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Epiisopiloturin–Hydroxypropyl-β-Cyclodextrin inclusion complexes: preparation, characterization, and application in neglected diseases

Complexos de inclusão de epiisopiloturina-hidroxipropil-β-ciclodextrina: preparação, caracterização e aplicação em doenças negligenciadas

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Abstract

Context: *Pilocarpus microphyllus* (Jaborandi) is widely used for extracting pilocarpine, generating biomass rich in secondary metabolites. Among these, epiisopiloturin (EPI) exhibits potential against neglected diseases, in addition to anti-inflammatory and antinociceptive effects. However, its poor aqueous solubility limits its pharmaceutical application. **Objective:** To enhance the solubility of EPI by forming an inclusion complex with hydroxypropyl-β-cyclodextrin (HPβCD) using the freeze-drying technique. **Methods:** A phase solubility study was conducted to determine the stability constant and stoichiometry. The inclusion complex was prepared via lyophilization and characterized by Differential Scanning Calorimetry (DSC), Fourier Transform Infrared Spectroscopy (FTIR), X-ray

Resumo

Contexto: *Pilocarpus microphyllus* (Jaborandi) é amplamente utilizado para extrair pilocarpina, gerando biomassa rica em metabólitos secundários. Entre eles, a epiisopiloturina (EPI) apresenta potencial contra doenças negligenciadas, além de efeitos anti-inflamatórios e antinociceptivos. No entanto, sua baixa solubilidade em água limita sua aplicação farmacêutica. **Objetivo:** Aumentar a solubilidade da EPI através da formação de um complexo de inclusão com hidroxipropil-β-ciclodextrina (HPβCD) utilizando a técnica de liofilização. **Métodos:** Foi realizado um estudo de solubilidade de fase para determinar a constante de estabilidade e a estequiometria. O complexo de inclusão foi preparado por liofilização e caracterizado por calorimetria diferencial de varredura (DSC), espectroscopia de infravermelho

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Diffraction (XRD), and in vitro dissolution testing. **Results:** Characterization confirmed the formation of the EPI:HPβCD complex, indicating strong interactions between components. The DSC thermogram showed the disappearance of the EPI melting peak, supported by FTIR results, suggesting successful complexation. XRD patterns revealed an amorphous structure. In vitro dissolution demonstrated a marked increase in solubility: 100% of the complexed EPI dissolved within 5 minutes, compared to only 19% of the free compound. **Conclusion:** Complexation with HPβCD significantly improved the solubility of EPI, reinforcing its potential for development into an innovative pharmaceutical formulation for the treatment of neglected diseases.

Keywords: Epiisopiloturin, Cyclodextrin, Inclusion complex, Increased solubility.

por transformada de Fourier (FTIR), difração de raios X (XRD) e testes de dissolução in vitro. **Resultados:** A caracterização confirmou a formação do complexo EPI:HPβCD, indicando fortes interações entre os componentes. O termograma DSC mostrou o desaparecimento do pico de fusão do EPI, apoiado pelos resultados da FTIR, sugerindo uma complexação bem-sucedida. Os padrões de XRD revelaram uma estrutura amorfa. A dissolução in vitro demonstrou um aumento acentuado na solubilidade: 100% do EPI complexado se dissolveu em 5 minutos, em comparação com apenas 19% do composto livre. **Conclusão:** A complexação com HPβCD melhorou significativamente a solubilidade do EPI, reforçando seu potencial para o desenvolvimento de uma formulação farmacêutica inovadora para o tratamento de doenças negligenciadas.

Palavras-chave: Epiisopiloturina, Ciclodextrina, Complexo de incluso, Maior solubilidade.

Introduction

Neglected tropical diseases (NTDs) predominantly affect low-income populations and receive limited investment from the pharmaceutical industry (Dias et al., 2013). It is estimated that they contribute to approximately 12% of the global disease burden, causing more than 35,000 deaths daily, mainly in developing countries (Conteh et al., 2010). Despite this, less than 5% of global R&D funding targets therapies for NTDs (Chatelain & Ioset, 2011).

