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Original article

Synthesis and characterization of binary and ternary complexes of diclofenac with a methyl-β-CD and monoethanolamine and *in vitro* transdermal evaluation

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ABSTRACT

Here, we describe the chemical characterization of the inclusion complex between diclofenac (DCF) and methyl-β-cyclodextrin (M-β-CD) in the presence or absence of monoethanolamine (MEA). Several techniques were used to analyze the complex both in solution and in the solid state. Solubility of DCF was increased by the addition of M-β-CD. However, the DCF solubility increase was more significant by the addition of M-β-CD in the presence of MEA. *In vitro* permeation experiments through excised human skin revealed that DCF was enhanced by M-β-CD. Nevertheless, further improvement in the flux and the permeability coefficient of DCF was obtained by the ternary system DCF-M-ß-CD-MEA.

1. Introduction

Cyclodextrins (CDs) consisting of several glucose units possess hydrophobic cavities. Naturally occurring CDs are six, seven, or eight-membered-1, 4-linked cyclic oligomers of D-glucopyranose (α-, β-, and γ-CD) and are generally described as shallow truncated cones with the primary hydroxyl rim of the cavity having a reduced diameter compared to the secondary hydroxy one [1]. Various hydrophilic, hydrophobic and ionic CD derivatives have been utilized to extend the physicochemical properties and inclusion capacity of natural CDs [2].

The sequestration of a hydrophobic molecule or some part of it, inside the cavity usually alters the physicochemical properties of the encapsulated molecule, which is protected against the aqueous medium from light, oxidants or reactive attacks. CDs and their derivatives have received considerable attention in the pharmaceutical field due to their extensive use in drug delivery processes. CDs have been used extensively to increase the aqueous solubility, stability, and bioavailability of drugs [3].

It should be stressed, however, that pharmaceutical dosage forms should contain as little CD as possible, because excess CD can cause some problems in formulation bulk or potential toxicity, as well as reducing bioavailability and preservative efficacy [4]. Therefore, in cases where low complexation efficiency would require a larger amount of CD than that acceptable for solid or liquid dosage forms, enhancement of the complexation capacity of the chosen CD is of practical importance.

Because of the interest in modifying the inclusion properties of CDs in several fields of science such as organic and analytical chemistry, enzymatic studies, and controlled delivery of drugs, attention has been paid to investigate the effect of adding a third component to binary systems consisting of a guest and a CD. Moreover, some works have demonstrated that the presence of a suitable third component can significantly improve CD complexation and solubilizing efficiency.

It is possible to increase drug availability in aqueous CD formulations by including small amount of a water-soluble polymer. Polymers enhance the CD complexation of the drug through the formation of ternary complexes or co-complexes; therefore reducing the amounts of CD needed in the formulation, while simultaneously enhancing the absorption of the drug from the CD complex [5,6]. Similar positive effects on CD solubilization due to the addition of certain low molecular weight acids or hydroxyacids have also been reported for base-type drugs, e.g., the synergistic effect on the hydrosolubility of econazole by the combined use of hydroxyacids and CDs [7–9].

Alcohols may alter the efficiency of CD complexation leading to either a decrease [10–12] or increase [13,14] of CD-guest association constants. Enhancement of the complexation efficiency has been frequently attributed to the formation of ternary complexes, where the third complexation agent replaced the water molecules in the void space of the CD cavity. In contrast, when the equilibrium

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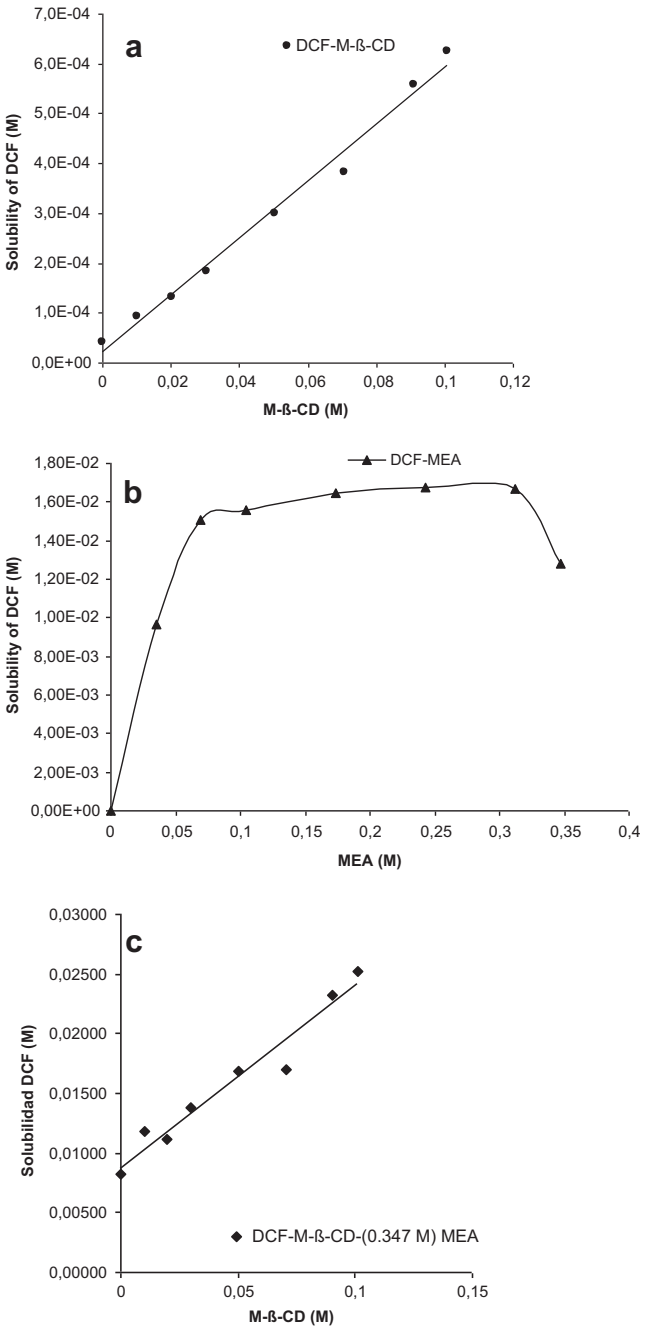

Fig. 1. Phase solubility diagram of DCF in the present of M-β-CD (a), MEA (b) or M-ß-CD-(0.35 M) MEA (c) in water under 32 °C. Data points are average values of triplicate measurements.

constants decrease the third complexation agent may partially displace the guest molecule within the CD [15].

