such interactions affect the transport of tetracaine, at different concentrations, across a model membrane. The kinetics and mechanism of TC release from HPMC gels is also evaluated giving an insight on the role of cyclodextrin on the tetracaine transport. HPLC, fluorescence and NMR spectroscopies provided solid physicochemical knowledge of these systems and in vitro studies were performed to obtain relevant data on the transport and mechanism parameters. HPLC and fluorescence spectroscopy data revealed that tetracaine interacts with both cyclodextrins on a 1:1 stoichiometry but it is observed that neutral tetracaine forms more stables complexes (ca. 1050 M^{-1} for both cyclodextrins) than in its ionized form (628 and 337 M^{-1} for β-CD and HP-β-CD respectively). Despite of that, no host–guest interactions take place as seen by ROESY. This study clearly demonstrates that both forms of tetracaine are successfully released from the formulations at a controlled rate, following a Super-Case transport mechanism and the transport of tetracaine can be tuned by using cyclodextrins.

1. Introduction

Tetracaine is a potent local anesthetic, primarily used for topical anesthesia and spinal block. It is generally believed that the interaction of the anesthetic with membrane lipids and proteins leads to the inactivation of neuronal ion channel activity (Ragsdale et al., 1994). Due to its high systemic toxicity tetracaine use in other anesthetic techniques is limited. Adverse effects of its topical administration include mild erythema at the site of the application and less frequently slight edema, pruritus and blistering of the skin. Tetracaine is commonly used as the hydrochloride form in solutions and creams and as the base form in ointments or gels. Tetracaine is reported to be about 15% bioavailable after application of a 4% gel to intact skin, with a mean absorption and elimination half-life of about 75 min (O'Brien et al., 2005). This drug can suffer hydrolysis, resulting in equilibria between three different absorbing species: tetracaine base form (TC) and tetracaine ionized forms (TCH^+ and TCH_2^{2+}) depending on the pH (Fig. 1). The ability of this drug to cross biological membranes is also pH-dependent. It has been reported that tetracaine in its base form can permeate the skin (Liu et al., 2005). The ionic form (TCH^+), is dominant at physiologic pH of the skin (pH ~ 4.2–6.5) and it is generally accepted that the cationic form binds to the sodium channels on the nerve membrane, blocking the initiation and transmission of nervous impulses (Chekirou et al., 2012).

The bioavailability of most topically applied drugs is generally very low and various approaches have been developed to enhance drug diffusion across the skin (Hadgraft, 1999; Thomas and Finnin, 2004). One of them involves the use of cyclodextrins (CD). These compounds are known to have the ability to form complexes with a wide range of organic molecules, both in solution and in solid state (Másson et al., 1999; Ventura et al., 2006). CD have been recognized as promising drug carriers and delivery systems but how they function to modify topical administration has not as yet been fully elucidated (McCormack and Gregoriadis, 1998). Natural CD possess

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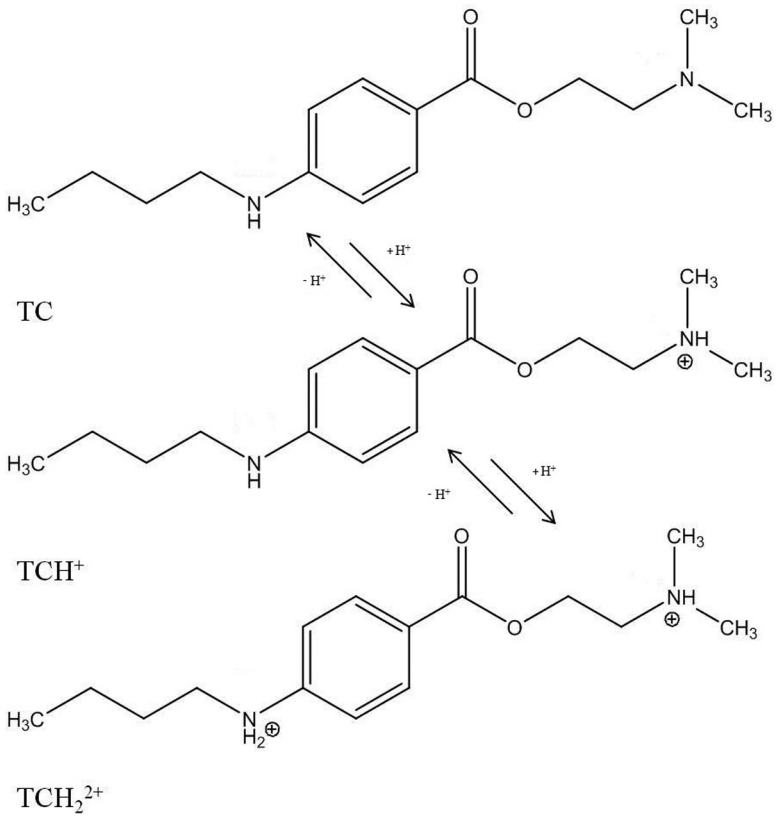

Fig. 1. Schematic representation of ionization equilibrium of tetracaine, exhibiting two ionizable groups (pK_a's 3.41 and 8.24) (Iglesias-García et al., 2010).

a cyclic structure containing six, seven or eight α (1–4) linked glucopyranose units denominated as α, β and γ, respectively. As a result of glucopyranose units in chair conformation, CD exhibits the form of a truncated cone or torus (Loftsson and Brewster, 1996). The hydroxyl groups are orientated to the exterior: the primary hydroxyl groups of the glucose residues at the narrow edge of the cone and the secondary hydroxyl groups are located at the wider edge, resulting in a hydrophilic outer surface. The central cavity is structurally composed of skeletal carbons and ethereal oxygen groups, providing a lipophilic environment, optimal for entrapping non-polar drugs (Arun Rasheed and Sravanthi (2008); Loftsson and Masson, 2001). Host–guest like complexes may improve the properties of the guest molecule, e.g. the drug, such as solubility enhancement (Loftsson and Brewster, 2012; Yuan et al., 2013) and stability improvement (Yuan et al., 2013). Additionally, CD act as efficient drug carriers, providing a controlled and sustained release, avoiding undesirable toxic effects (Castronuovo and Niccoli, 2006; Mosher and Thompson (2002)). Although the naturally occurring CD (in particular the β-CD) and their complexes are hydrophilic, their aqueous solubility is limited. The synthesis of new CD derivatives (such as HP-β-CD) has allowed overcoming the solubility issue (Booij, 2009; Loftsson et al., 2007). As previously mentioned, the neutral TC permeates the skin, however that occurs at pH higher than 8, clearly above the skin physiological pH. This work reports a comprehensive analysis regarding the transport of tetracaine across a model membrane. Studies using a simple inert barrier model are very useful for optimization of formulations in earlier stages of research. The dialysis membrane was selected as a model membrane because it is isotropic and homogeneous. In these conditions, release data can be described by simple mathematical models. Essentially, we focused on the complexation phenomenon occurring between aqueous solutions of two forms of tetracaine and β- and HP-β-CD and how the CD concentration on the formulation alters the release profile of tetracaine. In vitro release studies across dialysis membrane using different tetracaine formulations were carried out at two distinct pH values (4.1 and 9.0). The mechanism of release was evaluated on the basis of the interactions established between tetracaine and HP-β-CD.