Consequently, affected populations lack adequate treatments and suffer from limited therapeutic options. Effective drug access is therefore crucial for addressing these public health issues (Venturini et al., 2008). *Pilocarpus microphyllus* (Jaborandi) contains various alkaloids of pharmaceutical interest. Among them, pilocarpine (PILO) and epiisopiloturin (EPI) demonstrate biological activities, particularly against parasites responsible for schistosomiasis (Veras et al., 2012; Véras et al., 2013), with some results surpassing praziquantel (PZQ), the reference drug. Additionally, EPI exhibits activity against *Leishmania spp*. (Guimarães, 2018) and anti-inflammatory effects.

However, the poor water solubility and low oral bioavailability of EPI limit its therapeutic application (Veras et al., 2012). Cyclodextrins (CDs), cyclic oligosaccharides capable of forming inclusion complexes with lipophilic molecules, are a well-established strategy to enhance solubility and dissolution rates of poorly soluble drugs (Del Valle, 2004; Sarabia-Vallejo et al., 2023).

Different techniques, such as freeze-drying, spray-drying, and co-precipitation, can be used to prepare inclusion complexes. Freeze-drying is particularly advantageous, producing amorphous powders with strong drug-CD interactions (Cunha-Filho & Sá-Barreto, 2008; Sobrinho et al., 2011).

The present study aimed to obtain and characterize the Epiisopiloturin–Hydroxypropyl-β-Cyclodextrin (EPI:HPβCD) inclusion complex, with the objective of overcoming its solubility limitations and advancing its potential as a therapeutic candidate against NTDs.

Theoretical Framework

The treatment of neglected diseases (NDs) continues to present urgent challenges. Many of the drugs currently available, such as those used in leishmaniasis therapy, are associated with high toxicity and adverse effects, which reduce patient adherence and therapeutic success. Moreover, the persistence of NDs is directly linked to the lack of investment in affordable drugs, reflecting the pharmaceutical industry's limited interest in populations with low purchasing power (Oliveira, 2006).

Within this context, natural products remain an important source for new therapeutic prototypes. Epiisopiloturin (EPI), an alkaloid derived from *Pilocarpus microphyllus* (jaborandi), has demonstrated significant schistosomicidal activity (Veras et al., 2012; Véras et al., 2013) and promising results against *Leishmania* spp., in addition to anti-inflammatory and antinociceptive effects (Guimarães, 2018).

Although EPI has shown strong pharmacological potential, its poor water solubility remains a major obstacle for pharmaceutical formulation development. One consolidated strategy to address this challenge is the formation of inclusion complexes with cyclodextrins (CDs), which has been widely employed to improve the solubility and bioavailability of several poorly soluble drugs (Wangsawangrung et al., 2022).

Methodology

Materials

Epiisopiloturin (EPI) was isolated and supplied by Anidro do Brasil Extrações S.A.® (Parnaíba, Brazil), following the procedure described by Véras et al. (2013). Hydroxypropyl-β-cyclodextrin (HPβCD) was kindly donated by Ashland Specialty Ingredients®. Distilled water was used as the vehicle in all experiments.

Methods

Phase solubility study

For the phase solubility study, excess EPI was added to aqueous solutions of HPβCD at different concentrations (1–50 mM). A control sample was prepared with distilled water only, to determine the saturation concentration of EPI. The suspensions were kept under constant agitation at 25 °C for seven days, followed by centrifugation. Supernatants were analyzed by UV–Vis spectrophotometry at 260 nm, as previously described for alkaloids with similar profiles (Véras et al., 2013).