Previous studies performed in our laboratory have demonstrated that the solubility capacity of the HP-β-CD (hydroxypropyl-β-cyclodextrin) is significantly enhanced when a basic compound, triethanolamine (TEA), is incorporated as a ternary component in the complexes of the acid drug sulfisoxazole [16]. Moreover, in our recent study, we have found that the simultaneous complexation and salt formation with TEA significantly increased the HP-β-CD solubilizing power for the sparingly water-soluble acetazolamide (ACZ) by forming a drug:HP-β-CD:TEA multicomponent system [17]. This ternary system (ACZ:HP-β-CD:TEA) reduced the intraocular pressure (IOP) in rabbits by about 30%. Also, the *in vitro* corneal permeation of ACZ was increased. The extended ocular hypotensive effect produced by the ternary system ACZ:HP-β-CD:TEA was attributed to the fact that the relative stability of the ACZ:HP-β-CD complex in the presence of TEA favors a higher amount of free drug which is readily available for absorption [18].

Table 1
Solubility of DCF in different formulations.

Solvent	Solubility (mg/mL)	pH
Water	0.0122 ± 0.0007	6.1
M-β-CD 0.10 M	0.156 ± 0.002	4.4
MEA 0.35 M	3.5 ± 0.5	10.8
M-β-CD 0.10 M:MEA 0.35 M	7.46 ± 0.01	11.4

In a previous study we also showed that the complexation of flurbiprofen with HP-β-CD and salt formation with ethanolamines [monoethanolamine (MEA), diethanolamine (DEA) and TEA] improves the solubility and dissolution properties of this drug [19].

Diclofenac (DCF), 2-[(2,6-dichlorophenyl)amino] phenylacetic acid, is a potent nonsteroidal anti-inflammatory drug (NSAID) with a very low aqueous solubility and gastrolesive actions. It is used in inflammatory and painful conditions of rheumatic and non-rheumatic origin [20]. Transdermal delivery of DCF would be advantageous since it would avoid hepatic first-pass metabolism and considerable gastrointestinal disturbances. However, DCF does not penetrate well through skin and cannot reach the effective concentration at the site of action after transdermal application. A possible mechanism of penetration enhancement could involve the complex formation between drug and components in the pharmaceutical formulation, thus altering the physicochemical properties of the active substance.

CDs are known to influence the percutaneous absorption of therapeutic agents both by a solubilizing action on the drug, thus increasing its availability at the absorption site, and by an interaction with the free lipids present in the stratum corneum [21,22].

To our knowledge, there is not any report to date concerning the influence of the sparingly methyl-β-cyclodextrin (M-β-CD), in the presence of MEA, on the percutaneous absorption of DCF.

In addition, the rational design of pharmaceutical CD formulations requires a good knowledge of the encapsulation process. Structural information, such as the stoichiometry and the geometry of the complex, and thermodynamic information of binding, are necessary to draw a complete picture of the driving forces governing the drug:CD interaction. The investigation of all these aspects is the objective of this work, focused on the analysis of the interactions between M-β-CD, in the presence or absence of MEA, and the drug DCF. Also, the present work focuses on evaluating the ability of M-β-CD in the presence of MEA to influence the percutaneous absorption of DCF through isolated human skin.

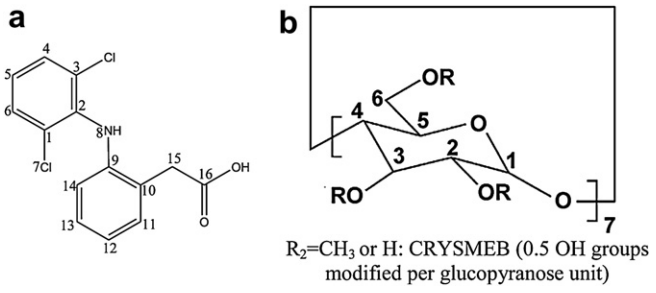

Fig. 2. Chemical structures of DCF (a) and M-β-CD (b).

2. Results and discussion

2.1. Phase solubility studies

Traditional phase solubility analysis of the effect of complexing agents on the drug compound can provide not only the stability constant of the complex but also insight into the stoichiometry of the complex at equilibrium. The phase solubility diagrams were obtained by plotting the apparent equilibrium concentration of the drug against complexing agent concentrations and are shown in Fig. 1.

For the DCF:M-β-CD system, CD enhances the aqueous solubility of DCF (Fig. 1a). This diagram showed a linear relationship between the amount of DCF solubilized and the concentration of CD in solution over the entire concentration range studied. This linearity was characteristic of an A_L-type system, as described by Higuchi and Connors [23], and suggested the formation of inclusion complexes in a 1:1 DCF/CD molar ratio, and the formation of a soluble complex. The stability constant (K_c) for the complex calculated from the slope of the initial straight portion of the solubility diagrams was 146.9 M^{-1}. There is ~13-fold enhancement in the solubility of DCF in the presence of 0.10 M M-β-CD in comparison with the DCF solubility in water in the absence of the CD.

For the DCF:MEA system, its solubility curve can be classified as B_s type as described by Higuchi and Connors [23] (Fig. 1b). B_s denotes complexes with limited solubility. Nevertheless, the complex exhibits higher solubility than does the guest molecule. The ion-pair formation of DCF with 0.35 M MEA increased the aqueous solubility of DCF by a factor of ~287.

In view of the obtained results it was interesting to investigate the combined effect of an alkalizing agent (MEA) and M-β-CD on the enhancement of the aqueous solubility of this compound by the multicomponent complexation method. It was found that these solubilizers acted in a synergic way (Table 1), an important increase in the DCF aqueous solubility values was observed as a function of the M-β-CD concentration, in the presence of 0.35 M MEA (Fig. 1c). The aqueous solubility of DCF increased by a factor of ~611 in the presence of 0.10 M M-β-CD and 0.35 M MEA. The solubility of DCF was linearly proportional to the concentration of the M-β-CD in the presence of a constant amount of MEA (0.35 M); thus this diagram can be classified as A_L-type according to Higuchi and Connors [23]. The Association constant for the DCF:M-β-CD:MEA system calculated from the slopes of this line was 20.54 M^{-1}. This value suggests that the presence of MEA weakens the interactions between DCF and M-β-CD. Nevertheless, it is interesting to note that the solubility of the ion-pair DCF:MEA increases beyond its solubility limit (~4.96 mg/ml in water at 25 °C) in the presence of M-β-CD (Fig. 1c) leading to the formation of a highly soluble complex.

