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Research Article

Preparation, Characterization, and *In Vitro* Intestinal Permeability Evaluation of Thalidomide–Hydroxypropyl-β-Cyclodextrin Complexes

Jadel M. Kratz,[1] Marina R. Teixeira,[1] Karine Ferronato,[2] Helder F. Teixeira,[2] Letícia S. Koester,[2] and Cláudia M. O. Simões[1,3]

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Abstract. Thalidomide is emerging as a therapeutic agent with renewed clinical importance, presenting anti-inflammatory, immunomodulatory, and antineoplasic properties. In this work, we studied the complexation of thalidomide with cyclodextrins as a strategy to circumvent the poor aqueous solubility of the drug. Thalidomide–hydroxypropyl-β-cyclodextrin complexes were obtained by kneading method and were characterized by differential scanning calorimetry, powder X-ray diffractometry, and scanning electronic microscopy. The aqueous solubility and *in vitro* dissolution of thalidomide were significantly improved through the complexation. Physicochemical analysis of the complexes in solid state revealed a decreased crystallinity of the complexed drug in comparison with free thalidomide. Thalidomide was able to dissociate from the complexes and permeates across intestinal epithelial Caco-2 cells with a favorable high permeability profile equivalent to that of the free drug. In summary, the present results suggest that thalidomide–hydroxypropyl-β-cyclodextrin complexes could be regarded as a promising strategy for improving the gastrointestinal absorption of thalidomide.

KEY WORDS: cyclodextrin; dissolution; intestinal permeability; solubility; thalidomide.

INTRODUCTION

Thalidomide (Fig. 1) is a glutamic acid derivative that was first synthesized in Germany in 1954. It was initially approved in many countries as a sedative and antiemetic drug. However, due to teratogenicity and neuropathy, it was withdrawn from the market in the early 1960s (1,2).

The pharmacological properties of thalidomide extended beyond the neurosedative effects. Its subsequent establishment as an anti-inflammatory, immunomodulatory, and antineoplasic compound inspired researchers to define its clinical range. Thalidomide was approved by the FDA for the treatment of erythema nodosum leprosum and, more recently, in association with dexamethasone, for multiple myeloma (3–5).

The drug is formulated as an equimolar racemate of two active enantiomers that rapidly interconvert under physiological conditions. The molecule contains a glutarimide moiety with a single chiral center and undergoes spontaneous hydrolysis in aqueous solutions at pH 7.4 (6). In addition to the chemical instability, thalidomide exhibits diverse polymorphs (7) and absorption rate-limited pharmacokinetics. The low solubility and poor dissolution of the drug in the gastrointestinal tract lead to an erratic and incomplete absorption (6,8). In order to circumvent instability and solubility issues, different pharmaceutical strategies have been employed which include: isolation of pure enantiomers (9), association with polymeric carriers (10), incorporation into nanoemulsions (11), and complexation with cyclodextrins (12–16).

Cyclodextrins are cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity which are able to form inclusion complexes (17). They have been extensively used in the pharmaceutical industry to improve bioavailability, especially of class II drugs (low solubility and high permeability), according to the Biopharmaceutics Classification System (18). Cyclodextrins may prove utility by both improving the solubility and the permeability (17). While some studies have shown the improvement of the aqueous solubility of thalidomide via complexation, there is a lack of information regarding the intestinal permeability of the complexes. In this view, this work reports the preparation and characterization of thalidomide complexes and the study of the effect of complexation over the aqueous solubility, dissolution rate, and *in vitro* intestinal permeability of thalidomide.