Mathematical models appear as useful tools to correlate material properties, interaction parameters, kinetic events, and transport behavior within complex hydrogel systems (Korsmeyer et al., 1983; Siepmann and Peppas, 2001, 2011).

Although the interactions between tetracaine and CD, at neutral pH, have already been reported elsewhere (Fernandes et al., 2007; Franco de Lima et al., 2012; Van Santvliet et al., 1998), no studies have been presented with acetate buffer at acidic pH (ca. pH 4). CD:TC complexes were characterized using different techniques: (i) stoichiometry, association constants, and HPLC, fluorescence and NMR spectroscopies to analyze topology of complexation of the inclusion complexes. This work gathers a comprehensive set of data and observables obtained from several complementary techniques regarding the nature of the association between two forms of TC and CD and how this interaction affects the transport across a model membrane. The TC concentration during the experiments was kept below the critical micelle concentration – cmc (128 mM), as reported elsewhere (Dukhin and Miller Loglio (2005)). The results obtained were rationalized and the conclusions are new, significant and provide solid physicochemical knowledge of these systems that are crucial for future delivery studies with local anesthetics and cyclodextrins.

2. Experimental section

2.1. Materials

Tetracaine base, acetic acid, 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP), ammonium acetate, sodium acetate and beta-cyclodextrin (β-CD) were acquired from Sigma–Aldrich (St. Louis, MO, United States of America). Millipore water was used to prepare sodium acetate buffer aqueous solution. Hydroxypropyl beta-cyclodextrin (HP-β-CD) with 97% of purity, with an average degree of substitution of 2–6 units of 2-hydroxypropyl (C_3H_7O) per glucose unit, and with an average molecular weight of 1380 g/mol, was purchased from Acros Organic (Geel, Belgium). Methanol (MeOH) and acetonitrile (ACN) HPLC grade were purchased from LiChroSolv Merck (Darmstadt, Germany). Phosphate buffer saline tablets (PBS) were purchased from TIC Gums (Belcamp, MD, USA). Dialysis membrane (Spectra/Por) with a 6-8.000MWCO, was bought from Spectrum Laboratories, Inc. (Broadwick St., Rancho Dominguez, US & Canada). Deuterated water (D_2O) (99.9%) for preparing NMR samples was purchased from Eurisotop (Saint-Aubin Cedex, France). Hydroxypropylmethyl cellulose (HPMC – METHOCEL K15M Premium, 19–24% methoxyl and 7–12% hydroxypropyl, $M_w = 4.3 \times 10^5$ Da) was a kind gift from Dow Chemical. All reactants were used as received.

2.2. Phase-solubility studies

Phase-solubility diagrams were carried out to assess the solubility of tetracaine and determine the association constants with the CD. Phase solubility studies were performed according to the procedure reported by Higuchi and Connors (Siepmann and Peppas, 2011), at room temperature, by adding an excess of TC base to deionized water (pH 9) and sodium acetate buffer aqueous solution (pH 4.1) containing increasing amounts of β-CD (1–15 mM) and HP-β-CD (1–45 mM), stirred during 72 h. The solutions were filtered using a MCE 0.45 μm filter syringe (Fioroni filters, Ingré, France) ($n = 3$). An aliquot of solution was removed, diluted in a filtered and degassed mobile phase (MeOH, ACN and ammonium acetate) and quantified by using HPLC (Shimatzu LC-20AD). The wavelength selected for tetracaine quantification was 311 nm. The injection volume was 25 μL and the mobile phase flow rate was set to 1.0 mL/min. The apparent stability constants (K) of the complexes were calculated from the slope of the straight lines of the phase-solubility diagrams and the drug solubility in the medium using Eq. (1) (Connors and Mollica, 1966),

$$K = \frac{\text{Slope}}{S_0(1 - \text{Slope})} \tag{1}$$

where S_0 is the intrinsic solubility of the drug in the medium, obtained from the origin intercept and the slope is obtain from the linear regression of the data points.

2.3. Fluorescence studies

In order to characterize the stability constants between TCH^+ and CD, the fluorescence emission spectra of TCH^+ were collected and analyzed. Changes in the intrinsic fluorescence of TC using different CD molar ratios of complexation were also investigated. A batch of buffered acetate aqueous solutions with a fixed concentration of TC (10 μM) were prepared ($n = 3$) by adding increasing amounts of β-CD and HP-β-CD. The pH of each solution was fixed at 4.1 and maintained constant during the experiments. The solutions were subsequently mixed at 25 °C during 72 h. The measurements were performed using a Horiba-Jobin-Ivon SPEX Fluorolog 3-22 spectrophotometer at 25 °C. The excitation wavelength was fixed in 311 nm and the emission spectra were collected from 320 to 500 nm. The emission and excitation slits used were fixed at 5 nm.

2.3.1. Modeling stoichiometry and association constants

Assuming that a 1:1 complex between β-CD and HP-β-CD with ionized tetracaine, TCH^+ (a proof for this assumption will be presented in the Section 3.1) is formed

$$CD + TCH^+ \overset{K}{\rightleftarrows} CD : TCH^+ \tag{2}$$

the stability of the complex CD:TCH^+ can be described in terms of an association constant, K, as defined in

$$K = \frac{[CD : TCH^+]}{[CD]_f [TCH^+]_f} \tag{3}$$

where $[CD]_f$ and $[TCH^+]_f$ represent the concentration of free (non-complexed) species, β-CD or HP-β-CD and ionized tetracaine, respectively, and $[CD:TCH^+]$ is the concentration of the 1:1 complex.