2.2. NMR spectroscopy

In an attempt to investigate the interaction of DCF with M-β-CD and evidence their complexation, ^{1}H NMR spectroscopy was employed. M-β-CD and DCF structures are shown in Fig. 2 with the numbering systems used to ^{1}H assignment. The ^{1}H resonances of M-β-CD and DCF were assigned according to the literature [24].

The formation of an inclusion complex can be proved from the changes of chemical shifts of DCF or CD in the ^{1}H NMR spectra. Table 2 lists the detailed variation of the chemical shifts of DCF and protons of M-β-CD before and after forming the inclusion complex.

Concerning the shift values of the proton resonances of DCF moieties, all protons were influenced by the macrocycle. The most significant changes have been observed on the H_{4-6} and H_{14} protons, which indicate a possible inclusion of the dichlorophenyl moieties and a partial inclusion of the phenylacetic acid ring. The downfield shifts observed for phenyl H_{4-6}, H_5, H_{13} and H_{12} of the drug could be attributed to changes in the local polarity or to a deshielding effect due to van der Waals forces between the aromatic moiety and carbohydrate chains, as a result of complexation within M-β-CD cavity. The upfield shifts observed for H_{11} and H_{14} protons could be due to a shielding effect produced by the oxygen atoms of the methoxyl group of the macrocycle that were in the proximity of the drug.

Table 2
Proton complexation shifts (Δδ ppm) for the 1:1 DCF:M-β-CD complex.

Compounds	Protons	*n*H	$\delta_{(free)}$	$\delta_{(complex)}$	$\Delta\delta$[a]
DCF	H_{4-6}	1	7.40	7.45	−0.05
	H_5	1	7.17	7.18	−0.01
	H_{11}	1	7.07	7.05	0.02
	H_{13}	1	7.03	7.04	−0.01
	H_{12}	2	6.88	6.89	−0.01
	H_{14}	1	6.40	6.35	0.05
M-β-CD	H_1	1	5.16	5.16	0.00
	H_2	1	4.97	4.96	0.01
	H_3	1	3.89	3.87	0.02
	H_5	1	3.77	3.76	0.01
	H_6	1	3.52	3.52	0.00
	H_4	1	3.46	3.46	0.00
	CH_3	3	3.29	3.29	0.00

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

The H_3 and H_5 protons of the glucose units are facing the interior of the CD cavity, both protons are located in the interior of the CD cavity, with H_3 protons near the wide side of the cavity and H_5 protons near the narrow side, whereas H_6 protons are located at the rim and H_2 and H_4 are at the opposite entrance of the cavity. High frequency shifts of the interior proton signals of CDs are indicative that aromatic guest molecules are located close to the protons for which a shift is observed. M-β-CD showed a moderate upfield shift for H_3 and H_5 protons in the presence of the drug (Table 2), this effect being stronger mainly for H_3. These results may indicate that DCF should be included in the M-β-CD cavity from the wide side.

The continuous variation plot (Job plot) reported in Fig. 3 shows a sharp maximum at r ([DCF]/([DCF] + [M-β-CD])) 0.5, which might indicate that the main stoichiometry is 1:1, in agreement with the stoichiometry suggested from the phase solubility study.

Two-dimensional ROESY spectroscopy is capable of revealing spatial relationships among protons in a molecule or in a complex. This experiment utilizes the dipolar interaction between protons at distances less than 5 Å. The 2D ROESY spectrum of the DCF:M-β-CD is reported in Fig. 4, including a partial contour plot (Fig. 4, inset). The ROESY spectrum of the DCF:M-β-CD complex shows an

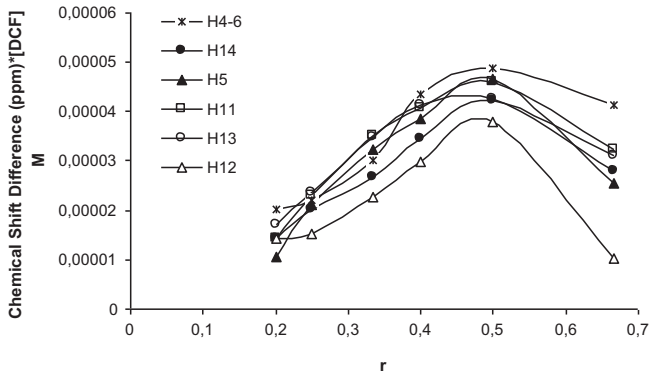

Fig. 3. Continuous variation plot for DCF obtained from the chemically induced shift displacement (CID) of selected NMR proton signals of DCF.