MATERIALS AND METHODS

Reagents and Chemicals

Thalidomide produced by Microbiológica Química e Farmacêutica (Brazil) was a kind gift from Fundação

[1] Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina–UFSC, Florianópolis, SC, Brazil.
[2] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul–UFRGS, Porto Alegre, RS, Brazil.
[3] To whom correspondence should be addressed. (e-mail: claudias@reitoria.ufsc.br)

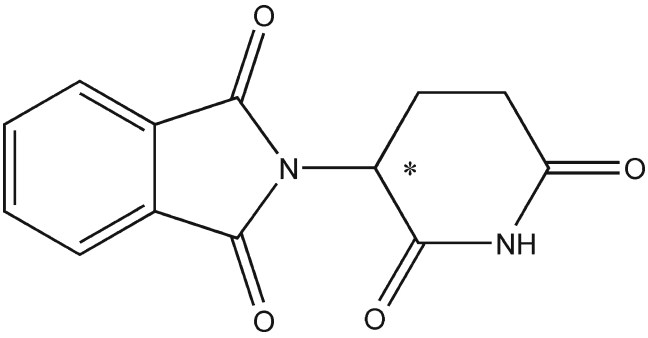

Fig. 1. Structure of thalidomide with the chiral center marked with an *asterisk*

Ezequiel Dias (MG, Brazil). All thalidomide raw materials used in this study consisted of an equimolar racemate of (+)-(R) and (−)-(S) enantiomers. α-Cyclodextrin (α-CD), γ-cyclodextrin (γ-CD), and phenacetin were purchased from Sigma (USA). β-Cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD), and methyl-β-cyclodextrin (ME-β-CD) were supplied by Roquette (France). Acetonitrile was obtained from Tedia (Brazil). All other reagents and solvents used in this study were of the highest purity commercially available.

High Performance Liquid Chromatography Analysis

The method employed in this study was developed based on previous reports (19,20). Quantitative determinations of thalidomide were performed by high performance liquid chromatography (HPLC) on a Shimadzu LC-10A chromatographer. Chromatographic separations were obtained using a 150×4.6-mm C18 column (Phenomenex, USA) at 40°C. Mobile phase consisted of acetonitrile, water, and phosphoric acid in the ratio of 24:76:0.1 (*v*/*v*) under an isocratic flow rate of 1.0 ml/min. The analytical wavelength was set at 237 nm and samples of 20 μl were injected into the HPLC system. Phenacetin was used as an internal standard. Under these conditions, the retention times of thalidomide and phenacetin were 4.86 and 6.77 min, respectively. The chromatographic method was validated according to ICH recommendations (21) and found specific, linear ($y=0.0073x+0.0012$, $r^2=0.9998$), precise (RSD <2.40%), and accurate (97.7–101.6%).

Phase Solubility Studies

Phase solubility studies were carried out in aqueous medium as described previously (22). Excess amounts of thalidomide (50 mg–7.7 mM) were added to 25 ml of water or aqueous solutions containing increasing concentrations of cyclodextrins (0–31 mM). Flasks were covered with aluminum foil and the pH of the dispersions was adjusted to 4–5 in order to avoid degradation. Dispersions were magnetically stirred for 6 h, at room temperature (25°C), filtered through 0.45-μm membranes (Millipore, USA), and appropriately diluted. Concentration of thalidomide in the filtrates was determined by HPLC. The presence of cyclodextrins did not interfere with the assay as previously evaluated (data not shown). The solubilizing potential of cyclodextrins was assessed through the determination of the complexation efficiency (CE), which can be obtained from the slope of phase solubility diagrams, according to the equation (23):

$$CE = \frac{\text{slope}}{(1 - \text{slope})}.$$

Preparation of the Solid Thalidomide–Cyclodextrin Complexes

Thalidomide–cyclodextrin solid complexes (KN) were obtained by the kneading method (24). A mixture of 0.50 g of thalidomide and an appropriate amount of cyclodextrin (molar ratio of 1:1) was wet in a mortar with a minimum volume of ethanol/water solution (1 ml, 1:1, *v*/*v*). The mixture was grounded thoroughly with a pestle for 30 min and afterward dried for 48 h at room temperature (25°C). The dried complex was pulverized into a fine powder. A physical mixture (PM) of thalidomide and cyclodextrin was prepared at the same molar ratio by the same procedure as described for the complexes. All products were stored in a dessicator until further evaluation.

Characterization of the Solid Complexes

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) measurements were performed on a DSC-60 calorimeter (Shimadzu, Japan). Samples of 1 to 2 mg were accurately weighed in aluminum pans and crimped. The operating conditions were 10°C/min of heating rate, from 35°C up to 350°C, 50 ml/min of nitrogen gas flow. The temperature calibration was performed using indium (melting point (mp) 157°C) and zinc (mp 420°C) as standards.