From Eq. (3) and the mass balance equations

$$[CD]_T = [CD : TCH^+] + [CD]_f \tag{4}$$

$$[TC^+]_T = [CD : TCH^+] + [TCH^+]_f \tag{5}$$

where $[CD]_T$ and $[TCH^+]_T$ represents the total (initial) concentrations of β-CD or HP-β-CD and TCH^+, respectively, the association constant, K, can be re-written as

$$K = \frac{f}{(1 - f)([CD]_T - f[TCH^+]_T)} \tag{6}$$

where f is the fraction of TCH^+ complexed with the β-CD and HP-β-CD.

Since the CD is not fluorescent, the observed fluorescence emission intensity, F, from free and complexed tetracaine is given by

$$F = k_1[TCH^+]_f + k_2[CD : TCH^+] \tag{7}$$

where k_1 and k_2 are constants. Considering F_0 and F_∞ the fluorescence intensity of TCH^+ in the absence and in an excess of cyclodextrin, respectively, the f can be represented by

$$f = \frac{F - F_0}{F_\infty - F_0} \tag{8}$$

combining Eqs. (6) and (8) we obtain

$$F - F_0 = \frac{(F_\infty - F_0)K[CD]_T}{1 + K[CD]_T} \tag{9}$$

The experimental data (see Fig. 4) can be perfectly fitted ($R^2 > 0.9965$) to the corresponding Eq. (9), using a non-linear least-square algorithm, to obtain the fitting parameters K and F_∞.

Eq. (9) can be simplified in order to have information on the stoichiometry of the association (Smith et al., 1991). Thus, a linear relationship of $(F - F_0)/[CD]_T$ as a function of $(F - F_0)$ is indicative of a 1:1 stoichiometry.

2.4. NMR/ROESY studies

^{1}H-NMR and ROESY experiments explore aspects of the geometry of the complex formed and provide robust evidence of the nature of the host–guest interaction. One-dimensional ^{1}H-NMR spectra for each different [CD]/[TC] ratios, R, were recorded using a 600 MHz Varian NMR spectrometer (Palo Alto, CA) at 25 °C using a 3 mm indirect detection NMR probe. The NMR samples were prepared using D_2O (99.9%) as solvent, and TSP, used as

internal reference, at tracer amounts. Solutions were prepared by weighting different amounts of CDs to a fix TCH^+ concentration at room temperature and mixed for 12 h to achieve the equilibrium. Spectra were obtained with residual solvent (HOD) pre-saturation and the experimental parameters included 24 k data points covering a spectral width of 8 kHz, a radiofrequency excitation pulse was 45° and an interpulse delay of 10 seconds to allow complete nuclei relaxation.

Two-dimensional (2D) rotating frame overhauser effect spectroscopy (ROESY) experiments were performed using the same NMR spectrometer and probe. The spectra were collected using 2048 data points in the F2 dimension and 512 increments defining the F1 dimension. The spectral width was 7.2 kHz in both dimensions and 32 free induction decays were acquired per increment. ROESY spectra were processed using the VNMR 6.1 software (Varian Inc., Palo Alto, CA, USA). Zero-filling and apodization Gaussian functions were employed in both dimensions before Fourier transformation, to improve resolution and signal to noise ratios, respectively. The cross-peaks volumes were directly correlated with inter-nuclear distance, r, of the two observed protons, via the known r^{-6} dependence. The fixed and well-known intramolecular distances between two vicinal aromatic protons (2.48 Å) of BZC – ortho and meta – were used for calibration.

2.5. Drug release studies

The in vitro release studies were conducted in vertical Franz diffusion cells (PermeGear, Inc., PA, USA) with a volume of 5.1 cm^3 and a surface area of 0.64 cm^2. The dialysis membranes were placed between the two chambers of the cell. The receptor compartment was filled with PBS solution (pH 7.4), maintained at 37 (±0.1) °C and under stirring during the diffusion experiments. Each donor compartment ($n = 3$) was filled with different TC hydrogel formulations (95 mM), composed of different amounts of CD and immediately covered with Parafilm® to prevent evaporation. Samples were taken at predetermined times (0, 1, 2, 3.5, 5, 6, 16, 24, 32, 46, 73, 110 and 142 h) from the receptor compartment and replaced by fresh PBS solution. In vitro studies provided the release profiles of TC and TCH^+ in the presence of different amounts of HP-β-CD, as a function of time. It is worth noticing that the HP-β-CD derivative was selected because it promoted the highest increase in solubility of TC.

2.5.1. Composition and preparation of the HPMC gel

Drug-loaded HPMC hydrogels were prepared by adding 2.5% (w/w) of TC with different amounts of HP-β-CD in water and sodium acetate buffer aqueous solution to 1% (w/w) of dry polymer powder (HPMC), at room temperature. The polymer solutions were kept stirring for 24 h before use.

2.5.2. Drug quantification

Tetracaine quantification was carried out using a Shimatzu LC-20AD apparatus equipped with a quaternary pump, an autosampler unit, and a L2450 UV/visible dual wavelength detector. The quantification of tetracaine was carried out using a Phenomenex (Torrance, CA, USA), reverse phase C_{18} column Luna (5 μm pore size, 250 mm × 4.6 mm) with a guard column as the stationary phase, at 25 °C. The mobile phase was prepared by mixing methanol, acetonitrile and ammonium acetate solution on the proportions 7:7:6 respectively. The solution was subsequently filtered using a 0.45 μm Nylon membrane (Supelco Analytical, Bellefonte, USA) and sonicated for 1 h prior to use. The detection was at 311 nm, the injection volume was 25 μL and the mobile phase flow rate was set to 1.0 mL min^{-1}. In these conditions the retention time of tetracaine was ca. 6.5 min.

2.5.3. Data analysis in the release studies

The steady state flux (J, μg cm^{-2} h^{-1}) which represents the rate transfer of diffusion substance through unit area of a section (A) was calculated by using the following equation:

$$J = \frac{V dc}{A dx} \tag{10}$$

where J is the cumulative drug amount permeated at time (t) calculated from the slope of the linear portion of the plot of cumulative drug amount permeated and V is the volume of the receptor compartment. Taking the Eq. (10) the permeability coefficient can be calculated by using Eq. (11):

$$P = J\frac{l}{Q_0} \tag{11}$$

where Q_0 is the initial amount of drug in the donor compartment and l the thickness of the membrane. Q_6 refers to the cumulative drug amount present in the receptor compartment solution after 6 h. Results are presented as mean ± standard deviation (SD).