Obtaining the physical mixture and inclusion complex by lyophilization

The physical mixture (PM) and the inclusion complex (IC) were obtained respecting the equimolar ratio (EPI:HPβCD), according to their respective molecular weights. Initially, to obtain the PM, the two constituents were weighed and then ground for one minute using a mortar and pestle and the resulting powder stored in an amber bottle in the desiccator.

To obtain the IC, the PM was poured into a two-liter beaker and distilled water was added, stirring at a constant temperature of 50°C for 6 hours or until completely solubilized. The resulting solution was kept in an ultrafreezer at a low temperature for 48 hours to achieve complete freezing. It was then subjected to the freeze-drying process at a pressure of around 30µHg and a temperature of approximately -55°C for a drying time of around 75 hours. The resulting powder was removed from the freeze-dryer (brand Liotop®, model L101) and stored in an amber bottle in a desiccator.

Physico-chemical characterization of the inclusion complex and the physical mixture

The obtained samples (pure EPI, physical mixture and inclusion complex) were subjected to physicochemical characterization to verify the efficiency of complexation, as recommended in previous studies (Figueiras et al., 2007). The selected techniques included Fourier-transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), X-ray diffraction (XRD) and dissolution profile analysis.

The reference data for pure EPI had been previously reported by Vieira (2017), serving as a comparative standard in this work.

Fourier Transform Infrared Absorption Spectroscopy (FTIR)

The infrared spectrum was obtained using PerkinElmer® equipment (Spectrum 400) with an attenuated total reflectance (ATR) device (Miracle ATR, Pike Technologies Spectroscopic Creativity) with a zinc selonide crystal. The samples to be analyzed were transferred directly into the compartment of the ATR device in triplicate. Scans were obtained from 400 to 4500 cm-1 with a resolution of 4 cm-1.

Thermal analysis - Differential Scanning Calorimetry (DSC)

The DSC curves for the thermal characterization of the samples were obtained using a Shimadzu® calorimeter, model DSC-60, in a temperature range of 30 to 300°C, under a dynamic nitrogen atmosphere with a flow of 100mL.min-1 and a heating rate of 10°C.min-1. A mass of 5mg (± 0.2) was used, packed in hermetically sealed aluminum sample holders. All tests were carried out in triplicate. The DSC was calibrated using the melting point of Indium (156.6 ± 0.3ºC) and Zinc (419.6ºC ± 0.3). Heat flow and enthalpy were calibrated using the melting point of Indium (18.59 J.g-1 ± 0.3), under the same conditions as the samples. The thermoanalytical data was analyzed using Shimadzu® software TA-60WS® (Therma Analysis) version 2.20.

X-ray diffraction (XRD)

The XRD analyses were carried out on Shimadzu® equipment, model XRD-600, using Cukα radiation of λ=1.5406Å. The time count was 0.6 seconds for each 0.02° step, with a scan interval of 5 to 50° (2θ). The diffractograms obtained were compared with the JCPD standard charts registered with the ICDD (International Center of Diffraction Data). These analyses were carried out at the Analytical Center of the Center for Strategic Technologies of the Northeast (CETENE) in Recife-PE.

In vitro dissolution tests

Dissolution tests were performed using USP apparatus II (paddle, 50 rpm) in 250 mL of phosphate buffer (pH 6.8, 37 ± 0.3 °C). Aliquots of 3 mL were withdrawn at predetermined time intervals (5–90 min) and immediately replaced with fresh medium to maintain constant volume. The procedure followed the general method described in the Brazilian Pharmacopoeia, 5th edition (Agência Nacional de Vigilância Sanitária, 2010). A sample amount equivalent to 10% of the EPI saturation solubility (5.5 mg) was used to ensure sink conditions.

At the end, the samples obtained were filtered through a 0.45 µm membrane filter and duly diluted for subsequent quantification of EPI by UV-vis spectroscopy at 220nm (Model B582, Micronal®), using the dissolution medium as the equipment's blank to eliminate possible interferents. The procedure was carried out in triplicate and the values were averaged. According to the results and with the help of the calibration curve, the EPI concentrations were determined at each collection time and the area under the curve (AUC) value was found using OriginPro® software.