Powder X-Ray Diffractometry

The powder X-ray diffractometry (XRD) patterns were recorded on a Siemens D5000 diffractometer (Siemens, Germany) equipped with a curved graphite crystal, using Cu Kα radiation. The scanning rate employed was 0.05° per second over a 2θ range of 5–70°. Generator tension and current were 40 kV and 30 mA, respectively.

Morphology Evaluation

Surface morphology was analyzed in a JSM-6060 scanning electron microscope (SEM) (JEOL, USA). Samples were fixed on a brass stub using double-sided tape and then coated with a thin gold layer under vacuum. The photomicrographs were taken at a voltage of 10–20 kV and magnification factor from ×500 to ×1,500.

Dissolution Studies

In vitro dissolution profiles were evaluated as described previously (25), with some modifications, using the USP basket apparatus. Capsules containing 50 mg of thalidomide, or its equivalent in KN or PM products, were added to 1,000 ml of dissolution media (0.225 M HCl and 1% of sodium lauryl sulfate) at 37.0±0.5°C and stirred at 100 rpm on standard dissolution equipment (Nova Ética, Brazil). About 5 ml of the

test medium was sampled at 10, 20, 30, 45, and 60 min with medium reposition, and thalidomide concentration was determined by HPLC.

In Vitro Intestinal Permeability Studies with Caco-2 Cells

Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection (ATCC # HTB-37, USA). Cells were maintained in a humidified 5% CO_2 air atmosphere at 37°C and were cultured in DMEM containing 4.5 g/l glucose (Gibco, USA) with 20% fetal bovine serum, 1% nonessential amino acids, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 25 μg/ml of amphotericin B (Gibco, USA). After reaching 80–90% of confluence, the cells were harvested and seeded into Millicell® polycarbonate inserts (0.6 cm^2, 0.4 μm pore size—Millipore, USA) at a density of 10^5 cells/insert.

Transport Experiments

The experiments were carried out under sink conditions, according to recommendations described previously (26). *In vitro* permeation studies were performed after 21–25 days of culture, using Caco-2 cells between passage 25 and 31. Hank's balanced salt solution (HBSS) buffered at pH 6.0 (10 mM of methanesulfonic acid) and at pH 7.4 (10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was used as transport buffers in the apical (AP—donor) and basolateral (BL—acceptor) side, respectively. Transepithelial electrical resistance (TEER) measurements (Millicell® ERS meter—Millipore, USA) and Lucifer Yellow (LY, Sigma, USA), a fluorescent paracellular permeability marker, were used to control the integrity of Caco-2 monolayers.

Sample solutions containing 50 μM of thalidomide or equivalents in KN or PM were added to the AP side and filters were incubated for 2 h at 37°C in an orbital shaker (100 rpm). At suitable time intervals, samples were collected from the BL side by moving the cell monolayers to a new well containing fresh HBSS. A sample was also collected from the AP side at the final time point in order to perform the mass balance calculation. The apparent permeability of LY was not affected by the incubation of cells with samples, and TEER values were stable before and after the experiments (data not shown). These findings show that cell monolayers were not destabilized during the permeability evaluation.

After sample collection, all aliquots were mixed with two volumes of cold acetonitrile/methanol mixture containing 2% acetic acid and 100 μM of phenacetin to prevent thalidomide from spontaneous degradation (20). Following, the samples were dried using a SpeedVac concentrator (Thermo, USA) and the residues were reconstituted in mobile phase. The mean recovery value obtained in this process was 87.3±2.5%. Thalidomide was assayed by HPLC and the apparent permeability coefficients (P_{app}, in centimeter per second) were calculated according to the following equation:

$$P\text{app} = \frac{(\text{dQ})}{(\text{dt})} \times \frac{1}{A \times C_0 \times 60}$$

where (dQ/dt) is the amount of thalidomide permeated in the unit of time, A is the surface area of the monolayers, and C_0 is the initial donor concentration (26).