2.5.4. Release Models

The release mechanism has been assessed using the power law equation (Korsmeyer et al., 1983).

$$\frac{C_t}{C_\infty} = kt^n \tag{12}$$

where C_t and C_∞ are cumulative concentrations of the drug released at time t and at infinite time, respectively, and k and n are fitting parameters, giving the latter useful information on the release mechanism; from Eq. (12), the mean dissolution time (MDT), which characterizes the drug release rate from a dosage form and to indicate the drug-release-retarding efficiency of the polymer (Sriamornsak and Sungthongjeeh, 2007) can be calculated using Eq. (13) (Möckel and Lippold, 1993).

$$MDT = \left(\frac{n}{n+1}\right)k^{-n^{-1}} \tag{13}$$

The application of Eq. (12) is restricted to cumulative release smaller than 60%.

The release kinetics was evaluated through first and second order rate law equations (Eqs. (14) and (15), respectively); we attempted to fit the experiments data to a zero order law equation but these fits were poor. For both cases the release rate is concentration-dependent.

$$\ln Q = \ln Q_0 - k_1 t \tag{14}$$

$$\frac{1}{Q} = \frac{1}{Q_0} - k_2 t \tag{15}$$

In equations (14) and (15), Q and Q_0 are the amount of drug remaining in the matrix at time t and at $t = 0$, respectively, Q_∞ is the total amount of all released drug and k_0 and k_1 are first and second order rate constants. Both the release kinetics and the mechanism of release were analyzed by using the Weibull function.

$$C_t = C_\infty[1 - \exp(-k_W t)^d] \tag{16}$$

where k_W and d are constants related to the release rate and mechanism, respectively (Papadopoulou et al., 2006).

3. Results and Discussion

3.1. Physicochemical characterization of the complexes

The formation of complex with CD alters the physicochemical properties of the drug. In general by adding CD to a poorly soluble

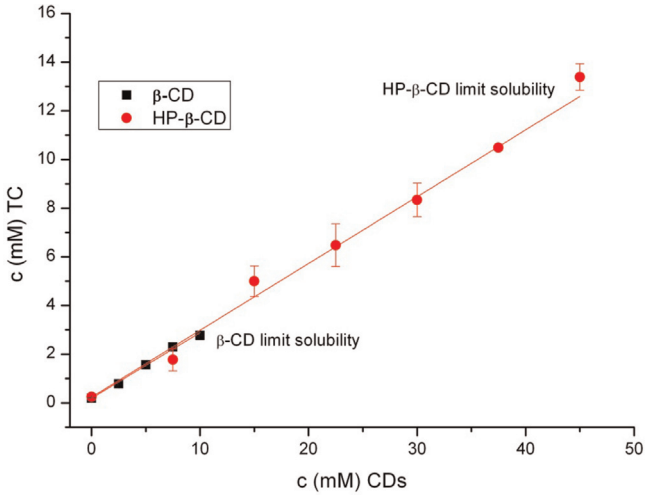

Fig. 2. Solubility of tetracaine in water aqueous solution (pH 9) as function of CD concentrations at room temperature.

drug aqueous solution it will increase of its solubility (Marques et al., 1990). The solubility phase diagrams (Loftsson et al., 2005; Loftsson and Masson, 2004) were obtained for tetracaine/β-CD and tetracaine/HP-β-CD mixed aqueous solutions at a pH of 9.0, at this pH, tetracaine occurs in its neutral form.

According with previous work an increase of either β-CD or HP-β-CD, leads to an increase in the concentration of TC dissolved in water (Sadlej-Sosnowska, 1997; Szejtli, 1998). Unionized TC is poorly soluble in water (51.4 μg/mL) and that the solubility was enhanced respectively by 14-fold and 69-fold respectively using β-CD (10 mM) and HP-β-CD (45 mM), as a consequence of formatting the drug-CD complexes (Connors and Mollica, 1966). The data shown in Fig. 2 fitted to a straight linear Eq. (1) with a good correlation coefficient $R^2 = 0.999$. By applying the fitting parameters mentioned in Table 1 into Eq. (1), the following stability constants, K, for the formation of TC:β-CD and TC:HP-β-CD complexes, in a 1:1 stoichiometry, were computed and are equal to 1052 M^{-1} and 1051 M^{-1}, respectively. These results are in agreement to those reported by other authors (Franco de Lima et al., 2012; Loukas et al., 1998).

Table 1 summarizes the association constants values (K_c), the maximum solubility and the efficiency of the complexation for TC:CD complexes, as computed from Fig. 2. Both CDs forms have similar stability constants (Table 1), however the HP-β-CD significantly improves the solubility of TC due to its higher solubility in water (Mura et al., 1995). From the analysis of tetracaine ionization constants it was noted that only approximately 9% of the total amount of TC was protonated at pH 9. The proportion was calculated considering the pKa of TC and according to Henderson-Hasselbalch equation (Becker and Reed, 2012). In order to investigate the effect of the protonated form of tetracaine in CD interactions, similar experiments were carried out at pH 4.1. At the acid pH, TC solubilized even in the absence of CDs (data not shown). At pH 9 the higher solubility of TC observed in the presence of CD was due to a more efficient complexation of the neutral form of TC with CD when compared with its ionized form. However, at pH 4, TC was completely ionized, the concentration of dissolved TC only slightly change as a function of CD concentration, indicating a more limited interaction between TC and CD. Similar results regarding the effect of ionization of drugs and its affinity for CD have been reported elsewhere (Brewster and Loftsson, 2007).

3.2. Stoichiometry and association constant as seen by fluorescence spectroscopy measurements

In order to further understand the interactions of CD with ionized TC, fluorescence spectrophotometry of TC/CD mix solutions, at different molar ratios at pH 4.1, were carried out (Fig. 3). Fluorescence spectroscopy is very sensitive to changes in the surrounding environment of the fluorophore. Therefore, this technique is able to provide accurate information of possible interactions occurring between TCH^+ and CD. This procedure has been reported in a previous study to calculate the concentrations of free (uncomplexed) ligands, needed for the calculation of the association constants (Sadlej-Sosnowska, 1997).