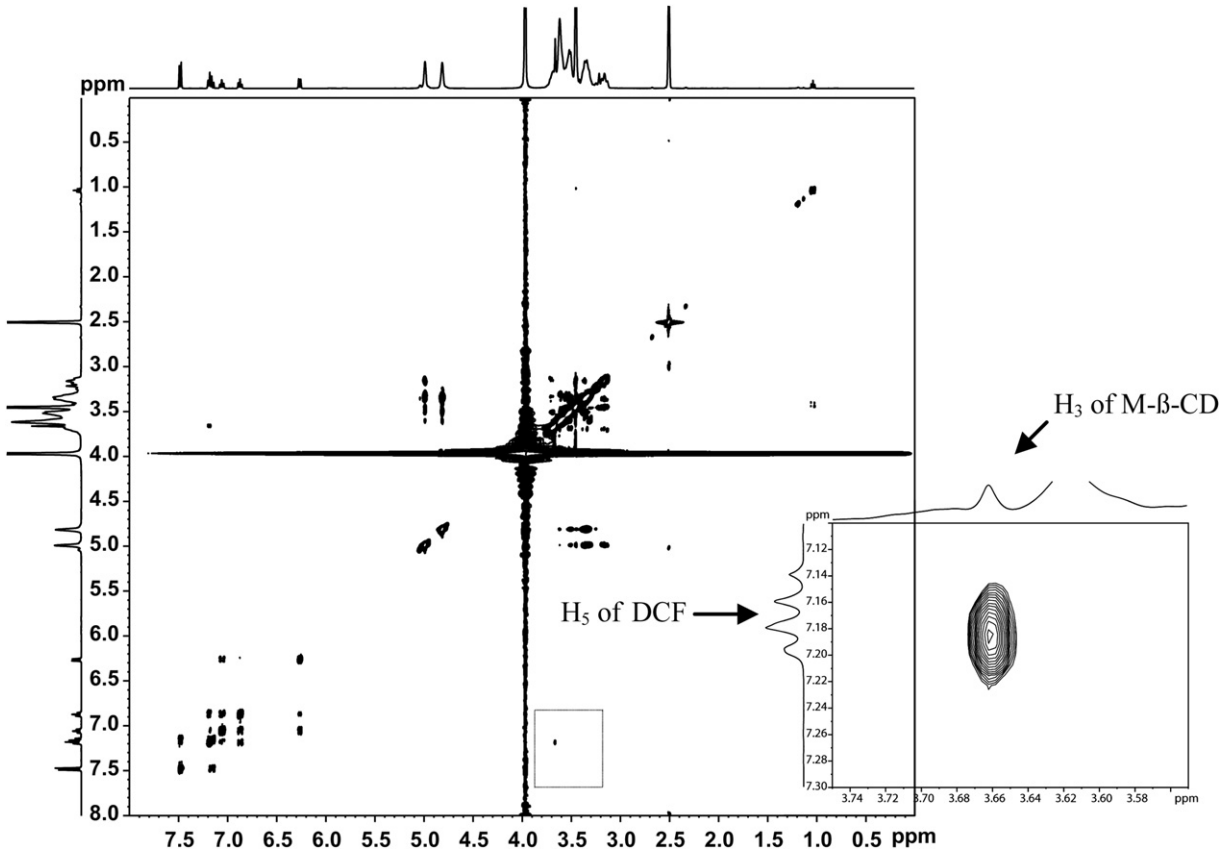

Fig. 4. Partial contour plot of a ROESY spectrum of the DCF complex with M-β-CD.

appreciable correlation of the H_5 proton of DCF with the H_3 protons of M-β-CD. No correlation is observed between DCF protons and the H_5 proton of the CD. These results indicate that the dichlorophenyl moites of DCF are included in the M-β-CD cavity. In combination with the 1:1 inclusion stoichiometry observed in the phase solubility diagram, a possible inclusion mode for the DCF:M-β-CD complex is proposed (Fig. 5).

2.3. *Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)*

The Differential scanning calorimetry (DSC) can provide a lot of information on drug/CD interactions in the solid state.

The DSC curves and the TG and DTG thermograms for pure DCF, M-β-CD and the physical mixtures, as well as, those of the freeze-dried binary complexes of DCF with M-β-CD and/or MEA are shown in Figs. 6 and 7.

DSC curve of DCF (Fig. 6a) showed the typical behavior of an anhydrous crystalline drug with a well-defined melting peak at 178.85 °C ($\Delta H_f = 82.79$ J/g). The DSC curve of pure M-β-CD (Fig. 6b) exhibited a very broad endothermal phenomenon between 50 °C and 150 °C ($\Delta H = 258.1$ J/g) due to the loss of water, indicative of its amorphous hydrate state. The DSC curve of pure M-β-CD did not show any endothermic peaks in the melting point region of DCF.

DSC analysis of the binary systems of DCF with MEA was performed in order to gain more insight about possible drug–ethanolamine solid state interactions. Fig. 6e–f shows the DSC curves and the TG and DTG thermograms of the physical mixture of DCF with MEA. The thermal curves for the binary system DCF:MEA prepared in ethanol are shown in Fig. 6g–h. DSC and TG analysis revealed the presence of water molecules in varying proportion in the salt of DCF and MEA obtained by the two methods of preparation. However, their DSC and TG patterns were found to differ in their peak position and relative intensities. The DSC curve

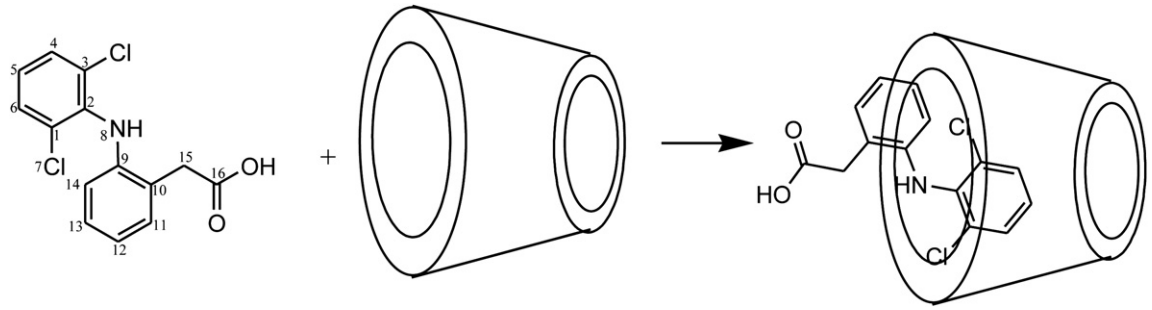

Fig. 5. Proposed structure of the DCF:M-β-CD complex.

