Studying the effect of solubilizing agents on drug diffusion through the unstirred water layer (UWL) by localized spectroscopy

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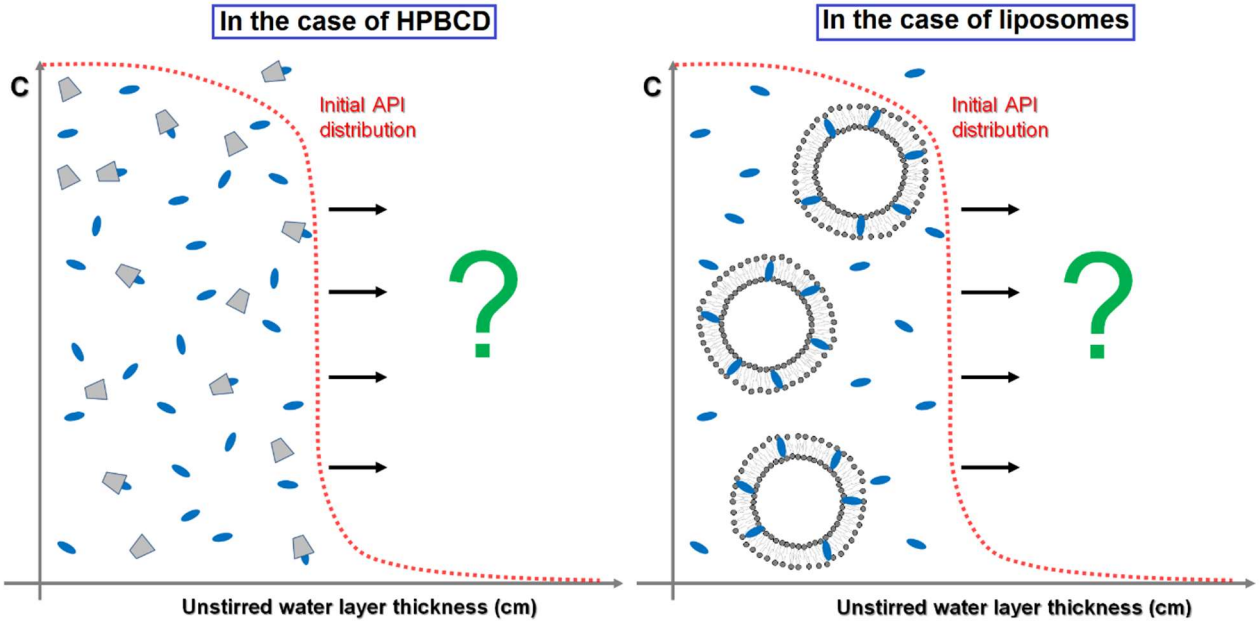
Abstract

An experimental/computational approach has been successfully applied in order to study the effect of solubilizing vehicles (cyclodextrins and liposomes) on the passive diffusion of four active pharmaceutical ingredients (API) of different nature (hydrophilic, ionizable and lipophilic) through an unstirred water layer (UWL) model. This approach allowed the measurement of flux changes through the UWL and the computational calculation of different parameters relevant to interpret the interplay within solubilizing vehicles and UWL diffusion. In the case of cyclodextrin, this approach allowed the determination of free drug diffusivity (D_f), bound drug diffusivity (D_b) and the equilibrium constant (K). In the case of liposomes, the experimental approach allowed the determination of the liposomes/water partition coefficient ($P_{lip/w}$) as well as relative API diffusivity ($\overline{(D)}$, i.e. the drug diffusion in the presence of solubilizing agents). This work demonstrates that the presence of solubilizing vehicles hampers the diffusion of API through UWL due to a combination of reduction in

relative diffusivity and concentration gradient. These results are highly relevant as they might help to explain why biological performance of API is affected by the presence of solubilizing/complexing agents.

Keywords: Unstirred water layer, passive diffusion, gradient of concentration, solubilizing agents, cyclodextrin, liposomes.

Graphical abstract



1. Introduction

More than 40% of marketed drugs and 90% of new chemical entities under development with promising pharmaceutical activities suffer from poor water solubility [1]. In an attempt to increase the biopharmaceutical performance of these compounds one approach that has been extensively studied in the recent year has been the employment of solubilizing agents [2] under the assumption that increased apparent aqueous solubility of the drug will result in increased bioavailability. Since their first descriptions in the middle of last century cyclodextrins and liposomes have been heavily studied in light of their strong ability to solubilize lipophilic compounds [3, 4]. Cyclodextrins are capable of solubilizing lipophilic entities due to inclusion complexes formation [5] whereas liposomes incorporate lipophilic as well as amphiphilic moieties in the phospholipid bilayers of which liposomes consist of [4]. Even though in most cases these entities are capable of solubilizing poorly soluble substances of orders of magnitude [6, 7] resulting in a positive enhancement of bioavailability, in some cases biopharmaceutical performances are reduced [8]. It has been suggested that the negative influence of some solubilizing agents (dose dependent effect [9]) on biopharmaceutical performance of drug is related to a reduction in API transport (i.e. mass transfer) through biological barriers. Furthermore, some studies have emphasized the role of the unstirred water layer (UWL [10, 11]) as the limiting step of the transport process through barriers [12, 13]. The UWL represents an additive aqueous layer that covers biological barriers where conditions of stagnation hold [14] and that drug molecules need to cross before entering in contact with the lipophilic environment represented by cell membranes [12, 13]. Considering the UWL as a homogeneous environment, where molecules will spontaneously diffuse through, the flux (j) of an API through this layer can be described by Fick's first low as Eq. 1:

$$j = -D\frac{dc}{dx}$$ Equation 1

In this equation, D represents the diffusion coefficient of API molecules in the UWL and dc/dx the local concentration gradient. Brewster et al. [15] investigated the *effective permeability* of different drugs and hydroxypropyl-β-cyclodextrin (HPBCD) through a parallel artificial membrane permeability assay (PAMPA) in the presence of UWL of different thickness. They found that, for molecules with high affinity for HPBCD (i.e. lipophilic) the permeability of the drug was reduced by increased cyclodextrin concentration, whereas, for compound with low HPBCD-API equilibrium constant (K), no significant reduction was observed. Dahan et al. [16, 17] tried to describe the interplay between permeability/complexing agents and UWL with the quasi-equilibrium mathematical model. In this case they utilized a cellular-based permeability assay (Caco-2), PAMPA and an animal model in order to investigate the *effective permeability* of drugs in the presence and absence of cyclodextrins in order to understand the role of UWL in drug permeability in presence of complexing agents. In accordance with Brewster at al. they have found a correlation between reduction in drug permeability and HPBCD concentration. Some mechanistic explanations have been suggested to describe this interesting phenomenon [15-17]. One hypothesis is that HPBCD reduces the amount of free fraction of drug available, decreasing the concentration gradient (dc/dx) and therefore reducing the net flux of drug molecules through the UWL (Eq. 1) [17]. Another explanation that has been proposed is related to partitioning and permeability. According to Fine-Shamir et al. [18] the presence of cyclodextrin should reduce the ability of API molecules to distribute through the lipophilic environment (i.e. reduction in apparent distribution coefficient) negatively affecting the net transport of the drug through the whole barrier. Stewart et al. (2017) introduced a new analytical method capable of discriminating the limiting step in permeability within UWL or the membrane in the presence of bioavailability-enhancing drug products [19]. They identify two main mechanisms of

permeation, in which the API flux through the barrier is influenced by the total concentration gradient of the drug (i.e. free drug and bound drug) only when the UWL is the limiting step of the permeation. Even though all these studies indicate UWL as responsible for the reduction of the overall mass transfer in the presence of solubilizing agents, a proper mechanistic explanation of the phenomena is still missing. The aim of this work is to experimentally measure and mathematically describe the diffusion of API molecules through an UWL in the presence of two types of solubilizing vectors: cyclodextrins and liposomes. In this work we applied the analytical/computational approach based on temporal resolution of diffusion profiles in UWL recently introduced by us [20] in order to effectively quantify the changes in API flux through an UWL in the presence of solubilizing vectors. In this way we could derive all parameters relevant for the characterization of diffusion process namely, diffusivities, equilibrium constants and concentration gradients. This new approach is quite unique as it allows the real-time measurement of relative flux changes, allowing the direct characterization of all relevant parameters in the UWL. The results obtained in this work highlight the role that the UWL plays in permeation of drugs, especially when solubilizing vehicles are present.

2. Materials and Methods

2.1 Materials

All buffering agents (sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), sodium chloride (NaCl) and sodium hydroxide (NaOH)), active ingredients (caffeine(caf), ibuprofen (ibu), ibuprofen sodium salt (Na-ibu) ketoprofen (ket) and hydrocortisone (hc), Table 1) and organic solvent employed in this work (methanol) were purchased form Sigma Aldrich Chemie GmbH (Steinheim, Germany). Soy phosphatidylcholine (S-100) was a generous gift form Lipoid GmbH (Ludwigshafen, Germany). 2-hydroxylpropyl β-cyclodextrin (HPBCD) with estimated

molecular weight of 1396 g/mol and average degree of substitution within 0.5-1.3 (defined as unit of 2-hydroxypropyl per glucose unit) was also purchased form Sigma Aldrich or, alternatively, from Roquette Freres (Lestrem, France).

Table 1: Molecular weight (mw), ionization constant (pKa) distribution coefficient at pH 7.4 ($LogD_{7.4}$), topological polar surface area (TPSA) and molar volume (V_m) of the investigated compounds.

Drug	**mw** **g/mol**	**pKa[21]**	**$LogD_{7.4}$**	**TPSA[21]** **Å^2**	**V_m[24]** **cm^3/mol**
caf	194.2	10.4	-0.03[22]	58.4	133
hc	362.5	-	1.51[22]	98.4	281
Ibu/Na-ibu	206.3/228.3	4.91/≈	1.00[23]/≈	37.3/≈	200/≈
Ket	254.2	4.45	0.19[23]	54.4	212

2.2 UV-visible localized spectroscopy

2.2.1 API solutions preparation

In order to obtain a 73 mM neutral (pH 7.4) and isotonic (280-290 mOsm) phosphate buffer saline (PBS), a solution of $NaH_2PO_4 \cdot H_2O$ (2.2% W/V) was mixed in a ratio 1:5 with a solution of $Na_2HPO_4 \cdot 12H_2O$ (1.8% W/V). The pH of was subsequently adjusted to 7.3−7.4 (pH meter Lab 744, Metrohm AG, Herisau, Switzerland) by the addition of NaOH solid pellets whereas the tonicity was brought to 280−290 mOsm (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) by the addition of NaCl solid crystals. Each of the API investigated was dissolved in the PBS solution in order to achieve a final drug concentration in the range 1- 6 mM.

2.2.2 Cyclodextrin-API samples preparation

The complexation studies were conducted following the basic principle of standard phase-solubility studies [25] therefore exposing the same amount of API to increasing concentration

of the complexing agent. For caffeine, hydrocortisone and ketoprofen, a stock solution of the complexing agent (in this work HPBCD) was prepared dissolving approximatively 3.6 g of cyclodextrin derivative in PBS in order to obtain a 50 mM HPBCD solution. One mL of drug solution was mixed together with increasing volumes of HPBCD stock solution (form 0 mL up to 1 mL) inside standard Eppendorf vials, in order to achieve a minimum of 5 samples with increased cyclodextrin concentration (ranging from 0 mM up to 25 mM) and constant API concentration (samples $caf_{0\text{-}4}$, $hc_{0\text{-}4}$, $ibu_{0\text{-}5}$, $ket_{1\text{-}5}$ in Table 2).

Table 2. Concentration of active pharmaceutical ingredient (API), 2-hyrdoxypropil β-cyclodextrin (HPBCD), soy phosphatidylcholine (SPC) and buffer in each of the samples investigated. Each sample was analyzed at maximum wavelength of absorption (λ_{max}) and the local concentration was calculated using its specific API absorptivity (ε).