At pH 4.1 TCH^+ is the main species in solution although a small percentage of diprotonated tetracaine TCH_2^{2+} (ca. 16%) is also present. Fig. 3 shows the fluorescence emission spectra of TCH^+ at different TC/CD molar ratios. The relative fluorescence emission intensity of tetracaine, at 363 nm, increases in the presence of the cyclodextrins.

The emission enhancement can be used to determine the stoichiometry and the association constant of the TCH^+:CD complexes. Fig. 3 shows the variation of the fluorescence intensity (F) as a function of CD concentration for solutions where the [TCH^+] concentration was kept constant and equal to 1.0×10^{-5} M. The experimental data of F can be fitted to Eq. (9) (see solid lines in Fig. 4), using a non-linear least-square algorithm, to obtain the fitting parameters K and F_∞, assuming a 1:1 complex was formed.

Further support for the 1:1 association assumption is observed from the linear relationship of $((F - F_0)/[CD]_T)$ as function of $((F - F_0))$ for β-CD and HP-β-CD respectively, indicating that a 1:1 interaction stoichiometry is occurring Fig. 5 (Smith et al., 1991). This stoichiometry was also in agreement with complexation studies between TCH^+ and CD reported elsewhere (Fernandes et al., 2007). The fitting parameters obtained by mapping the data to Eq. (9) (Fig. 4) are: $K = 628$ (±24) M^{-1} and $F_\infty = 292$ (±3), and $K = 337$ (±11) M^{-1} and $F_\infty = 503$ (±4), for β-CD and HP-β-CD, respectively. It can be concluded from fluorescence spectroscopy studies, that TCH^+ form more stable complexes with β-CD than with HP-β-CD. Since both TCH^+ and HP-β-CD are highly soluble in water, it should be expected that a possible complexation would

Table 1
Effect of cyclodextrins on the solubility of tetracaine (TC) ($n = 3$).

System	Solubility (μg/mL)[a]	Efficiency of the complex[b]	Slope = [TC]/[CD]	R^2	K (M^{-1})
TC: β-CD	734[c] ± 0.05	14	0.2671	0.9947	1052 ± 118
TC: HP-β-CD	3539[d] ± 0.14	69	0.2874	0.9923	1051 ± 46

[a] Maximum of solubility of TC in the CD complexes.
[b] Ratio between TC solubility in the CD aqueous solution and the intrinsic solubility (51.4 μg/mL).
[c] Solubility computed by taking [β-CD] = 10 mM.
[d] Solubility computed by taking [HP-β-CD] = 45 mM.

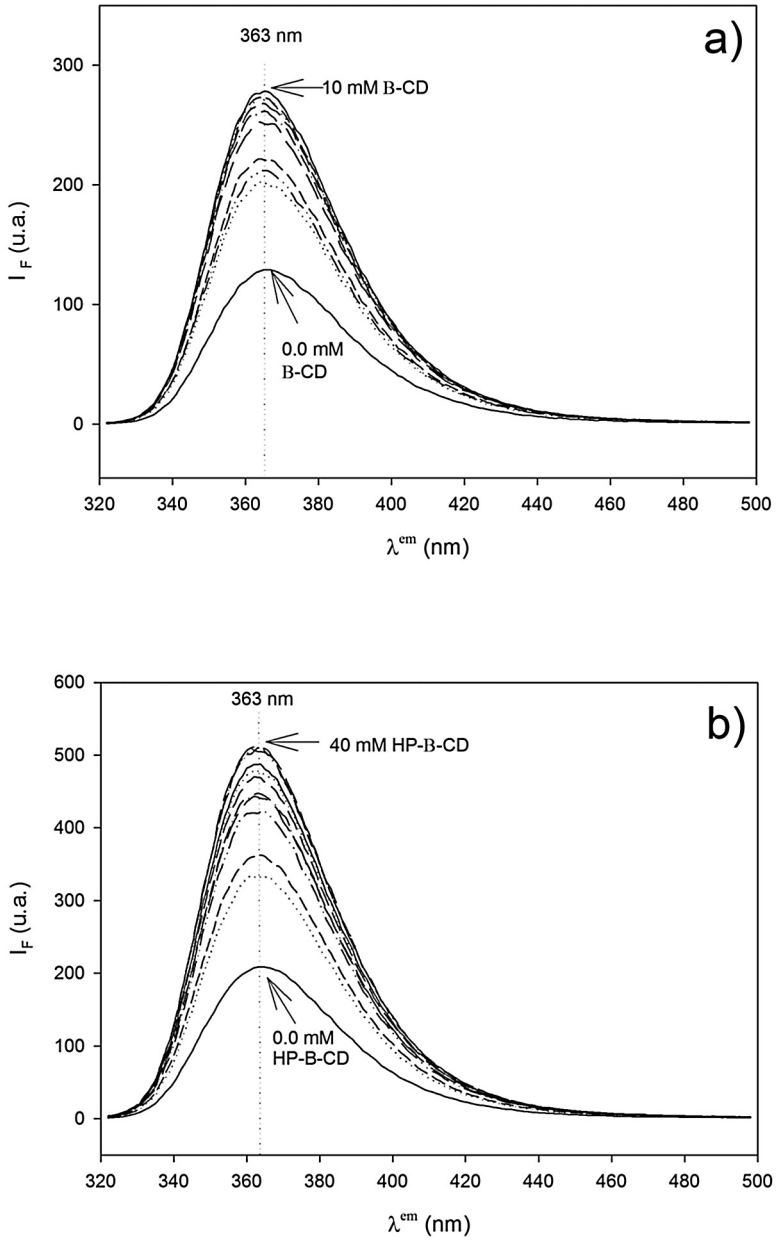

Fig. 3. Effect of the normalized fluorescence relative intensity by the initialconcentration of cyclodextrin as a function of fluorescence relative intensity forassessment on the stoichiometry of association. (o) β-CD and (□) HP-β-CD.

not cause a significant gain in the free energy of complexation. The same is not observed for β-CD, which can be justified by the relatively low solubility of this CD in water and consequently, the TCH^+ induces a stabilization effect on the β-CD in water interaction. These K values are lower than those obtained for neutral TC by using phase solubility diagrams but indicate that although interactions between CD: TCH^+ are weaker they should not be neglected. The TCH^+ structure could be incorporated inside CD cavity and if this was the case then the positive charge located at secondary amine induces a weak interaction between TCH^+ and CD (when compared with the corresponding neutral structure). Alternatively the TCH^+ may only interact with hydroxyl groups located at the extremities of the CD cavity. Although it is known that CD cavities can be penetrated by positive charged structures (Nilsson et al., 2008), the later hypothesis seems to be more reliable.