CALIBRATION CURVE

The analytical curve used was obtained from the following concentrations: 260, 600, 800, 1000, 1600µg/mL, starting from a mother solution at 5000µg/mL. The absorbance values were determined using a spectrophotometer at a wavelength of 220nm, using the dissolution medium itself as a blank. The results obtained were to obtain the calibration curve. The straight line equation was obtained by linear regression using the OringPro® software.

Results and Discussion

Phase solubility study

The phase solubility method described by Higuchi & Connors (1965) is widely used to evaluate the formation of inclusion complexes. In this approach, the solubility of the compound is determined in solutions containing progressively higher concentrations of cyclodextrins, which makes it possible to estimate the stability constant and define the stoichiometric ratio of the complex at equilibrium (Higuchi & Connors, 1965). In the case of EPI, its poor absorption after oral administration has been reported, and one strategy to overcome this limitation is the preparation of inclusion complexes (Melo, 2015). As shown in Figure 1, the solubility of EPI increases as the concentration of HPβCD rises, displaying an AL-type profile as described by Higuchi & Connors, which is associated with the formation of a soluble complex (Cunha-Filho & Sá-Barreto, 2008).

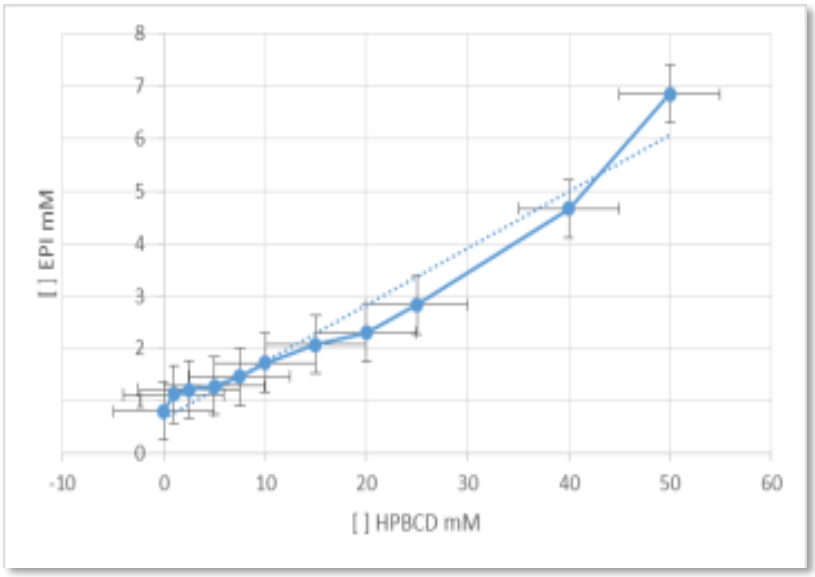

Figure 1. Phase solubility diagram of epiisopiloturine, with HPβCD in water at room temperature (25°C ± 0.5°C).
Source: Own authorship, 2018

The slope of the diagram was less than 1 (0.108191), suggesting the formation of a complex with a 1:1 ratio (m:m). Thus, there was an increase in solubility, which was initially 220.63 µg/mL and reached 1873.34 µg/mL at a concentration of 50 mM, an increase of 849.08%.

This behavior can be attributed to the presence of methyl substituents, which expand the cyclodextrin cavity and contribute to a structure that is more hydrophilic on the outside and hydrophobic on the inside. Such characteristics facilitate the accommodation of the active compound with greater flexibility (Castillo et al., 1999). In addition, the amphiphilic properties of cyclodextrins decrease the interfacial tension between the drug and the dissolution medium, which enhances the dissolution rate of the molecule (Mura, 2015).