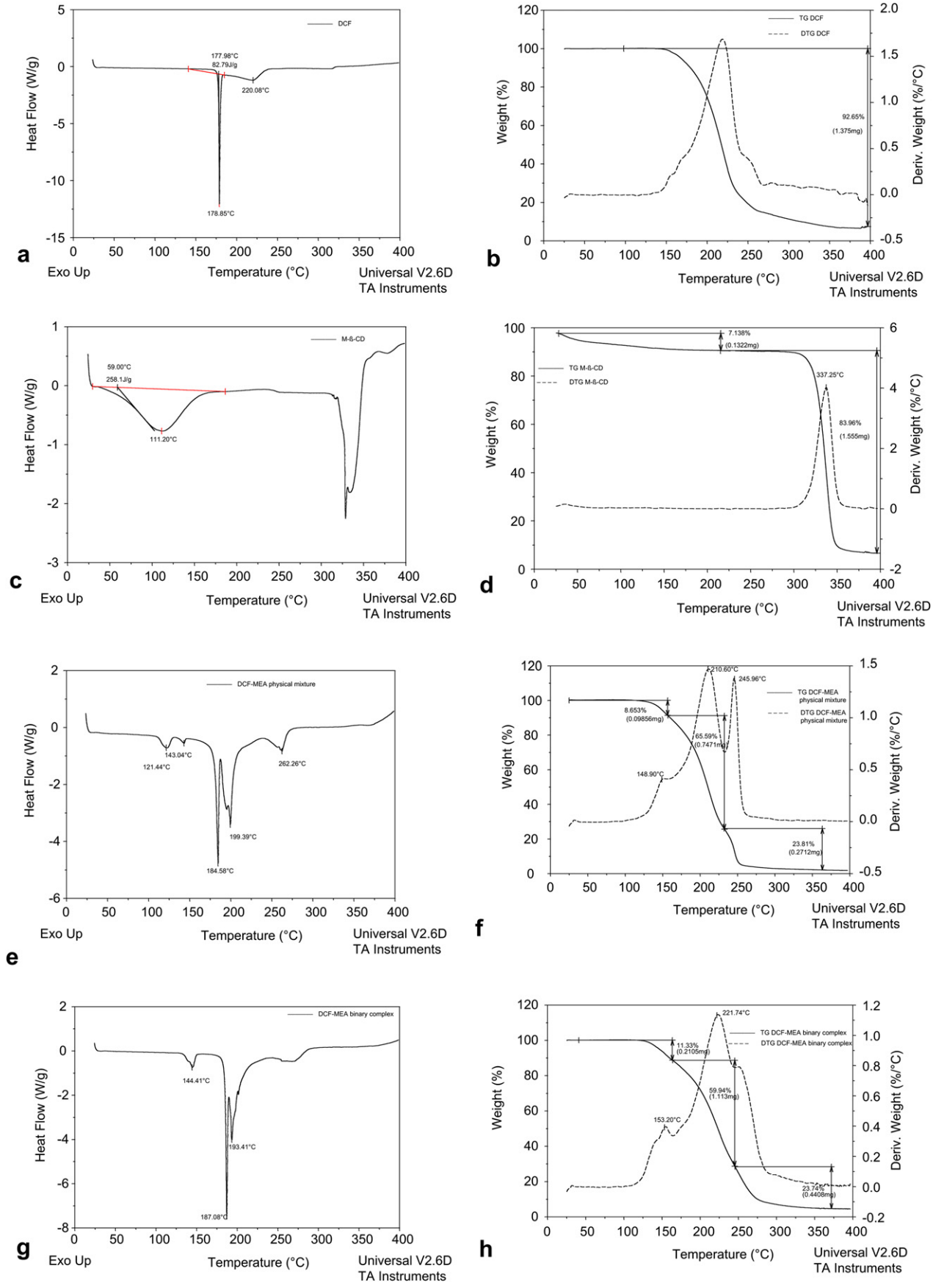

Fig. 6. DSC and TG/DTG curves of DCF (a–b), M-β-CD (c–d), DCF:MEA physical mixture (e–f) and DCF:MEA binary complex (g–h).