Statistical Analysis

Results were expressed as the mean ± SD of three independent experiments, unless otherwise stated. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($p<0.05$). Calculations were performed with GraphPad Prism software (GraphPad, USA).

RESULTS AND DISCUSSION

Phase Solubility Studies

A preliminary phase solubility study was performed with α-CD, β-CD, and γ-CD. An excess of ten times the solubility of thalidomide (2 mM) and two molar ratios (1:1 and 1:4) were used in this screening. Additionally, two different stirring times were employed (6 and 24 h), but no significant differences between stirring times were found ($p>0.05$).

The apparent aqueous solubility of free thalidomide determined at pH 5 was 51.4±1.5 μg/ml. Among the three natural cyclodextrins, β-CD showed the most promising results (Fig. 2). The complexation with this cyclodextrin increased the solubility of thalidomide by 1.3- and 1.8-fold for the 1:1 and 1:4 molar ratios, respectively ($p<0.001$).

Accordingly, a more robust phase solubility study was performed with β-CD and two other β-derivatives, HP-β-CD and ME-β-CD (Fig. 3). HP-β-CD and ME-β-CD presented higher solubilization potential than β-CD ($p<0.001$). These complexes presented A_L-type diagrams (21), where a linear increase of thalidomide solubility was observed as function of cyclodextrins concentration ($r^2=0.9749$ and 0.9974, respectively). In general, modified cyclodextrins, such as HP-β-CD and ME-β-CD, form A-type phase solubility

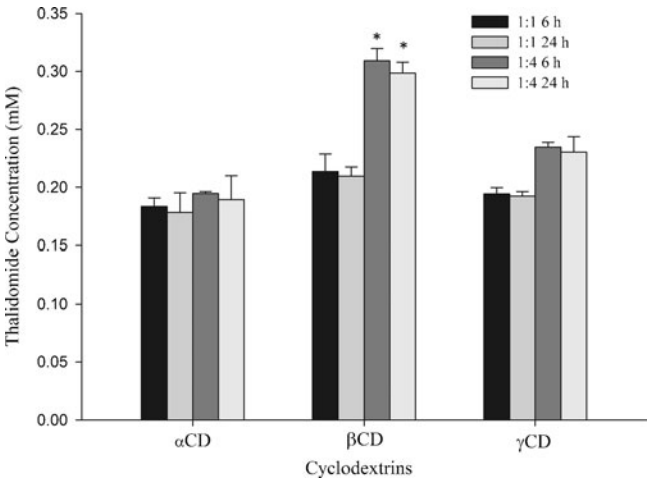

Fig. 2. Preliminary phase solubility study of thalidomide and α-, β-, and γ-cyclodextrins. Experimental conditions: stirring time of 6 and 24 h at room temperature (25°C), molar ratio of 1:1 and 1:4 and excess amount of thalidomide (2 mM). Results are expressed as mean ± SD ($n=3$). *Asterisk* Significantly different from other cyclodextrins and/or molar ratios (ANOVA/Tukey—$p<0.001$)

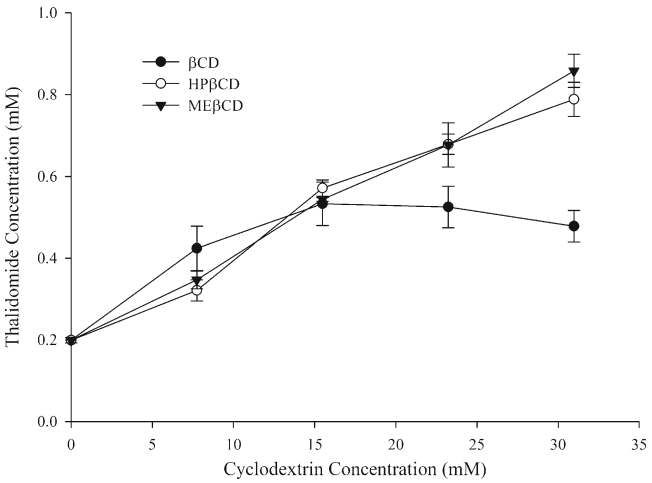

Fig. 3. Phase solubility diagrams of thalidomide and β-CD, HP-β-CD, and ME-β-CD. Experimental conditions: stirring time of 6 h at room temperature (25°C) and excess amount of thalidomide (7.7 mM). Results are expressed as mean ± SD (n=3)

profiles, whereas less soluble cyclodextrins frequently form B-type profiles, indicative of the formation of complexes with limited solubility, as observed for β-CD (Fig. 3). In this second set of experiments, a different excess of drug was used (7.7 mM), since parallel experiments showed an improved solubilization of thalidomide with this setup (data not shown).