3.3. NMR analysis of TCH^+: CD complexes by ^{1}H-NMR and ROESY

In order to test if the TCH^+ did penetrate the CD cavity the interactions between TCH^+ and CD was characterized by ^{1}H-NMR and ROESY analysis on 1:1 and 1:2 TCH^+:CD mixtures. Fig. 6 shows the ROESY spectrum for a 1:1 TCH^+:HP-β-CD mixture. The attribution of each resonance on the 1D ^{1}H-NMR spectrum is given on the projection. A full analysis of the cross-correlations present in the ROESY spectrum shows intramolecular nuclear Overhauser interactions between TCH^+ protons (dotted lines) and HP-β-CD protons (solid lines), but not even a single intermolecular interaction between TCH^+ and HP-β-CD protons. The absence of any intermolecular cross-correlation in ROESY spectra is evidence of the TCH^+ inclusion by the CDs at pH 4.1, thus the TCH^+ and CD interactions were assayed to the formation of fast equilibria hydrogen bonds.

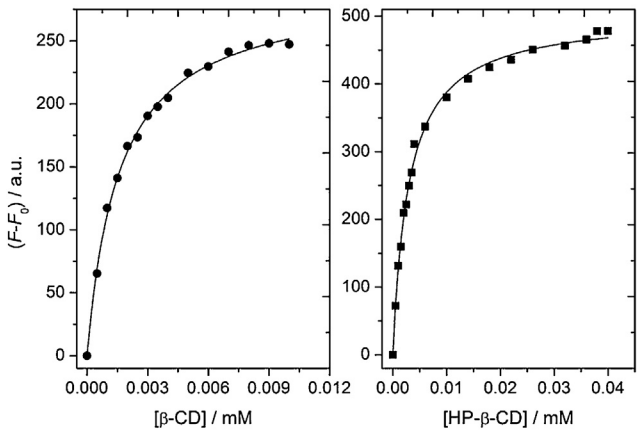

Fig. 4. Relative fluorescence intensity of tetracaine in the presence of β-CD and HP-β-CD. $[TCH^+] = 1.0 \times 10^{-5}$ M. Solid lines were obtained by fitting the Eq. (9) to experimental data.

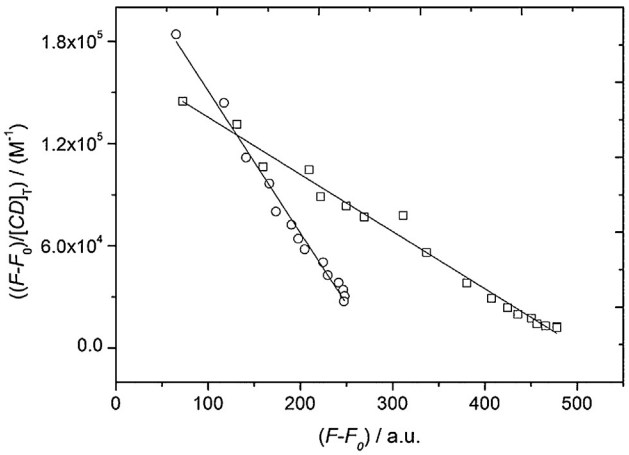

Fig. 5. Effect of the normalized fluorescence relative intensity by the initial concentration of cyclodextrin as a function of fluorescence relative intensity for assessment on the stoichiometry of association. (o) β-CD and (□) HP-β-CD.

3.4. Effect of cyclodextrins on the tetracaine delivery: In vitro release studies

3.4.1. Steady-state transport

The transport of TC from the HPMC gels, in the presence of different amounts of HP-β-CD, across the dialysis membrane, shows a steady state flux in the first 6 h of the experiment (see inset in Fig. 7). It can be seen that Q_6 (Table 2) decreases linearly with the increase of CD concentration, showing that CD controls the release of the drug.

Such effect can be ascribed to the corresponding formation of less mobile supramolecular structures (Loftsson et al., 2007), which might be explained by the occurrence of complexation equilibrium between the drug molecules and hydroxyl groups located at both ends of cyclodextrins cavities. The role of CD (either in the complex or free forms) on the transport of tetracaine can be assessed through the analysis of equilibria equations and the corresponding K values. Thus, for TC:HP-β-CD systems an increase of initial cyclodextrin concentration from 190 to 518 mM leads to an increase of free CD concentration from 96 to 406 mM, which corresponds to an increase of free CD from 51 to 81%, respectively. On the other hand such increase of CD concentration only leads to a decrease of free TC from 1 to 0.2%, giving indication that for both systems the amount of complexed TC is higher than 99%. Similar values are obtained for the ionized TC system. Once TC aggregation can be ruled out, it can be hypothesized that the increase of free CD plays an important role on the depletion of TC flux.

3.4.2. Release kinetic models

In the previous section we have found that the release of TC, either neutral or protonated, across the dialysis membrane, at short-range times, follows a steady-state flux. However, that is not valid for long-time range (i.e., for $t > 6$ h) (Fig. 7). On the basis of the discussion carried out in the previous section it is expected that the release mechanism of TC can be described by a power law equation with the exponential factor around to 1. In fact, taking into account the cumulative release of TC at equilibrium, and applying Eq. (11) in its linear form, we obtain n values ranging from 0.95 to 1.09 (Table 3), for TC:HP-β-CD (190 nm) and TCH^+:HP-β-CD (190 nm), respectively. Due to the cylindrical shape of the donor Franz cell compartment, those values are indicative of a complex mechanism, known as Super-Case II transport; such mechanism indicative of coupling of diffusional and relaxational mechanism (Costa et al., 2011; Polishchuk and Zaikov, 1997), occurs for processes with n values higher than 0.89 (Costa et al., 2010).