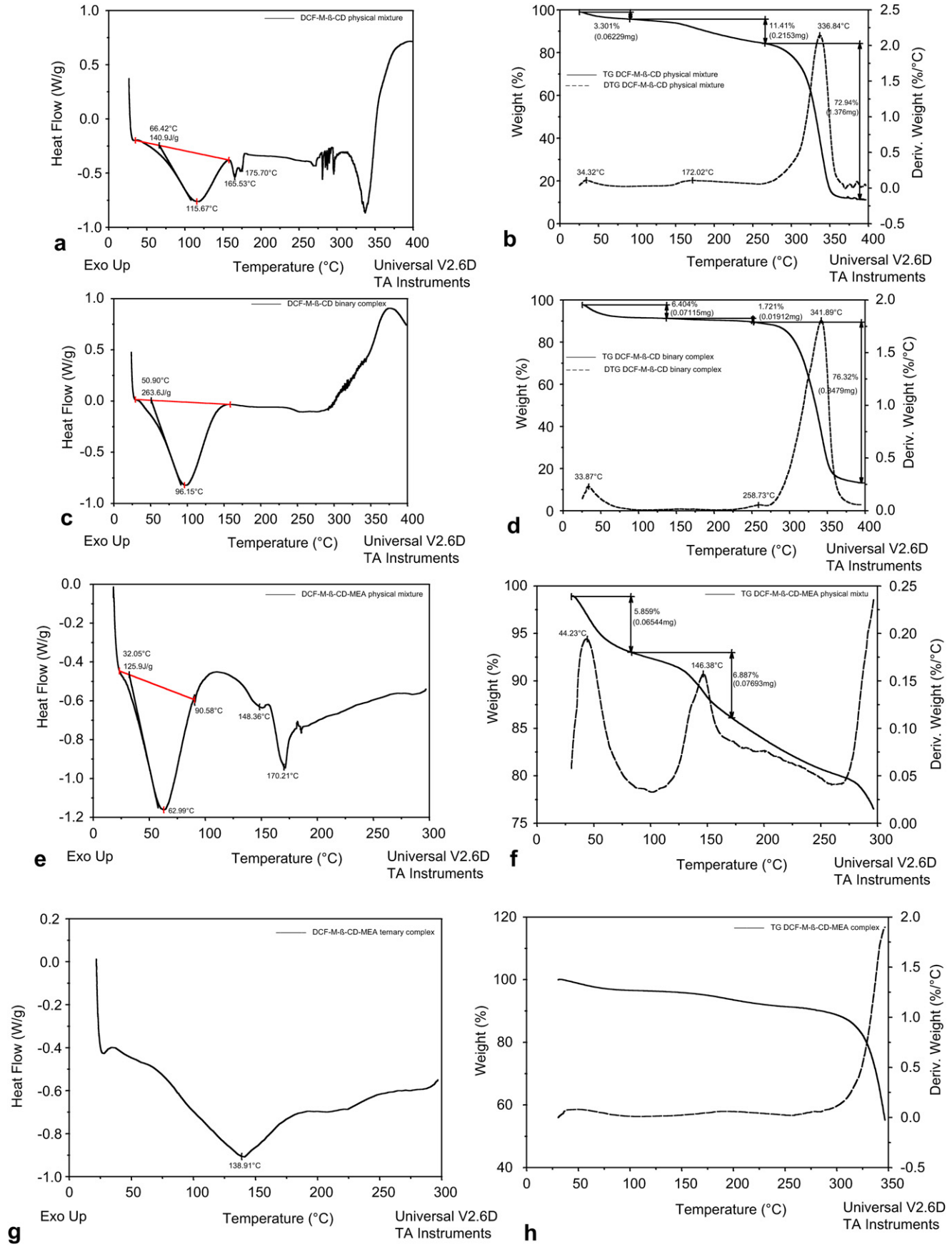

Fig. 7. DSC and TG/DTG curves of DCF:M-β-CD physical mixture (a–b), DCF:M-β-CD freeze-dried system (c–d), DCF:M-β-CD:MEA physical mixture (e–f) and DCF:M-β-CD:MEA freeze-dried system (g–h).

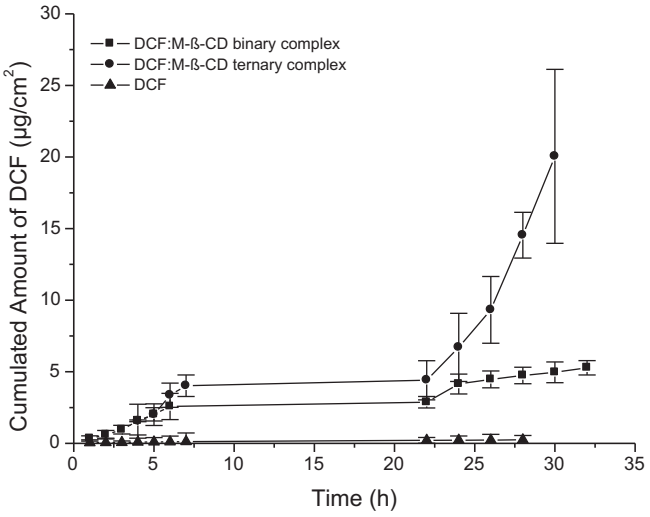

Fig. 8. Permeation profiles through human skin of DCF in different formulations. Each point represents mean ± SD ($n = 3$).

of the salt formed in ethanol displays four endothermic peaks (Fig. 6g). The first endotherm at about 144 °C is associated with the presence of one water crystallization molecule (11.33% by TG measurement). The second one is associated with the melting of the anhydrate form (187.08 °C), followed by a polymorphic transition to a more stable form, which melts at 193.41 °C, and with a melting endotherm overlapping the decomposition peak (at about 200 °C). The TG profile for the MEA salt shows three steps for the weight loss. The first one is associated to dehydration. The subsequent steps represent different degrees of decomposition that starts with the polymorph transition, continues with melting and terminates in correspondence of the broad endotherm. In the case of the salt obtained by simple physical mixture of DCF with MEA, the dehydration process appears to be stepwise, since an additional endothermal peak at a lower temperature (~121.44) was observed, which can be attributed to the partial dehydration of the salt.

In the DSC curve of the physical mixture of the binary system DCF:M-β-CD (Fig. 7a) it is possible to observe three endothermic peaks in the range of 25–250 °C. The first one occurred at ~115.67 °C, which was associated with water losses from M-β-CD, followed by two more peaks in the ranges of 150–200 °C, one at 165.53 °C and the other at ~175 °C. The appearance of a new peak at 165.53 °C and the broadening of the fusion peak of DCF were evident. This thermal behavior suggests that during DSC scans, some type of heating induced interaction takes place between DCF and M-β-CD, which apparently results in broadening of the melting endotherm and loss of DCF crystallinity and the formation of a new solid phase, which melts at lower temperature with respect to the free drug. Such an interaction can be interpreted by assuming that the hydrogen bonds of the crystalline drug embedded in the amorphous CD matrix are weakened and other hydrogen bonds involving water molecules and hydroxyl or alkyloxy groups of the carrier may be established.

As concerns the binary DCF:M-β-CD freeze-dried complex, its DSC curve (Fig. 7c) shows the absence of the characteristic endothermic melting peak of DCF (178.85 °C). Its disappearance may be attributed to the amorphous state and the inclusion complexation of the drug inside the cavity. Also, the decreasing in the endotherm peak associated with the M-β-CD dehydration (96.15 °C) in comparison with the pure M-β-CD (111.20 °C) has been observed, indicating that all drug molecules interacted with the CD.

The DSC profile of the ternary systems, physical mixture and freeze-dried products, of DCF with M-β-CD and MEA are despite in Fig. 7. DSC thermogram of the physical mixture (Fig. 7e) shows one peak corresponding to M-β-CD (62.92 °C, $\Delta H = 125.9$ J/g) and two peaks attributed to the DCF:MEA salt (148.36 °C and 170.21 °C), respectively. It was observed a decreasing and broadening of the DCF:MEA ion-pair endothermic peaks, probably due to interactions between DCF:MEA salt and M-β-CD. There is also a diminution of the dehydration effect of M-β-CD, indicating that a lower amount of water molecules are present at the internal cavity of M-β-CD. In the thermogram obtained for this ternary system using the freeze-dried processing method (Fig. 7g), the endothermic peaks attributed to the fusion and decomposition of the DCF:MEA salt cannot be observed. This fact could indicate a complexation of the salt into the cavity of M-β-CD, although it also could be attributed to the amorphization of the sample, due to the processing method employed.

Table 3
Permeation parameters across human skin.

Formulation	Flux ($\mu g\ cm^2\ h^{-1}$) 10^{-3}	K_p ($cm\ h^{-1}$) 10^{-3}	Permeated amount of DCF at 30 h ($\mu g/cm^2$)	t_{lag} (h)
DCF	6.4 ± 0.9	0.5	0.3 ± 0.1	10
DCF:M-β-CD binary complex	138 ± 4	11.6	5.3 ± 0.2	6
DCF:M-β-CD:MEA ternary complex	2680 ± 7	223	20 ± 12	22

2.4. Skin permeation studies

The cumulative permeation of DCF from different formulations through human skin is shown in Fig. 8. The analysis of data from Table 3 indicated that the ternary system DCF:M-β-CD:MEA was the most efficient in DCF permeation according to the amount drug permeated at 30 h, steady-state flux and K_p values (Table 3).