Sample	API conc. mM	HPBCD conc. mM	SPC conc. mM	Buffer conc. mM	λ_{MAX} nm	ε $cm^2/\mu mol$
			Caffeine			
caf_0	0.9	-	-	72.8	272	9.7
caf_1	//	1	-	//	//	//
caf_2	//	5	-	//	//	//
caf_3	//	10	-	//	//	//
caf_4	//	25	-	//	//	//
caf_5	//	-	25	//	//	//
			Hydrocortisone			
hc_0	0.5	-	-	72.8	247	11.9
hc_1	//	1	-	//	//	//
hc_2	//	2.5	-	//	//	//
hc_3	//	10	-	//	//	//
hc_4	//	25	-	//	//	//
hc_5	//	-	25	//	//	//
			Ibuprofen			
ibu_0	1.3	-	-	72.8	221	9.0
ibu_1	//	0.5	-	//	//	//
ibu_2	//	1	-	//	//	//
ibu_3	//	2.5	-	//	//	//
ibu_4	//	5	-	//	//	//
ibu_5	//	10	-	//	//	//
ibu_6	//	-	25	//	//	//
			Ketoprofen			
ket_0	1.4	-	-	72.8	260	16.5

ket_1	//	0.5	-	//	//	//
ket_2	//	2.5	-	//	//	//
ket_3	//	5	-	//	//	//
ket_4	//	10	-	//	//	//
ket_5	//	25	-	//	//	//
ket_6	//	-	25	//	//	//

For ibu, 0.4 mL of API stock solution in PBS (6.32 mM) were mixed inside standard 2 mL Eppendorf vials with increasing volumes (form 0.0 mL to 1.0 mL) of a 20 mM HPBCD PBS solution (Table 2). PBS was used in order to fill in the missing volume up to two mL. Samples were stored at room temperature prior to analysis.

2.2.3 Liposomes-API samples preparation

A liposomal dispersion was prepared following the standard thin-film hydration method [26]. In brief, approximatively 2 g soy phosphatidylcholine (S-100) were dissolved into 50 mL of methanol in a round bottom flask. The organic solvent was removed by controlled vacuum evaporation (25°C; 1 hour; 60–65 mBar final vacuum) employing a Büchi rotary evaporator system (model R-124), equipped with a water bath (model B-480) and vacuum pump (model V-500; Büchi Labortechnik AG, Flawil, Switzerland). Large liposomes dispersion was obtained by reconstituting the lipid film obtained after solvent removal with 50 mL PBS. The liposomal dispersion was subsequently extruded throw 800 nm (4 cycles) and 400 nm (4 cycles) polycarbonate filters (Whatman International Ltd., Bucking-hamshire, UK) in order to obtain a homogeneous dispersion of medium-sized liposomes (average diameter approximately 400 nm). Prior to analysis, one mL of the liposomal dispersion was mixed with 1 mL of API solution inside an Eppendorf vial (samples caf_5, hc_5, ibu_6 and ket_6 in Table 2). Samples were incubated for 10 min prior to analysis.

2.2.4 Analytical method

The analytical method recently introduced by us [20] was employed in this work to investigate the influence of cyclodextrins and liposomes on API diffusion in aqueous media.

For the spectrophotometric measurements, a double array VWR (VWR International, Radnor, USA) UV-visible spectrophotometer (model UV-6300 PC) equipped with a Hellma® Suprasil® (Sigma-Aldrich) quartz absorption cuvettes (chamber volume of 700 µL and path length of 10 mm) was employed. Both reference and sample cuvette were filled with the same volume of distilled water (675 µl and placed in the respective compartment of the spectrophotometer). At time (t) = 0 sec (starting of the experiment), 25 µL of one sample were gently injected in the bottom of the sample cuvette by a needle syringe. In order to avoid evaporation of water, the sample cuvette was sealed with parafilm right after sample injection. Absorbance readings were recorded at fixed wavelength (corresponding to the λ_{MAX} of each of the compounds, Table 2) at regular time intervals (120 sec) for 18 hours at room temperature (23-24°C). Absorbance was recorded at 0.51 cm from the bottom of the cuvette (h_m).

2.2.5 Mathematical data treatment

The mathematical approach previously described by us [20] was employed in order to calculate both reference diffusivity (D_0, the diffusivity of the API in absence of solubilizing vehicles) and apparent diffusivities ($\overline{D}$, the diffusivity measured in the presence of solubilizing vectors). In brief, the spontaneous process of molecules migrating through a homogeneous medium (in this case water) is described by Equation 2 as:

$$\frac{\partial c(x,t)}{\partial t} = D\frac{\partial^2 c(x,t)}{\partial x^2} \qquad \text{Equation 2}$$

In this equation, c represents the concentration of the substance (in this case the API concentration), t the time, x the position, and D the diffusivity.

Assuming times (t) and positions (x) such that $t \ll h^2/D$ and $x \ll h$ (where h is 3.30 cm, the full length of the cuvette occupied by water), eq. 2 can be solved analytically as:

$$c(x,t) = \frac{A}{\sqrt{\pi}} \frac{e^{\frac{-x^2}{2\sigma^2+4Dt}}}{\sqrt{2\sigma^2+4Dt}} \quad \text{Equation 3}$$

Where σ represents the width of the initial distribution (considered to be a half gaussian curve) and A represents the initial amount of the API. Equation 3 was fitted to the experimental data in order to find the best solutions for both D, A and σ.