Obtaining inclusion complexes by lyophilization

Figure 2 shows the process of obtaining the IC. Figure 2a shows the powder already dried and still in the equipment (Figure 2b), showing some of its macroscopic characteristics. It looks like a light powder that is easy to remove from the container. After the material has dried completely, a white agglomerate with a cotton-like appearance is formed, requiring maceration to break up this structure, obtaining a white powder with characteristics referring to the CD used (Figure 2c).

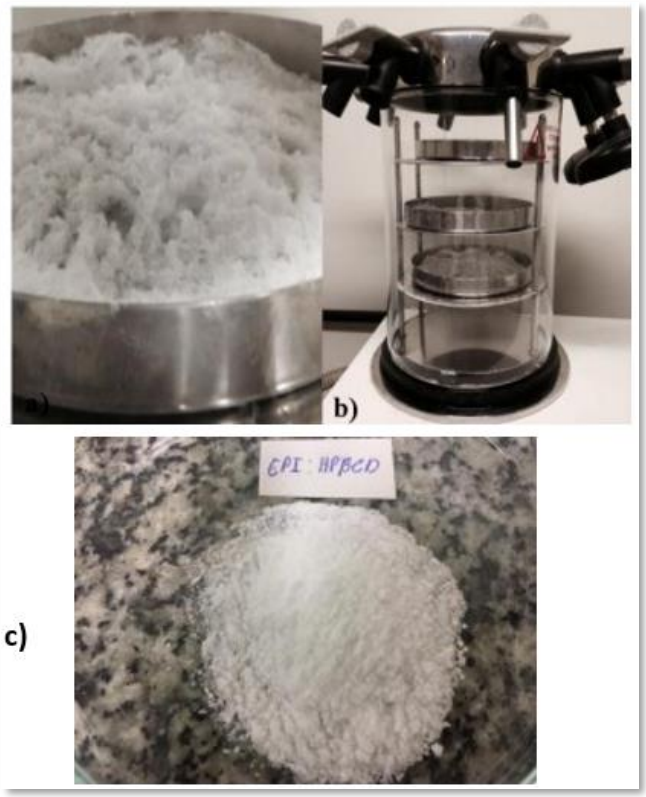

Figure 2. Images of the EPI:HPβCD inclusion complex, showing its macroscopic characteristics (Figure 2a), still in the lyophilizer (Figure 2b), white powder after grinding (Figure 2c).
Source: Own authorship, 2018

Physico-chemical characterization of the inclusion complex and the physical mixture

Absorption spectroscopy in the infrared region

Figure 3 has been divided into 4 parts which correspond to the most obvious bands in the EPI. Initially, it can be seen that the IC almost completely assumes a profile similar to that of the CD in question, serving as a strong indication of efficient complexation. Regions 2, 3 and 4 lack the defined, high-intensity bands present in EPI. For IC, region 1 still shows the presence of the characteristic band, but to a lesser extent, even compared to PM.

Moreover, the disappearance of minor peaks in the EPI spectrum suggests additional interactions between the compound and cyclodextrin, reinforcing the indication of complex formation (Figueiras et al., 2007).

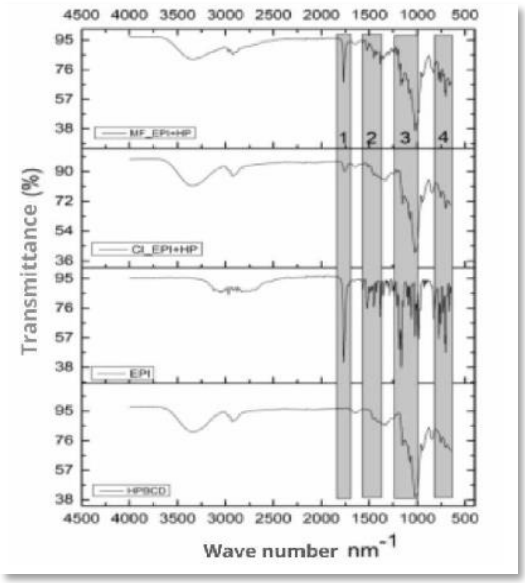

Figure 3. Infrared spectrum of the EPI:HPβCD inclusion complex and physical mixture samples, compared to the EPIISOPILOTURIN and HPβCD samples alone.
Source: Own authorship, 2018