The MDT parameter characterizes the drug release rate from a dosage form, indicating drug release delaying efficiency of the polymer; the MDT of the drug increases with cyclodextrin concentration, from 32 to 41 h when the drug is in the neutral form (TC) and from 14 to 22 h when it is in the ionized form (TCH^+). However, by comparing these two forms of tetracaine, the effect is more pronounced for TCH^+ (38%) than for TC (23%). It can be concluded from release data and mechanism analysis that TC release kinetics is dependent on the protonation state of TC and CD concentration. C_∞ was determined for each formulation. By fitting of first- and second-order kinetics equations (Eqs. (13) and (14)) to the experimental release data, we can conclude that, in general, good correlation coefficients were found for both cases. Assuming the analysis of those correlation coefficients as a condition to conclude about what kinetics law characterizes a system, it was found that, for the release of TC in the presence of less concentrated CD, the release follows a first-order kinetics. However in the presence of the highest concentration of CD, the release of TC is characterized by a second-order kinetics law. This is close agreement with the previous discussion. When the CD increases from 190 to 431 mM, the percentage of free (not bound) CD decreases from 47% to 24% for TC-containing solutions (46–23% for TCH^+-containing solutions). Consequently, for the most concentrated mix solution, the release is controlled by free and complexed TC, whilst for solutions with 190 mM CD, the release kinetics seems to be only dependent on the TC concentration. Considering the analysis of the rate constants, they are in close agreement with such a CD concentration effect (by decreasing with an increase of CD concentration).

The equation that best fits the entire set of TC release data is the Weibull function (Eq. (15)). This empirical equation provides, simultaneously, information on the mechanism (d) and rate k_W of release. d values are higher than 1 (except for TCH^+ with the lower CD concentration), characteristic of a sigmoid S-shaped, with upward curvature followed by a turning point concomitantly, and of a Super-Case II release. With respect to rate constants (k_W), they are quite similar to those calculated through a first order rate law equation. It is also worth noticing that the release rate constants for the whole time range follows the same trend than that found for the first 6 h. Consequently, the Weibull function seems to be reliable for an assessment of both mechanism (d) and rate k_W of release of TC.

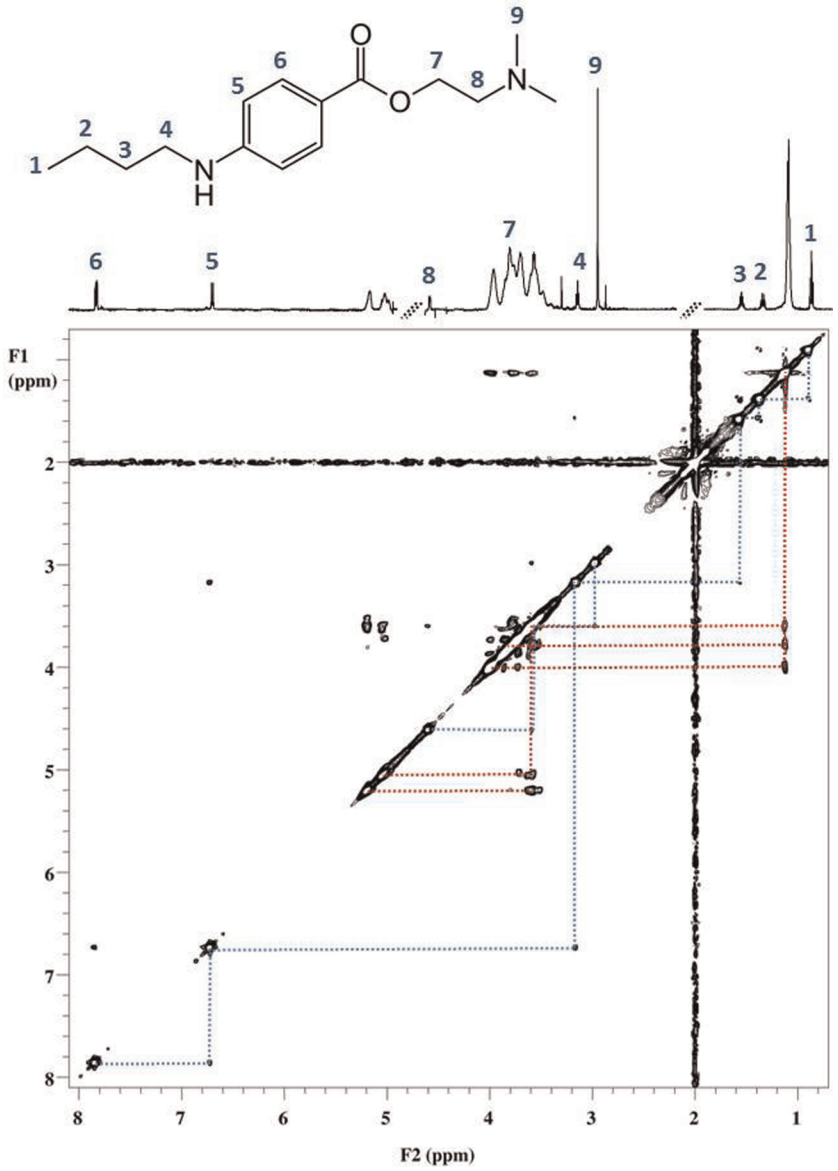

Fig. 6. 600 MHz ^{1}H-NMR and ROESY spectra of a 1:1 complex of TCH^{+}:HP-β-CD complex. Intramolecular nuclear Overhauser effects are detected for TCH^{+} (dotted lines) and HP-β-CD protons (solid lines). No intermolecular correlations are observed.

4. Conclusion

The work presented in this manuscript focuses on the effects of CD on the transport of TC across a model membrane. The results obtained show that the interaction between CD and TC causes changes in water solubility (14 and 69-fold for β-CD and HP-β-CD, respectively) and release profile of the latter. HPLC and fluorescence spectroscopy data revealed that TC interacts with both CD (β-CD and HP-β-CD) on a 1:1 stoichiometry. An interesting finding arises from analysis of the stability constants between TC and both CD. These values are very similar for TC in its neutral form (1052 and 1051 M^{-1} for TC:β-CD and TC:HP-β-CD respectively) but different when TC is ionized (628 and 337 M^{-1} for TC:β-CD and TC:HP-β-CD respectively), indicating that the TC forms more stables complexes with CD than with TCH^{+}. Additionally, ROESY experiments provided specific information concerning the non-inclusion

Table 2
Effect of the HP-β-CD concentration on tetracaine (95 mM) delivery across dialysis membrane.