The calculation of constant of equilibrium (K) was based on the assumption that for the 1:1 complex (L·S) formation between API molecules (i.e. the substrate, S) and a ligand (Eq. 4):

$$L + S \leftrightharpoons L \cdot S \quad \text{Equation 4}$$

For an ideal diluted solution, it can be assumed that the equilibrium constant (K) of complexation is given by:

$$K = \frac{[S \cdot L]}{[S][L]} \quad \text{Equation 5}$$

Knowing the initial concentration of the ligand (L_0), the substrate (S_0) and the equilibrium concentration of the complex (Q), equation 5 can be re arranged as:

$$K = \frac{Q}{(S_0 - Q)(L_0 - Q)} \quad \text{Equation 6}$$

Solving this expression yields two values for Q, whereof only one lies in the range $0 \leq Q \leq \min(L_0, S_0)$ (Eq. 7):

$$Q = \frac{1}{2K}\left(1 + (L_0 + S_0)K - \sqrt{1 + 2(L_0 + S_0)K + (L_0 - S_0)^2K^2}\right) \quad \text{Equation 7}$$

Assuming fast exchange between API in the free and the bound states, the measured value for diffusion ($\overline{D}$) will be the weighted average of the diffusions of the free and bound molecules (D_b and D_f respectively). The relationship between the different diffusivities is described by Eq. 8:

$$\overline{D} = MF_bD_b + MF_fD_f = \frac{Q}{L_0}D_b + \left(1 - \frac{Q}{L_0}\right)D_f = D_f + \frac{Q}{L_0}\left(D_b - D_f\right) \quad \text{Equation 8}$$

Where MF_b and MF_f represent the molar fractions of the bound and free substrate respectively. Inserting Eq. 7 in Eq. 8 gives a final expression of $\overline{D}$ as a function of L_0 that can be fitted to the experimental data (keeping S_0 constant, see section 2..2.2 and Table 2) and allows for the quantification the diffusivities of bound and free API (D_b and D_f respectively). Partitioning of API into liposomes ($P_{lip/w}$) was calculated using the following equation:

$$P_{lip/w} = \frac{(A_{lip} - A_0)}{A_0} * \frac{V_0}{V_{lip}} \qquad \text{Equation 9}$$

Where A_0 represents the initial API amount in the reference experiments (i.e. no liposomes), A_{lip} the amount in the liposomes experiments and V_0 and V_{lip} represent the liposome-free volume fraction of the injected volume (estimated to be 22 µL) and the volume occupied by the liposomes (estimated to be 3 µL) respectively.

2.3 Nuclear magnetic resonance (NMR) spectroscopy

10 µL of a 7.5 mM Na-ibu non-isotonic PBS solution (10% deuterated water) were added to 590 µL of a 5.7 mM HPBCD non isotonic PBS solution (10% deuterated water) in a standard 5 mm NMR tube, yielding a final solution of concentrations of 0.1 mM and 5.6 mM for Na-ibu and HPBCD respectively. The NMR experiments were performed employing an Agilent DD2 NMR (Agilent Technologies, Santa Clara, USA) spectrometer functioning at a proton frequency of 599.671 MHz. Temperature was stabilized at 30℃ during all experiments. Diffusion constants were measured using a standard DgsteSL sequence with convection compensation and treated with the DOSY package.

3. Results and discussion

3.1 quantification of diffusion coefficients in absence of binding agents

In table 3 the results from the diffusion studies of the API (caf, hc, ibu and ket) in PBS solutions without binding agents (i.e. neither HPBCD nor liposomes) are reported. In all experiments, the data recording position (h_m) was used as fixed parameter (0.51 cm) whereas A_0 and D_0 were fitting parameters. The nominal equilibrium concentration (c_{eq}) correlates

very well (R^2 of 0.99) with A_0, indication of very good correspondence between experimental and computational data.

Table 3. Nominal equilibrium concentration (c_{eq}), initial amount (A_0), width of the initial distribution (σ) and reference diffusivities (D_0) of the reference drug (caf, hc, ibu and ket) samples. All parameters were obtained by fitting the analytical solution of diffusion equation (Eq. 3) to experimental data of API solutions recorded at x=0.51cm.

Sample	**c_{eq} (mM)**	**A_0**	**σ**	**D_0 (10^{-6} cm^2/sec)**
caf_0	0.03	0.231	0.104	9.120
hc_0	0.02	0.145	0.111	6.442
ibu_0	0.05	0.331	0.101	7.788
ket_0	0.05	0.408	0.055	7.724

The Stokes-Einstein equation relates the diffusion constant (D) to the radius of a hypothetical sphere (r), the temperature (T) and the viscosity (η) via (Eq. 10):

$$D = \frac{k_B T}{6\pi\eta r} \qquad \text{Equation 10}$$

Where k_B is Boltzmann's constant. Assuming that all the experiments are performed at the same temperature (T) and that concentration of the API is so low that the viscosity (η) is not affected we can expect a linear correlation between molar volume (Vm, Table 1) and D_0. Diffusion coefficient values are consistent with previous finding [20] and indeed, fitting D_0 to the estimated molar volumes yields a straight line (R^2=0.99). Hydrocortisone is the largest molecule within the investigated series (V_m of 281 cm^3/mol, Table 1) and because of that it shows the lowest D_0 (6.4 *10^{-6} cm^2/sec) whereas caffeine, that is the smallest of the investigated compounds (V_m of 133 cm^3/mol), expresses the highest D_0 (9.2*10^{-6} cm^2/sec). Ibuprofen and ketoprofen have very similar V_m (200 and 212 cm^3/mol) in between caffeine and hydrocortisone and this is reflected in similar diffusivities (7.8 and 7.7 * 10^{-6} cm^2/sec

respectively) comprised between the other two compounds (Table 3). The data reported in Table 3 are fundamental as they are the reference data to which experimental data collected from samples with solubilizing vehicles should be compared with.

3.2 Diffusion coefficients in the presence of HPBCD

Table 4. Nominal equilibrium concentration (c_{eq}), calculated initial amount (A), width of the initial distribution (σ) and relative diffusivities ($\overline{D}$) of the investigated compounds (caf, hc, ibu and ket) in the presence of increasing concentration of HPBCD.