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is a widely applied technique for investigating inclusion complexes in the solid state, since it provides thermodynamic evidence of interactions between the drug and cyclodextrin. Typically, modifications such as shifts in transition temperatures or the disappearance of melting peaks of the pure compound are considered indicative of complexation (Bayomi et al., 2002; Badr-Eldin et al., 2008; Mura, 2015).

Figure 4 shows the DSC thermograms of the physical mixture (PM) and the inclusion complex (IC) of EPI:HPβCD. The curve of pure EPI exhibits a sharp endothermic melting event between 216 and 223 °C, which is in agreement with data previously reported in the literature (Tiwari et al., 2010). In contrast, HPβCD presents a broad and asymmetric endothermic peak in the range of 124.6–145.15 °C, corresponding to the loss of water molecules commonly observed in cyclodextrins, and the absence of a distinct melting event due to their non-crystalline nature (Mura, 2015; Tiwari et al., 2010; Mendhe et al., 2016).

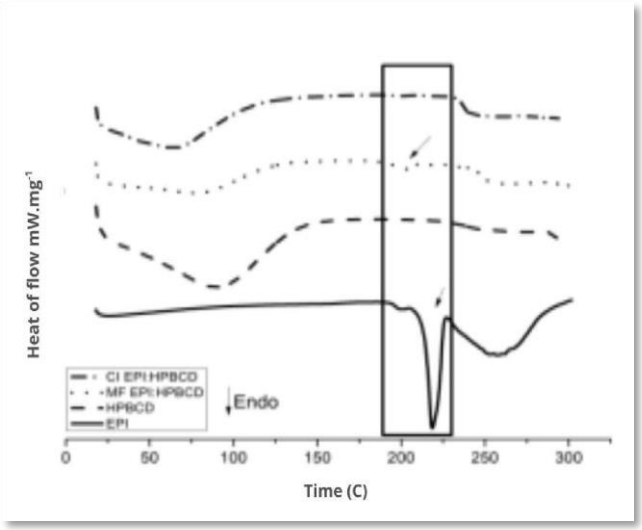

Figure 4. Differential exploratory calorimetry curves of epiisopiloturira, physical mixture and inclusion complex EPI:HPβCD, under a heating ratio of 10°C.min-1 and nitrogen flow of 50 mL.min-1.
Source: Own authorship, 2018.

For the physical mixture, a broad endothermic signal around 72 °C is observed, associated with dehydration of the CD, followed by a second event related to the melting of EPI. The reduced intensity of this melting peak indicates that simple mixing does not promote complexation, as the crystalline characteristics of the drug are still present (Tiwari et al., 2010; Mendhe et al., 2016; Haimhoffer et al., 2019).

In the case of the inclusion complex, the DSC curve does not show the melting event of EPI, which strongly suggests that the drug molecules are completely incorporated into the CD cavity. The absence of this transition is consistent with the formation of an amorphous system and provides evidence of effective interaction between the components (Figueiras et al., 2007; Sathigari et al., 2009; Sarabia-Vallejo et al., 2023). Complementary characterization techniques are nevertheless recommended to reinforce this interpretation.

X-ray diffraction

X-ray diffraction (XRD) is a standard method for evaluating the crystalline nature of compounds and has been extensively applied in the study of cyclodextrins and their inclusion complexes. By comparing the diffractograms of the pure drug, the physical mixture, and the inclusion complex, it is possible to detect modifications in solid-state properties, indicating molecular interactions between the components (Mura, 2015).