The drug permeation enhancement obtained from the DCF:M-β-CD complex is probably mainly due to two different mechanisms, on the one hand, M-β-CD acts as a carrier, keeping the hydrophobic DCF molecules in solution and delivering them to the surface of the skin, and on the other hand, due to a direct action of the M-β-CD on the stratum corneum (SC). Lipophilic cyclodextrins, such as M-β-CD, can permeate biomembranes, interact with the lipids and increase drug uptake [25]. It is interesting to note that the lag-time (t_{lag}) obtained with the binary system DCF:M-β-CD was shorter than that one of the saturated aqueous solution of DCF, which suggested some effect of the M-β-CD on lipids in the SC.

It was also observed that the ternary system DCF:M-β-CD:MEA showed longer t_{lag} value in comparison with that one of the binary system DCF:M-β-CD. The longer t_{lag} value obtained with the ternary system could be attributed to it had a less invasive action on SC of human skin than the binary system because the presence of MEA may, to some extent, impede the inclusion of the skin lipids with M-β-CD. Therefore, the permeation could reach the pseudo-state slower, but the amount permeates at 30 h and the flux were higher because of the greater availability of free DCF after the dynamic equilibrium between the free drug molecules and inclusion complex is achieved. The presence of MEA in the system might affect the dynamic characteristics for the complexation reaction between DCF and M-β-CD. From the phase solubility diagrams, it was found that binary DCF:M-β-CD and ternary DCF:M-β-CD:MEA systems exhibited widely different stability constant (K_c) values, showing the ternary system a lower stability constant than that of the binary one. It is clear that MEA can weaken the association between DCF and M-β-CD cavity. The drug permeation enhancement obtained with the ternary DCF:M-β-CD:MEA complex is

probably mainly due to the fast free drug release from the complex toward the surface of the skin and, consequently, permeation across the skin is improved.

Also, we found that the t_{lag} value of the saturated aqueous solution of DCF was shorter than that one of the ternary system. These results might be explained because at the beginning of the permeation experiment, the activity of DCF in the ternary system is lower than that in the aqueous saturated solution of DCF. However, after the lag-time, the flux of DCF increased rapidly.

3. Conclusion

The results of this study showed that DCF:M-β-CD binary and DCF:M-β-CD:MEA ternary complexes can be formed in solution and in solid state. Spectroscopic results confirmed the drug inclusion in the CD. The 2D ROESY NMR experiment showed that the inclusion complex between DCF and M-β-CD is formed by the insertion of the dichloropheyl moites of the drug in the CD cavity. From the phase solubility diagrams the stoichiometry of the DCF:M-β-CD complex was found to be 1:1. The association in the DCF:M-β-CD:MEA system was found to be less stable than in the DCF:M-β-CD system.

Skin drug delivery offers an alternative to conventional oral administration for drugs that show low stability at acidic conditions of the stomach and a strong first hepatic effect. However, the skin represents an effective absorption barrier and new strategies must be found to overcome it. The effect of cyclodextrins to increase the DCF permeability through the skin was studied. The *in vitro* transcutaneous permeation of DCF was enhanced in the presence of M-β-CD. Nevertheless, the complexation of DCF with M-β-CD in presence of MEA increases the amount of drug permeated at 30 h 67-fold, suggesting that MEA favors drug permeation due to the interaction between M-β-CD and MEA might prevent complexation of DCF with M-β-CD, allowing a larger amount of free drug generating a supersaturated solution of DCF, and thus favoring the skin absorption of this drug.

The results presented here demonstrate that complexation of DCF with M-β-CD in presence of MEA may be a promising approach to increase drug permeation through the skin, that ultimately can result in improving drug bioavailability.

4. Experimental

4.1. Materials

Diclofenac free acid (DCF) was obtained from diclofenac sodium salt (UniFarma, Buenos Aires, Argentina) by precipiting it with HCl. The obtained diclofenac free acid was filtered and dried at room temperature under a vacuum. M-β-CD (Methyl-β-cyclodextrin, CRYSMEB®, Mw ~1190, with an average degree of substitution of 0.5) was kindly donated by Ferromet S.A. (agent of Roquette in Argentina). Monoethanolamine (MEA) was obtained from Aldrich® (98%, USA) and used as received. All other materials and solvents were of analytical reagent grade. A Milli-Q Water Purification System (Millipore®, USA) generated the water used in these studies.

4.2. Solid systems preparation

The preparation of the solid complex of DCF-M-ß-CD in 1:1 M ratio or the multicomponent complexes of DCF with M-β-CD and MEA in 1:1:1 M ratio was performed by the freeze-drying method [26]. DCF, M-β-CD and/or MEA were accurately weighed and dissolved in distilled water. The whole solution was stirred on a magnetic stirrer for 24 h. After filtration, the solution was frozen overnight and then lyophilized over a period of 30 h using a freeze-drier, Labconco freeze dry system. The DCF binary complex with MEA was prepared by dissolving equimolar amounts of acidic DCF and MEA in ethanol by mixing, and ethanol was remover in vacuum after treatment with ultrasound for 1 h. The DCF content of the complexes was determined by dissolving an accurately weighed quantity followed by UV–visible spectroscopy. The physical mixtures were prepared by simple mixing DCF with M-β-CD in 1:1 (w/w) or DCF with M-β-CD and MEA in 1:1:1 (w/w) ratio in a mortar for 15 min.

4.3. Phase solubility studies

Solubility diagrams were obtained according to Higuchi and Connors (1965) [23]. Excess of DCF was added to vials containing various concentrations of M-β-CD (0–0.10 M) or MEA (0–0.011 M). In addition, the effects of the M-β-CD on the solubility of DCF were studied with the presence of the ethanolamine (0.35 M). All the suspensions were sonicated in an ultrasonic bath for 1 h and then, placed in a 25.0 ± 0.1 °C constant-temperature water bath until equilibrium was reached (72 h). The content of each vial was filtered [0.45 μm membrane filter (Millipore, USA)] and the concentration of DCF in the filtered solutions was measured by UV Spectrophotometry (Shimadzu UV 260 UV–Vis spectrophotometer) at 246 nm. The presence of M-β-CD and MEA did not interfere with the spectrophotometric assay of the drug. The equilibrium pH of each solution was measured (ORION SA 520 pHmeter). Each experiment was repeated at least three times and the results reported were the mean values.