Sample	HPBCD conc. (mM)	C_{eq} (mM)	A	σ	$\overline{D}$ (10^{-6} cm^2/sec)
		Caffeine			
caf_1	1	0.03	0.233	0.105	9.089
caf_2	5	//	0.223	0.104	8.335
caf_3	10	//	0.220	0.103	8.070
caf_4	25	//	0.221	0.104	7.924
		Hydrocortisone			
hc_1	1	0.02	0.132	0.119	5.149
hc_2	2.5	//	0.134	0.110	4.092
hc_3	10	//	0.135	0.122	3.415
hc_4	25	//	0.132	0.121	3.400
		Ibuprofen			
ibu_1	0.5	0.05	0.319	0.115	5.760
ibu_2	1	//	0.327	0.125	5.265
ibu_3	2.5	//	0.312	0.134	3.787
ibu_4	5	//	0.323	0.125	3.554
ibu_5	10	//	0.334	0.122	3.110
		Ketoprofen			
ket_1	0.5	0.05	0.389	0.062	6.828
ket_2	2.5	//	0.372	0.096	5.468
ket_3	5	//	0.342	0.114	4.796
ket_4	10	//	0.357	0.122	4.061

ket_5	25	//	0.326	0.125	3.213

Data recorded at x=0.51 cm

Fig. 1 reports the experimental data (blue line) and fit (red line) of ibuprofen in the presence of increasing HPBCD concentration (Fig. 1, ibu_0-ibu_5). The other compounds show similar behavior. The diffusion profiles change when the concentration of the binding agent is increased. Specifically, the slope of the rising section of the curves decreases whereas the curvature at the top becomes more gentle and the time where the maximum occurs (t_{max}) increases. The fitting of Eq. 3 to the experimental data was very good in all circumstances and in accordance with our previous work (fitting error below 1%, [20]). In Table 4 the initial amount (A) and diffusivities ($\overline{D}$) obtained from the data fitting are reported for each of the API investigated.

It should be highlighted that, for all drugs, increasing the HPBCD concentration results in a decrement in diffusivities, showing that all compound bind to HPBCD. The magnitude of the variation depends on the binding constant and varies significantly between the investigated compounds. For instance, in the case of caffeine, even at the highest concentration of HPBCD (25 mM) the relative diffusion identified is only 14 % lower than D_0 (Table 3 and 4). For all the other compounds, the impact of cyclodextrins on API diffusion is much more severe. At the highest concentration of HPBCD (25 mM), the decrease in diffusivities exceeds 50 % in the case of ibuprofen and hydrocortisone whereas for ketoprofen it is 47%. This data gives a picture of what is happening when cyclodextrins bind an API. As the HPBCD-API complex is larger than the API alone, we expect the complex to diffuse slower than the free API, as indeed is the case. In other words, binding with cyclodextrins has a negative effect on the net drug transport through the UWL. This results are in agreement with previous findings [15-17]. The data obtained in this work give also a better and clearer picture of the reason why drug transport of drugs through UWL is affected by the presence of solubilizing vehicles such as

cyclodextrins. From the data obtained in this work it is evident that for hydrocortisone, but also for the ionizable compound ibu, the gradient of concentration is produced by both free and complexed API molecules. This is demonstrated by the fact that the estimated initial drug amount of API (A) does not change significantly with increased concentration of HPBCD (Table 4, and therefore with increased API-HPBCD complexation) in the UWL. Moreover, these findings are in partial agreement with Stewart at al. [19] where they found that the net flux of itraconazole through a biomimetic barrier was proportional to the total apparent solubility of the drug in the donor (i.e. both bound and unbound API fraction in solution). However, in the present work, the total flux of all APIs investigated resulted reduced through the UWL and not improved by the presence of a solubilizer. This is already an interesting findings that exclude the role of concentration gradient as the responsible for the reduction of API flux observed. In the case of ketoprofen, there is a clear trend in reduction in A with increased HPBCD concentration (Table 4) and this could indicate that there is a decrease in ket molecules available with increased HPBCD. This could be explained by the formation of macromolecular aggregates [27] that reduces the initial concentration gradient (driving force of passive diffusion). From these data it is clear that, especially with compounds forming stable complex with HPBCD, the complex API-HPBCD is maintained also in diluted conditions (i.e. after injection), and this fact produces the reduction of API diffusing through the UWL measured. It is evident from these data that, and agreement with previous findings [15-17], cyclodextrins clearly hamper the diffusion of API through the UWL. In partial disagreement with previous reports [15] however, it appears that also hydrophilic compounds (in this case caffeine), are affected in their diffusion through the UWL at high concentration of HPBCD, even though the binding constant of caffeine to HPBCD is low [28].

3.3 calculation of K, D_f and D_b

The decrease of the relative diffusion coefficient measured when the API are complexed with cyclodextrin depends on the binding constant. After injection at the bottom of the cuvette, free API molecules, free HPBCD and API-HPBCD complex will start to diffuse. In accordance with Stokes-Einstein equation (Eq. 10), assuming similar experimental conditions (absolute temperature (T) and viscosity of the media (η)) in each experiment, the free API and API-HPBCD complexes will show different diffusivities (D_f and D_b respectively) determined by their size (hydrodynamic radius (r)).

In Fig. 2 the relationship between apparent diffusivity ($\overline{D}$) and HPBCD concentration is reported for all the compound investigated. Fitting the experimental data with equation 8 and 9 (red line, Fig. 2) it is possible to obtain numerical values for the equilibrium constant (K) and the diffusivities of bound and free API (D_b and D_f respectively). The results are reported in Table 5.

Table 5. Equilibrium constant (K), diffusivity of free API (D_f) and complexed API (D_b) identified for each of the investigated compound (caf, hc, ibu, ket) in the experiment performed in the presence of HPBCD.