In this case, evaluating the diffractograms (Figure 5), EPI (5d) shows numerous diffraction peaks with high intensity and well defined, characteristic of crystalline material. This behavior was also observed for PM (5c), with a decrease in intensity related to the reduction in particle size during the preparation of PM, which can be considered an indication of interaction between the constituents of the mixture, but its crystalline character remains.

In contrast, the diffractogram of HPβCD (5b) exhibits broad and diffuse peaks, consistent with its amorphous nature (Silva et al., 2023). A similar pattern was observed for the inclusion complex (5a), supporting the occurrence of molecular encapsulation. Once incorporated into the CD cavity, EPI molecules cannot maintain intermolecular interactions necessary for crystallization, resulting in an amorphous final product. This finding is consistent with the DSC data, which also indicated the absence of crystallinity.

The tests were carried out in partner laboratories and on different equipment, so unfortunately it was not possible to standardize the scales, but this did not affect the results.

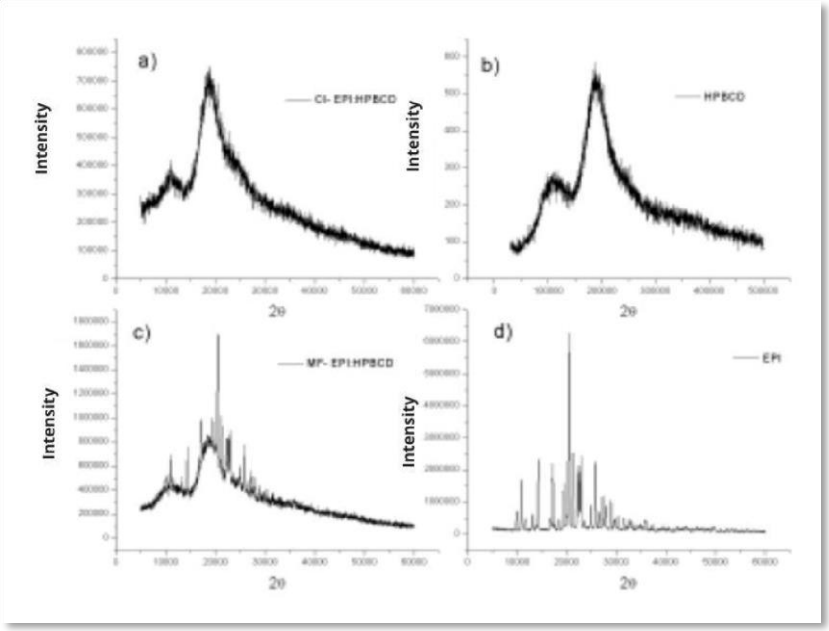

Figure 5. Diffractograms of epiisopiloturin (d), hydroxypropyl-beta-cyclodextrin (b), physical mixture (c) and inclusion complex (a).
Source: Own authorship, 2018.

Evaluation of the dissolution profile of Epiisopiloturine versus inclusion complexes.

Poorly water-soluble drugs usually present limited oral bioavailability, since dissolution is a key step for their absorption (Tiwari et al., 2010). Cyclodextrins have therefore been extensively explored as carriers to improve solubility and dissolution rates of such compounds. The positive effects of inclusion complexation are often related to modifications in the crystalline state of the drug, improved chemical stability, and increased wettability, which together enhance apparent solubility and pharmacological activity (Haimhoffer et al., 2019; Sathigari et al., 2009; Sarabia-Vallejo et al., 2023; Kumar et al., 2013; Sousa et al., 2020).

For the development of immediate-release formulations, dissolution testing can be conducted at single or multiple time points, or by constructing complete dissolution profiles. The latter approach, which follows the percentage of drug dissolved over time, provides the most reliable evaluation of dissolution efficiency and allows more robust comparisons between formulations (dos Santos, 2012).

It is possible to see the increase in its solubility, taking into account that in the first 5 minutes 100% of EPI is dissolved when it is in the form of IC EPI:HPβCD and approximately 19% when it is isolated.