The complexation efficiency, which characterizes the solubilizing power of the cyclodextrins, was calculated in order to select the most promising cyclodextrin for the preparation of solid thalidomide complexes. β-CD, with a CE value of 0.009, showed the lowest solubilizing power in the analysis, while HP-β-CD and ME-β-CD presented similar potential (CE=0.020), even though all three values are considered relatively low (23).

Preparation and Characterization of Solid Thalidomide Complexes

Since no significant difference between the amount of thalidomide solubilized by HP-β-CD and ME-β-CD was found (p>0.05), HP-β-CD was selected as the most advantageous candidate. This cyclodextrin is a widely studied alternative to β-CD, with improved water solubility, and toxicological studies have pointed out that HP-β-CD is well tolerated via intravenous and oral administrations (17,27,28).

Initially, a thalidomide–HP-β-CD freeze-dried complex was obtained by lyophilization method. This technique generated solid complexes containing <1% of the drug (data not shown), with little pharmaceutical usefulness. Therefore, the solid 1:1 thalidomide–HP-β-CD complex was obtained by the kneading method. The characterization of solid samples was carried out by several methods. DSC thermograms of free thalidomide, HP-β-CD, KN, and PM are shown in Fig. 4. Thermogram of thalidomide presented a sharp endothermic peak at 276.7°C corresponding to the melting point of the drug, while HP-β-CD showed only a broad endothermic peak near 80°C, corresponding to the release of water from the cyclodextrin cavity. The complete disappearance of thalidomide endothermic peak in the KN thermogram indicates a strong interaction of thalidomide with HP-β-CD in the solid state. The thermogram of PM presented a mixed profile, with characteristics from both HP-β-CD and thalidomide. The fusion peak of the drug was broaden, with lower onset temperature (270.5°C for PM and 275.3°C for thalidomide), indicative of a slight interaction in this simple mixture.

Additional evidence of the complexation of thalidomide was obtained by XRD patterns (Fig. 5). Free drug presented sharp and intense peaks, indicating the crystalline nature of the powder. In contrast, HP-β-CD presented a characteristic hollow pattern, representative of amorphous structures. Both