API	**K** $\mathbf{M^{-1}}$	**D_f** $\mathbf{10^{-6}\ cm^2/sec}$	**D_b** $\mathbf{10^{-6}\ cm^2/sec}$
caf	243 ± 151	9.2 ± 0.1	7.6 ± 0.3
hc	1028 ± 246	6.5 ± 0.1	3.2 ± 0.1
ibu	4058 ± 2890	7.6 ± 0.3	3.1 ± 0.3
ket	381 ± 102	7.5 ± 0.2	2.9 ± 0.3

For all compounds, D_f is very similar to D_0 (Table 3, discrepancy of 1%). Moreover, the equilibrium constants obtained are in good agreement with literature data [15, 28-31]. Ibu and hc are the compounds with the strongest equilibrium constant and therefore their diffusion through the UWL is most affected. For ibu, ket and hc, D_b is close to $3*10^{-6}cm^2/sec$. This value seems very reasonable, as the size of the inclusion complex API-HPBCD is mostly due

317 to the cyclodextrin (Mw of 1.4 kDa) and DOSY NMR results showed that the diffusion
318 constant for HPBCD in water is $2.9*10^{-6}$ cm^2/s. Moreover, NMR results with Na-ibu
319 evidenced that API-HPBCD complex diffuses at the same rate as HPBCD alone. Caffeine
320 expresses a much higher value for D_b (over $7*10^{-6}$ cm^2/sec). Theoretically, this value should
321 be much lower and close to $3*10^{-6}$ cm^2/sec (as with the other API investigated). It is quite
322 plausible that the Caf-HPBCD complex is more affected than the others by rapid on-and-off
323 kinetics (due to poor complex stability, see equilibrium constant values in table 5). This fact
324 makes a correct estimation of D_b impossible with the current technique, and this might be an
325 issue for all complexes with low K. We are aware that the obtained value lies outside the
326 expected range and will investigate the system further in the near future. From these data we
327 can anyway conclude that measured reduction in API flux through the UWL in the presence
328 of HPBCD is not due to a reduction in the concentration gradient but it is mostly due to the
329 reduction in relative diffusivity of API. In fact, API-HPBCD complexes diffuse much slower
330 through the UWL then free APIs ($D_b << D_f$, see Table 5), therefore $\overline{D}$ will decrease with
331 increasing concentration of HPBCD. This reduction in apparent diffusivity is, in practice,
332 directly corresponding to a reduction in the amount of API passing through the UWL. It
333 appears also clear from our results that the more stable the complex API-HPBCD is (i.e.
334 higher is K), the more significant this phenomenon will be.

335 **3.4 Partitioning and relative diffusivities**

336 The experiments involving liposomes were conducted similarly to the cyclodextrins ones but
337 in this case each of the API was incubated for 10 minutes previous injection in the cuvette
338 with a liposomal dispersion containing 25 mM phosphatidylcholine S-100 of 400 nm average
339 diameter. In this case, liposomes due to their sizes (dm. = 400 nm) were located on the bottom
340 of the cuvette for the duration of the experiment, differently from the cyclodextrin
341 experiments where the API-HPBCD complexes were also diffusing. For all compounds

342 investigated, a reduction in apparent mass transport of API through the UWL was measurable
343 when liposomes were present. The fitting to the experimental data in this case reveals that,
344 differently from cyclodextrins, the initial amount of API measured (A) was reduced after 10
345 min incubation with liposomes for ketoprofen, ibuprofen and hydrocortisone but not for
346 caffeine. Since the experimental set up used was a closed system (i.e. mass preservation) it
347 can be assumed that all the material that did not diffuse through the cuvette was sequestered
348 by the phospholipid bilayers. Interestingly, liposomes did not only incorporate significant
349 amount of API molecules, but they also affect the apparent diffusivity ($\overline{(D)}$ of each of the
350 compounds investigated (i.e. liposomes strongly retain API). This indicated that, as drug
351 diffusion occurs, the drug is release again, but with a kinetics proportional to the affinity of
352 the API for the phospholipid bilayers (indicatively expressed by the $LogD_{7.4}$, Table 1). In Fig.
353 3, the liposome/water partition coefficient ($P_{lip/w}$, gray column) calculated accordingly to Eq.
354 9 as well as the apparent diffusivities measured (blue dots) are reported for each of the drugs.
355 As it can be seen, hc is the most incorporated compound into the phospholipid bilayer (Fig.
356 3), with an almost 4 times higher distribution of API molecules in the lipophilic bilayer in
357 comparison to the water phase. Ibu and ket show very comparable behaviors, as expected
358 from the molecular physicochemical properties (comparable pK_a (Table 1) and chemical
359 structure). For both drugs, molecules distribute approximately two times more in the lipid
360 phase than in the water phase. Caffeine is quite hydrophilic (negative $logD_{7.4}$, Table 1) and
361 therefore its very low partition into lipophilic environment is not surprising. The experimental
362 approach utilized in this work gives additive information on the relative diffusivities of the
363 API in the presence of liposomes. From the results reported in Fig. 3, it is evident that also
364 relative diffusivities of API are reduced by the presence of liposomes, also for hydrophilic
365 compound. For example, caf relative diffusivity is reduced by approx. 20% in comparison to
366 D_0 (Table 2) whereas hc diffusivity is reduced down to 55% of its reference diffusion (Table

2). These data allow to make some interesting considerations. First, interaction of API molecules with phospholipid bilayers are extremely fast as equilibrium is reached within 10 min. Second, in the case of phospholipid vesicles, it is clear that the reduction in apparent flux of API through UWL is affected by the reduction in concentration gradient induced (dc/dx) by the segregation of drug molecules into liposomes. Unfortunately, in the case of liposomes it was not possible to estimate a real equilibrium constant API-liposome as in the case of HPBCD, since the stoichiometry of reaction API-liposomes was unknown. However, using as parameter the variation within relative diffusivity ($\overline{D}$) and reference diffusivity (D_0) it is possible to estimate that the binding between hc and the phospholipid bilayers should be approx. two-times stronger than ibuprofen and ketoprofen and almost three-times stronger than with caffeine.

4. Conclusion

In this work the interaction of four APIs with classical solubilizing vehicles (cyclodextrins and liposomes) has been successfully studied in unstirred aqueous conditions. The transport through the UWL of drug molecules is significantly affected by the presence of both cyclodextrins and liposomes. The extent is connected to the intrinsic physicochemical properties of API molecules. Specifically, the diffusivity of small hydrophilic compounds such as caffeine is not strongly hampered by the presence of solubilizing vehicles whereas, for compounds with higher lipophilicity (ibuprofen, ketoprofen and hydrocortisone), the reduction in transport rate results quite remarkable. In both cases (HPBCD and liposomes) the diffusion of drug through UWL is limited by drug sequestration and consequent reduced mass flux. In the case of cyclodextrins, empirical data are the results of the diffusion of both free drug and drug-HPBCD complex whereas, in the case of liposomes, the experimental data reassemble the diffusion of the free drug only, as we can assume that the liposomes are stationary (on the relevant time scales). This is due to the much slower diffusivity of

392 liposomes in respect to drug molecules. In both cases however, the mathematical approach
393 used results efficient in order to obtain reliable information on passive drug diffusion through
394 UWL in presence of solubilizing agents.