After 15 minutes, the dissolution curve (Figure 6) reached a plateau, indicating an equilibrium between free and complexed EPI molecules in solution. Such equilibrium may influence the drug's absorption spectrum, leading to shifts or variations in maximum absorbance peaks. These changes are comparable to polarity effects observed with different solvents and support the hypothesis that the guest molecule is transferred from the aqueous medium into the hydrophobic cavity of cyclodextrins (Tiwari et al., 2010).

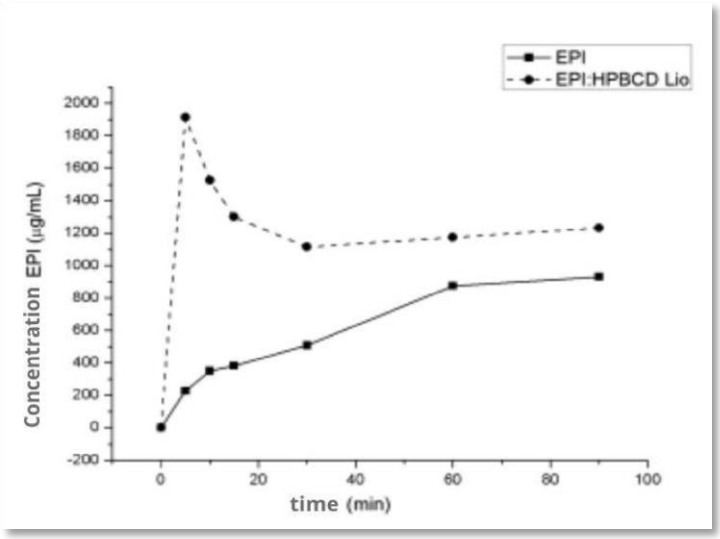

Figure 6. Dissolution profile of epiisopiloturine and EPI:HPβCD inclusion complex, evaluated by AUC values.
Source: Own authorship, 2018

An additional parameter for evaluating dissolution data is the calculation of the area under the curve (AUC). An increase in AUC values indicates enhanced dissolution efficiency, which may be associated with improved in vivo performance, since higher dissolution extent is generally correlated with greater drug bioavailability (Bhalani et al., 2022).

In the same figure, you can see the AUC value obtained for the EPI curve (AUC = 58427.5), while that of CI is almost double (AUC = 109017.5). This evaluation model correlates EPI concentration with time. Overall, it can be concluded that the formation of IC with HPBCD was able to increase solubility for better absorption of EPI and consequently promote an increase in its maximum concentration.

The physical mixture (PM) was not considered in this part of the discussion, since the inclusion complex (IC) results from the complete solubilization of EPI and HPβCD in aqueous medium under agitation. Under these conditions, the dissolution test itself mimics the complexation process, promoting higher apparent solubility of EPI. This effect is attributed to improved wettability and a reduction of interfacial tension

between solid particles and the solvent, which accelerates the dissolution rate (Haimhoffer et al., 2019; Sathigari et al., 2009; Sarabia-Vallejo et al., 2023; Kumar et al., 2013; Sousa et al., 2020; dos Santos, 2012; Bhalani et al., 2022; Rashid et al., 2019. Previous studies have shown that both PM and IC improve the dissolution profile of EPI compared to the pure drug, although the IC demonstrates superior dissolution efficiency and faster release kinetics (Melo, 2015).

Conclusions

It can be concluded that obtaining the complexation of EPI in HPβCD was satisfactory, which can be confirmed by the different techniques used, where the absence of the prototype in the results is strong evidence of interactions between them. The use of freeze-drying to obtain IC promoted a significant increase in the dissolution of EPI compared to pure and physically mixed forms.

In this way, the study successfully increased the solubility of EPI, but further analysis and dosing of EPI should be carried out in order to complement the information already found, with a view to obtaining the pharmaceutical form in the future.

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