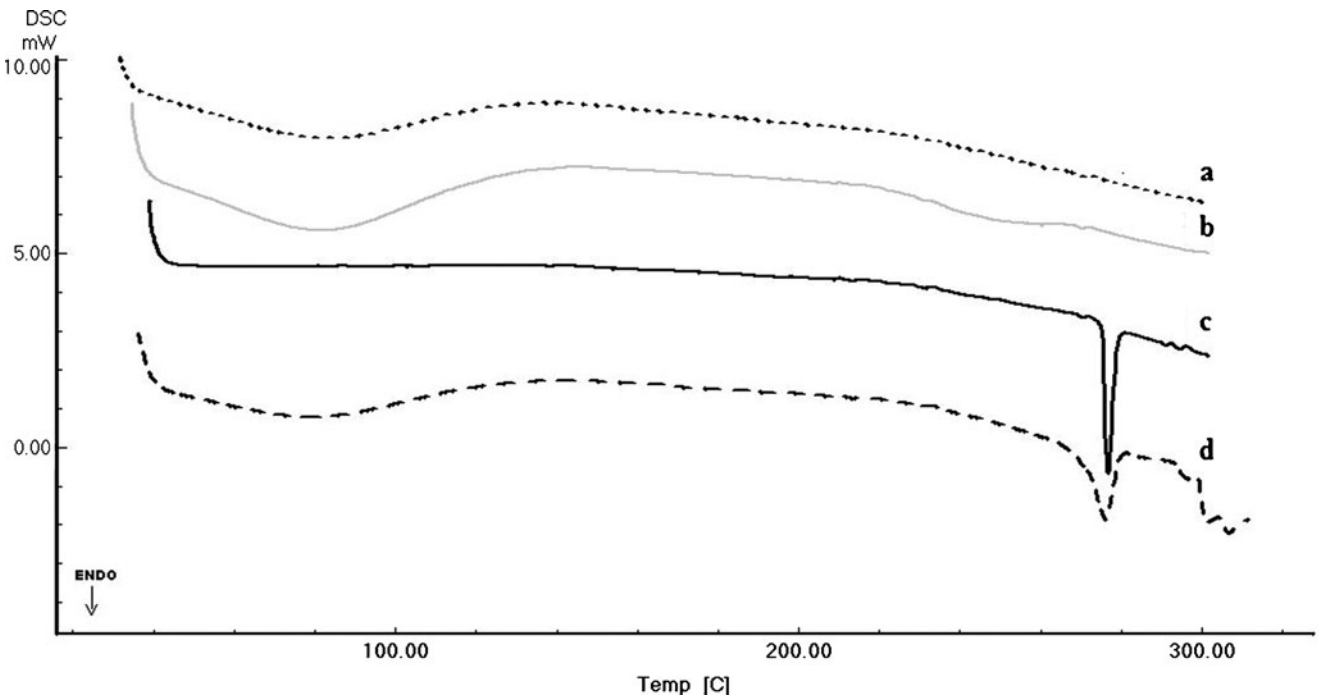

Fig. 4. DSC thermograms of **a** HP-β-CD, **b** thalidomide–HP-β-CD complex (KN), **c** thalidomide, and **d** physical mixture (PM)

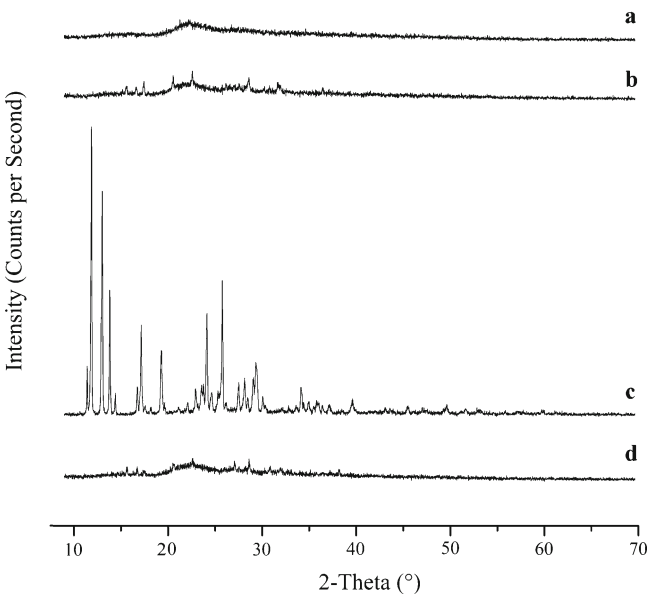

Fig. 5. Powder X-ray diffractograms of **a** HP-β-CD, **b** thalidomide-HP-β-CD complex (KN), **c** thalidomide, and **d** physical mixture (PM)