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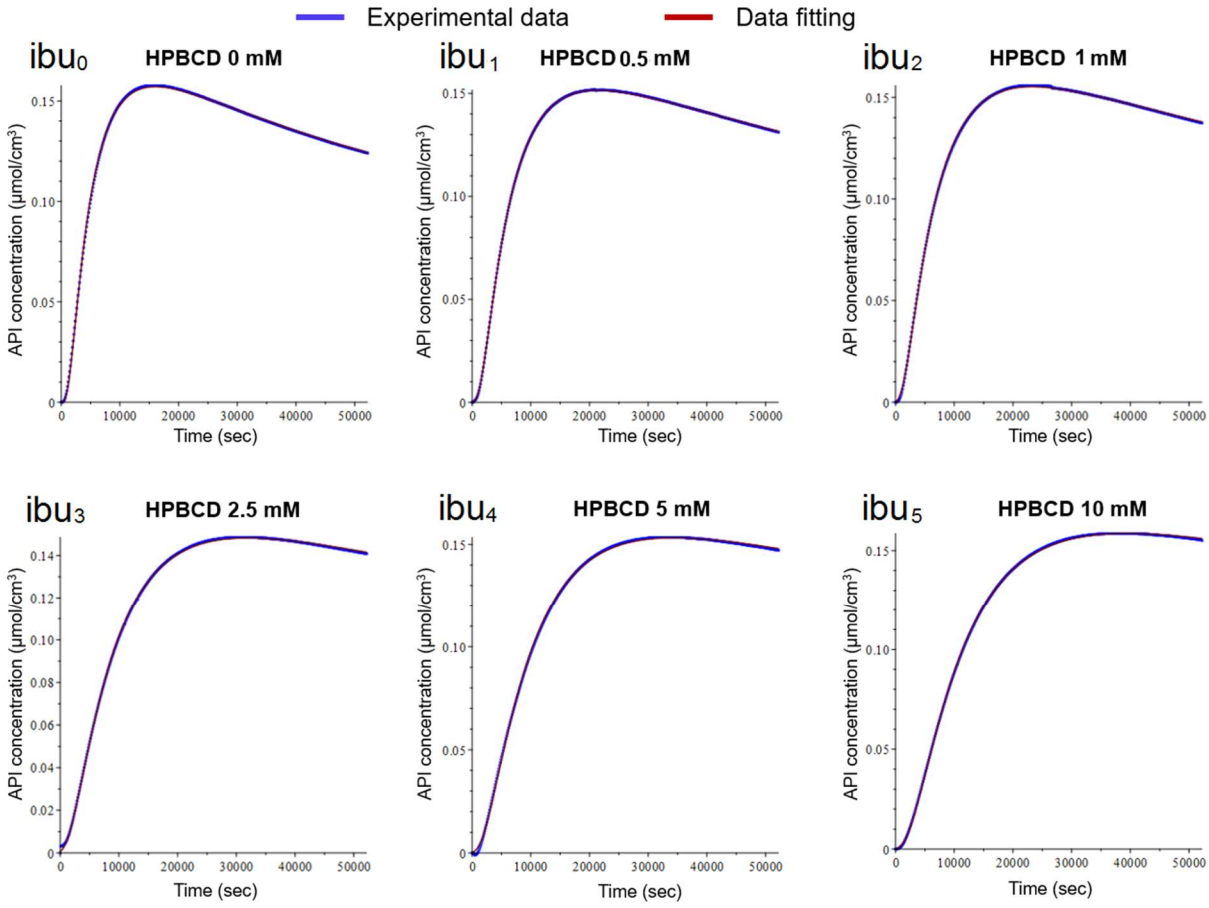


473

474 **Figure 1.** Diffusion profiles of ibuprofen through the unstirred water layer in absence (ibu_0)
475 and in the presence (ibu_1 to ibu_5) of increasing concentration (from 1 mM to 10 mM) of
476 hydroxypropyl-β-cyclodextrin. The blue lines represent the experimental data recorded at 0.51
477 cm from origin of diffusion whereas the red lines represent the data fitting.