Formulation	J ($\mu g\,cm^{2}\,h^{-1}$)	Q_6 ($\mu g\,cm^{2}$)	P ($10^{-3}\,cm^{2}\,h^{-1}$)
TC: HP-β-CD (190 mM)	203 ± 7	1215 ± 41	8.2 ± 0.3
TC: HP-β-CD (431 mM)	152 ± 5	923 ± 34	6.1 ± 0.2
TC: HP-β-CD (518 mM)	122 ± 5	740 ± 27	5.0 ± 0.2
TCH^{+}: HP-β-CD (190 mM)	362 ± 22	2168 ± 146	14.5 ± 0.9
TCH^{+}: HP-β-CD (431 mM)	234 ± 14	1415 ± 68	9.3 ± 0.6
TCH^{+}: HP-β-CD (518 mM)	204 ± 6	1247 ± 41	8.1 ± 0.2

Table 3
Fitting parameters of Eqs. (12)–(16) to experimental release data of tetracaine (95 mM) from HPMC matrices to receptor phase, at 37 °C.

		TC: HP-β-CD 190 mM	TC: HP-β-CD 431 mM	TCH⁺: HP-β-CD 190 mM	TCH⁺: HP-β-CD 431 mM
	C_∞ (μM)	5801	4016	7521	7253
PL[a]	n	0.95 (±0.05)	0.97 (±0.04)	1.09 (±0.03)	1.01 (±0.04)
	k	7.94×10^{-6} ($\pm 3.12 \times 10^{-7}$)	5.09×10^{-6} ($\pm 1.90 \times 10^{-7}$)	3.86×10^{-6} ($\pm 9.80 \times 10^{-8}$)	5.17×10^{-6} ($\pm 1.61 \times 10^{-7}$)
	R^2	0.9874	0.9888	0.9982	0.9945
	MDT (h)	31.6	41.1	13.9	22.3
FO	$k_1(h^{-1})$	3.01×10^{-6} ($\pm 8.47 \times 10^{-8}$)	$1.45x10^{-06}$ ($\pm 4.04 \times 10^{-8}$)	6.18×10^{-6} ($\pm 3.92 \times 10^{-7}$)	2.74×10^{-6} ($\pm 1.35 \times 10^{-7}$)
	R^2	0.99294	0.99304	0.9649	0.97843
SO	$K_2(M^{-1}h^{-1})$	4.37 ($\pm 0.18 \times 10^{-10}$)	1.76 ($\pm 0.03 \times 10^{-10}$)	1.32 ($\pm 0.13 \times 10^{-9}$)	3.794 ($\pm 0.128 \times 10^{-10}$)
	R^2	0.98539	0.99684	0.91521	0.98989
W	$k_W(h^{-1})$	6.51×10^{-6} ($\pm 3.02 \times 10^{-7}$)	4.75×10^{-6} ($\pm 2.1 \times 10^{-7}$)	1.13×10^{-5} ($\pm 1.16 \times 10^{-6}$)	8.61×10^{-6} ($\pm 4.23 \times 10^{-7}$)
	D	1.06 (±0.09)	1.12 (±0.086)	0.87 (±0.134)	1.03 (±0.093)
	R^2	0.9876	0.98827	0.96447	0.98798

[a] Fitting has been performed to cumulative release <60%. R^2: correlation coefficient; PL: power law Eq. (12); FO: first order, Eq. (13); SO: second order, Eq. (14); Weibull function, Eq. (15).

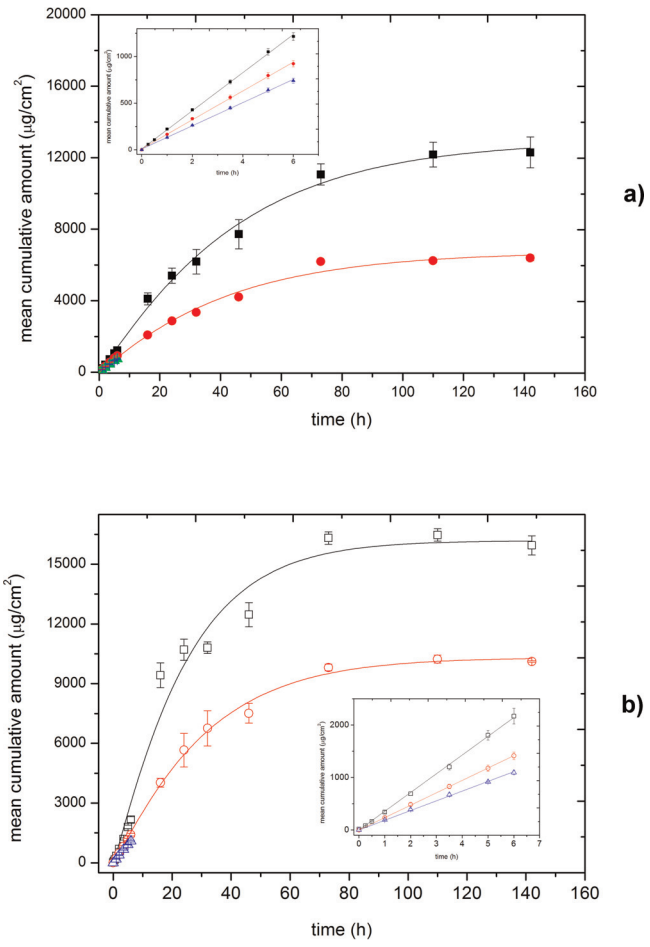

Fig. 7. Cumulative release of neutral (a) and ionized (b) tetracaine across dialysis membrane as a function of time, at different initial cyclodextrin concentrations: (■,□) 190 mM HP-β-CD, (●,○) 431 mM HP-β-CD and (▲,Δ) 518 mM HP-β-CD. Solid lines show the fitting of Eq. (16) to experimental data. Inset figures show the steady state flux of TC (A) and TCH⁺ (B) occurring in the first 6 h experiments.

nature of the interaction between TCH⁺ and cyclodextrins. Despite the stability constant values are relatively low, in vitro studies data show that the flux of TC and TCH⁺ across the dialysis membrane decreased with cyclodextrin concentration, following a Super-Case II transport mechanism. We have found that such behavior does not depend on the formation of inclusion host-guest compounds, but due to the formation of weak supramolecular compounds. The conclusions obtained are very important for future delivery studies, as the CD concentration used was found to be critical; it should be sufficiently high to improve bioavailability of the drug, but not excessively as it could result in drug retention within the formulation.

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