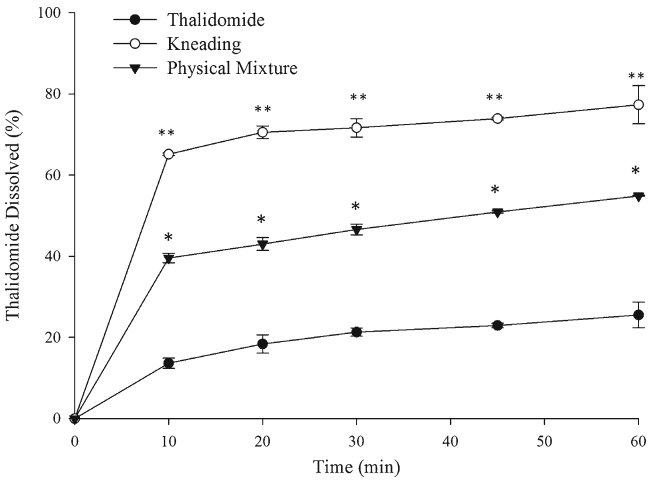

Fig. 7. Dissolution profiles of free thalidomide, thalidomide–HP-β-CD complex (KN), and physical mixture (PM). Dissolution media, 0.225 M HCl+1% sodium lauryl sulfate at 37°C. Results are expressed as mean ± SD (n=3). *Asterisk* Significantly different from free thalidomide (ANOVA/Tukey—p<0.001). *Double asterisks* Significantly different from free thalidomide and physical mixture (ANOVA/Tukey—p<0.001)

diffraction patterns of KN and PM correspond to the superimposition of those of the pure components, with lower intensities of the diffraction peaks. These profiles were observed due to particle size reduction during grinding and dilution of the pure crystalline components, indicating partial amorphization of the material.

Figure 6 shows SEM photomicrographs of thalidomide, HP-β-CD, and KN. The crystalline structure of the drug is evidenced in Fig. 6a, which presents characteristic format of big pointed plates, indicating that the raw material is formed mainly by the β polymorph (25). This hypothesis was confirmed by Fourier transformed infrared spectroscopy analysis (data not shown). Alternatively, curved particles of various sizes with some concavities on the surface were revealed for HP-β-CD, whereas aggregate formations occurred in the kneading process, as many small adhered formations can be observed in complexed product (Fig. 6c, d).

Even though physicochemical characterization results provide strong evidence of interaction between thalidomide and HP-β-CD, in this study, we were not able to demonstrate the inclusion of the drug molecule into the cyclodextrin cavity. It is well known that cyclodextrins form both inclusion and non-inclusion complexes, and that several different types of complexes can coexist in aqueous solutions. They can also aggregate and solubilize drugs and other hydrophobic molecules through micellar-type mechanisms (23,28). Nuclear magnetic resonance spectroscopy analyses were inconclusive, probably due to the low complexation efficiency (data not shown), and the molecular mechanism by which thalidomide and HP-β-CD interact remains yet to be clarified.

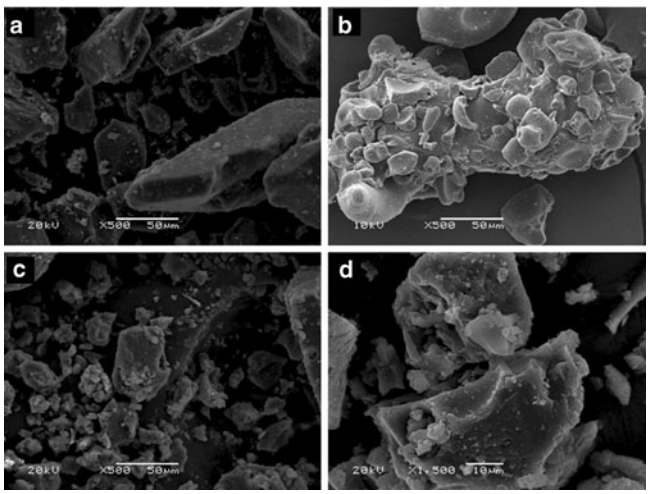

Fig. 6. Scanning electron microscopic (SEM) photomicrographs of **a** thalidomide, **b** HP-β-CD, and **c**, **d** thalidomide–HP-β-CD complex (KN). Magnification ×500 **a**, **b**, **c** and ×1,500 **d**