478

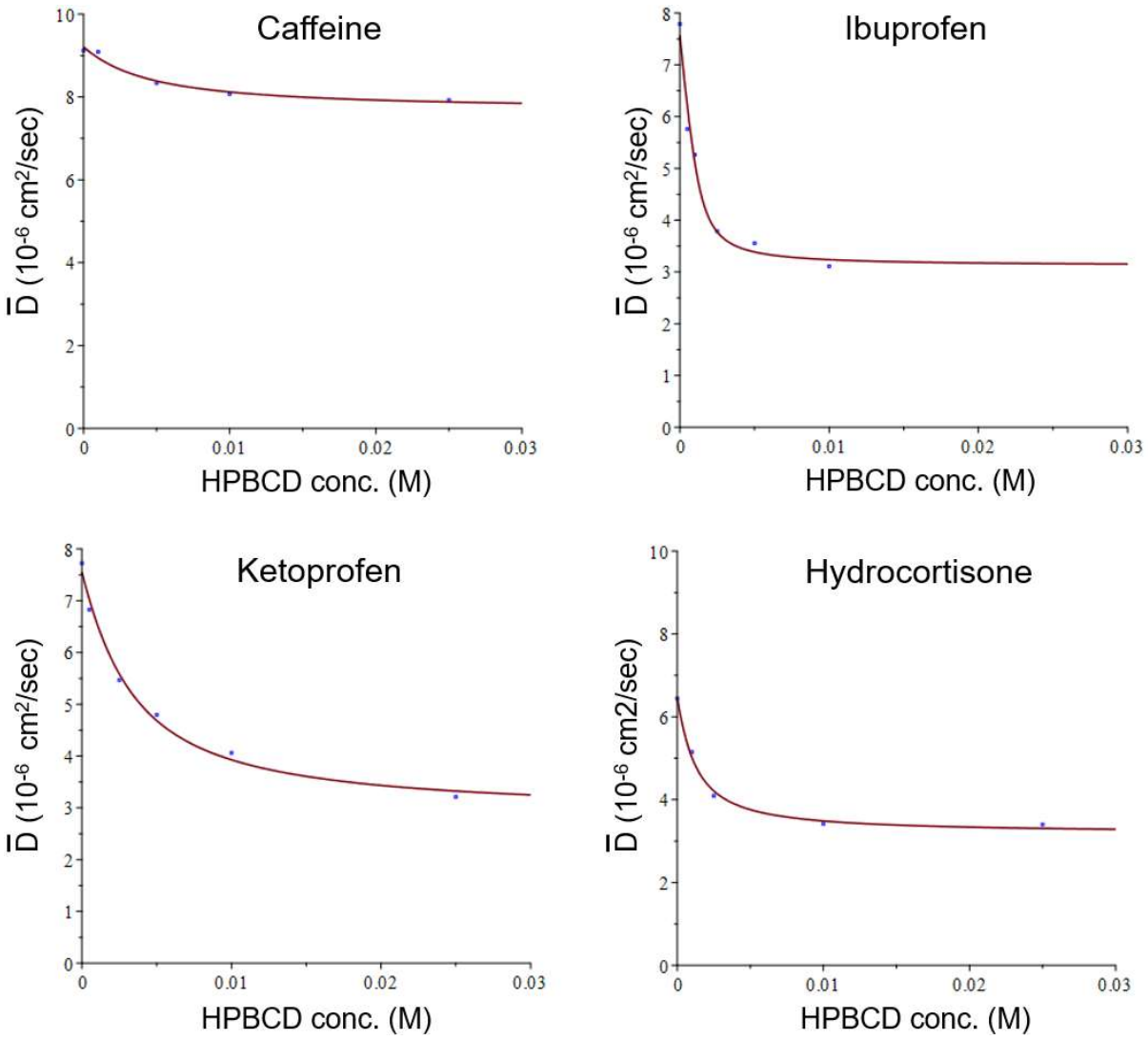
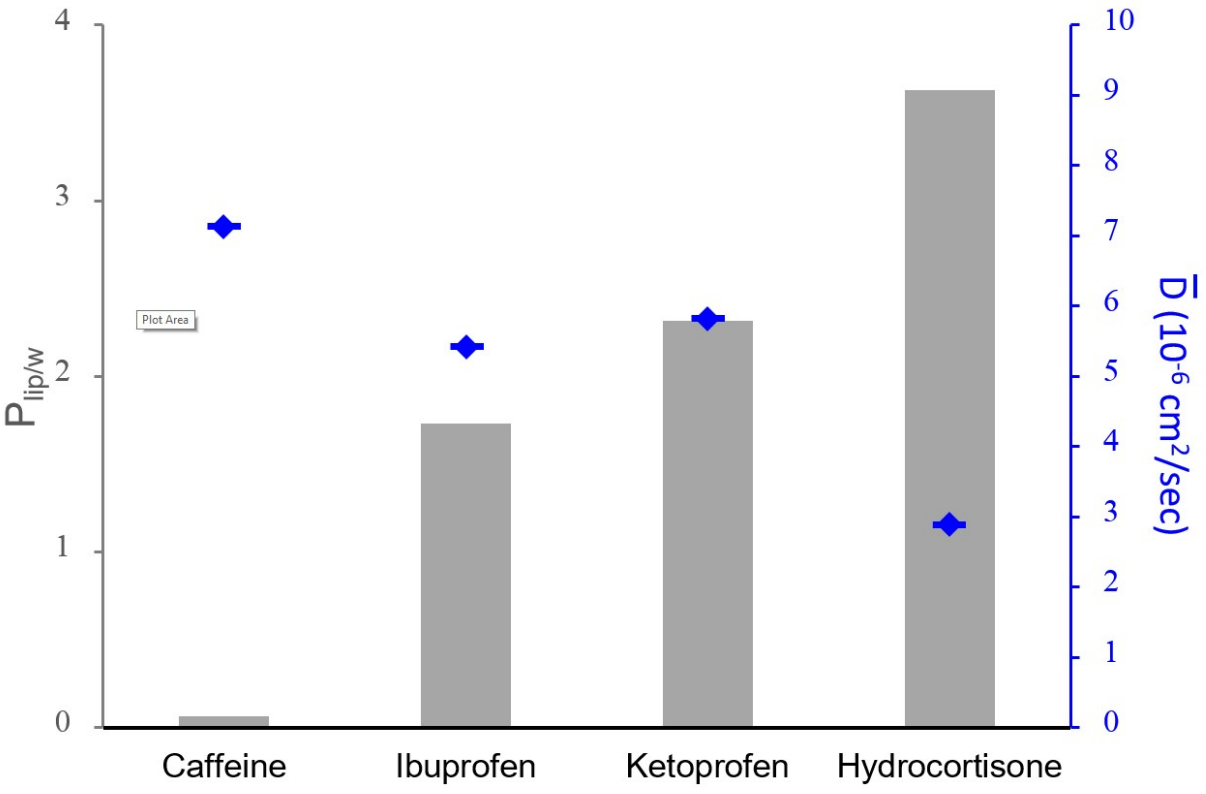


Figure 2. Relationship between relative diffusion coefficient ($\overline{D}$) and hydroxypropyl-β-cyclodextrin (HPBCD) concentration for all the investigated compounds. The red line represents the data fitting of Eq. 7-8 to experimental values.



484

485 **Figure 3.** Partition coefficient liposomes/water ($P_{lip/w}$) and relative diffusion coefficients ($\overline{D}$)
486 measured for all investigated compounds in the experiments performed in the presence of
487 phospholipid vesicles (25 mM phosphatidylcholine S-100 concertation, liposomes diameter of
488 400 nm).

489

490