The apparent stability constant (K_c) of the 1:1 (guest:host) complex was calculated from the slope of the phase solubility diagrams and the solubility of DCF in water (S_o):

$$K_c = \frac{\text{slope}}{S_o(1 - \text{slope})} \quad (1)$$

4.4. NMR spectroscopy

The ^{1}H NMR spectrum of pure DCF in D_2O could not be determined due to its very low aqueous solubility. Therefore, ^{1}H NMR signal assignments for DCF were performed in 1% (v/v) DMSO-d_6/D_2O mixtures to ascertain the negligible influence of DMSO at low concentrations.

All ^{1}H NMR experiments were performed using a Bruker Avance II 400 spectrometer, operating at 400.16 MHz ^{1}H NMR spectra were carried out in 1% (v/v) DMSO-d_6/D_2O mixture solutions at 298 K with the residual HOD as an internal standard and standard 5 mm NMR tubes were used. ^{1}H NMR chemical shifts ($\Delta\delta$) caused upon complexation were measured to confirm the inclusion of DCF and calculated according to the following formula: $\Delta\delta = \delta$ (free) $- \delta$ (complex).

The stoichiometry of the complex was studied by applying the continuous variation method, The NMR experiment was carried out as described below with solutions of DCF and M-β-CD in 1% (v/v) DMSO-d_6/D_2O mixtures. The total molar concentration of the two components concentrations was kept constant at 10 mM, but the mole fraction of M-β-CD {i.e., [M-β-CD]/([M-β-CD] + [DCF])} varied from 0 to 1. Chemical shifts of proton signals were observed for preparation of the plot. After registering the corresponding changes in the most sensitive NMR signals, the stoichiometry of the inclusion complex was calculated.

The geometry of the complex was studied by two-dimensional Rotating frame Overhauser experiments (2D ROESY), with spinlock for mixing phase sensitive, using 180 × 180 × pulses for polarization transfer. The spectra were measured with a relaxation delay

of 2 s, p15 pulse for ROESY spinlock of 20 ms and l4, spinlock loop, (p15/p25 × 2) = 400. Before Fourier transformation, the matrix was zero filled to 4096 (F2) by 2048 (F1) and Gaussian apodization functions were applied in both dimensions.

4.5. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)

The DSC curves of the different samples were recorded on a DSC TA 2920 and the TGA curves on a TG TA 2920, both by applying a heating rate of 10 °C min^{-1}. The thermal behavior was studied over a temperature range of 25–300 °C, by heating 1–3 mg of samples in aluminum-crimped pans under nitrogen gas flow. Data were obtained and processed using the TA Instruments Universal Analysis 2000 software.

4.6. In vitro drug skin absorption studies

4.6.1. Skin samples

Excised human skin from healthy patients, who had undergone abdominal plastic surgery, was used to get full-thickness skin. Immediately after excision the subcutaneous fatty tissue was removed using scissors and scalpel. The skin was cut into 10 × 10 cm pieces, wrapped in aluminium foil and stored in polyethylene bags at −26 °C until use. Under these conditions the skin is stable with regard to the penetration of drugs as well as the thickness of the stratum corneum over a time period of 3 and 6 months, respectively.

4.6.2. Skin permeation studies

The full-thickness skin was mounted in a Franz diffusion cell and clamped between the donor and the receptor chambers (available diffusion area 1.54 cm^2) with the stratum corneum side in contact with the donor phase. The skin membranes were first hydrated in PBS at 4 °C for 2 h. The donor compartment contained 2 mL of saturated aqueous solution of DCF. Donor phases containing DCF: M-β-CD complexes, in the presence or absence of MEA, were used in an equivalent amount of 2×10^{-5} M of DCF. The DCF content of the complex was determined by dissolving an accurately weighed quantity in ethanol/water solution (50/50, v/v) followed by spectrophotometric determination. The receptor compartment was filled with 10 mL of degassed PBS to prevent the formation of air bubbles at the skin-receptor fluid interface. The temperature of the diffusion cell was maintained at 32 ± 1 °C with a circulating water bath. A magnetic bar was used to stir the receptor phase to ensure uniform mixing. The solution in the receptor compartment was replenished after each withdrawal with an equal volume of fresh solution, allowed maintaining sink conditions in the experiments at scheduled intervals for a period of 30 h. The receptor fluid samples were analyzed by HPLC for DCF content. The donor vehicle was changed periodically to avoid a reduction in the drug thermodynamic activity and a change in vehicle components in the system throughout the experiment. The sampling arms and the donor compartment were occluded to prevent evaporation and therefore changes in the concentration.

4.6.3. Determination of DCF flux and permeability in the skin

The cumulative amount of DCF permeating across the skin was plotted against time. Drug flux (μg/h/cm^2), J_{ss}, at steady-state was calculated by dividing the slope of the linear portion of the curve by the area of the exposed skin surface (1.54 cm^2). The permeability coefficients, K_p (cm/h), were calculated by dividing the steady-state DCF fluxes by the concentration of the drug in the vehicle, C_o. The lag-time was determined by extrapolation of the linear portion of the cumulative amount of drug permeated versus time plot to the abscissa.

4.6.4. High performance liquid chromatography (HPLC) detection of DCF

The HPLC system consisted of an AGILENT 1100 HPLC pump, AGILENT 1100 HPLC detector (Agilent Technologies, USA,) set at 280 nm. The samples were chromatographed on a reversed-phase Supelcosil LC-18 (250 × 4.6 mm, 5 μm, USA). The mobile phase, at 1 mL/min flow rate, consisted of a mixture of methanol/PBS (66:34 v/v), which was filtered and degassed before use. The column was thermostated at 35 °C, and under these experimental conditions, the run time was 8.4 min.

4.6.5. Data analysis

Statistical data analysis was performed using the *t*-test with $p < 0.05$ as the minimal level of significance.

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