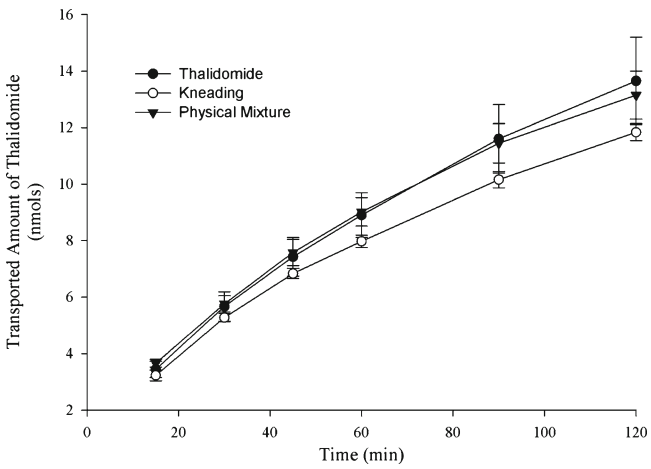

Fig. 8. Comparative *in vitro* permeability of free thalidomide, thalidomide–HP-β-CD complex (KN), and physical mixture (PM) across Caco-2 cells in the apical to basolateral direction. Results are expressed as mean ± SD (n=6). No significant difference among permeability profiles (ANOVA/Tukey—p>0.05)

Dissolution Studies

Dissolution profiles of capsules containing 50 mg of free thalidomide, or its equivalent amount in KN or PM products were assessed and the results expressed as the percent amount dissolved *versus* time. Figure 7 shows that only 25.5±3.2% of the free thalidomide was dissolved after 60 min, while the percentage of thalidomide dissolved from the PM was higher, 54.9±4.7% ($p<0.001$). The enhancement in the drug dissolution rate from PM could be due to the surfactant-like properties of cyclodextrins, thus improving the wettability and dissolution of the drug, as already reported (24). The kneaded product presented higher dissolution rate when compared to both PM and free drug ($p<0.001$), with 77.3±0.1% of thalidomide dissolved after 60 min. This enhancement has been attributed to the formation of stable complexes in the solid state, improving the hydrophilicity of the molecule and reducing its crystallinity, as indicated by DSC, XRD, and SEM studies. The *in vitro* dissolution results obtained with the free drug in this study were slightly different from those obtained by Kale and co-workers (13). This variability was attributed to the use of different thalidomide samples and experimental conditions. Raw materials may be composed of different polymorphs and present unrelated dissolution profiles (25). Furthermore, different formulations and dissolution techniques may produce diverse results, as already described (29).

In Vitro Permeability Data

Cyclodextrins are potential absorption enhancers and may alter epithelial barrier properties (30,31). Both positive and negative outcomes have been achieved with the complexation of drugs with cyclodextrins in relation to the *in vitro* permeability (32–37). To investigate if the complexation may also affect the *in vitro* intestinal permeability of thalidomide, transport experiments were performed on Caco-2 cells. In these experiments, thalidomide was stable in the transport buffer throughout the entire period of 2 h. The acidification of samples was successful in preventing the hydrolysis of thalidomide since 93.6±2.2% of the initial drug was detected at the end of the experiment (sum of acceptor and donor concentrations).

After 120 min of incubation, the accumulated transported amount of thalidomide for all three samples presented a similar linear profile (Fig. 8). Free thalidomide presented a P_{aap} value of $5.33\pm0.78\times10^{-5}$ cm/s, which categorizes thalidomide as a highly permeable drug (38). Our data corroborate previous reports showing that thalidomide has favorable properties for intestinal permeation, being rapidly transported through cells (39,40). KN and PM showed P_{app} values of $4.84\pm0.11\times10^{-5}$ and $4.98\pm0.77\times10^{-5}$ cm/s, respectively. No statistically significant difference among samples was detected ($p>0.05$). These findings show that the complexation of thalidomide with HP-β-CD, or the simple presence of the cyclodextrin in solution, did not influence the intestinal permeability. The high permeability profiles found suggest that the bioavailability of the complexed drug would not be limited by the intestinal permeability.

CONCLUSION

Our results showed that the complexation of thalidomide with HP-β-CD improved the aqueous solubility and the *in vitro* dissolution rate of the drug through the enhancement of its apparent solubility and reduction of crystallinity, both resulting from the formation of stable complexes in the solid state. Additionally, this is the first report demonstrating that thalidomide was able to dissociate from the complexes and permeate across intestinal epithelial Caco-2 cells with a favorable high permeability profile equivalent to that of the free drug. In summary, the present results suggest that thalidomide–HP-β-CD complexes could be regarded as a promising strategy for improving the gastrointestinal absorption of thalidomide.

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