FORMULATION AND CHARACTERIZATION OF INCLUSION COMPLEXES USING HYDROXYPROPYL- β -CYCLODEXTRIN AND FLORFENICOL WITH CHITOSAN MICROPARTICLES

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ABSTRACT

The interaction between Florfenicol (FF), Hydroxypropyl- β -cyclodextrin (HP β CD), and Chitosan (CH) has been studied in aqueous solution and in solid state, using 3 preparation methods (Evaporation, Lyophilization, Spray Drying) for HP β CD and only Spray Drying for Chitosan. The phase solubility study shows that the complex is formed with 1:1 stoichiometry and 181,4 M⁻¹ as the association constant. The analysis with Differential Scanning Calorimetry (DSC) together with Scanning Electron Microscopy (SEM) micrographs evidenced the formation of inclusion complexes, mainly with the product prepared by spray drying. Studies *in vitro* showed that FF solubility was improved almost to double, with a better dissolution profile exhibited by the product prepared by spray drying.

INTRODUCTION

Cyclodextrins (CD) are cyclic oligosaccharides made up by units of glucopyranose links with α -1,4 bonds. This creates an externally hydrophilic molecule with a non-polar cavity that provides a hydrophobic interior, so that inclusion complexes can be formed. The β -cyclodextrin (made up by 7 units of glucopyranose) has been widely used at the beginning of pharmaceutical applications due to its fast availability and proper size of its cavity. But its limited aqueous solubility and nephrotoxicity have restricted its use. Therefore, CD derivatives have been prepared by chemical modification in order to improve physicochemical properties (increased aqueous solubility, physical and microbiological stability, and decreased toxicity) and inclusion properties, being Hydroxypropyl- β -Cyclodextrin (HP β CD) one of the most commonly used¹⁻³.

On the other hand, Chitosan (CH), cationic polysaccharide obtained by deacetylation of Chitin, is a hydrophilic polymer, biocompatible and biodegradable, with low toxicity that has been widely researched for pharmaceutical and medical use. In the context of drug delivery, it has been used to prepare microparticles and nanoparticles together with CD⁴⁻⁷.

Florfenicol (FF) is a fluorinated Thiamphenicol analogue and structurally similar to Chloramphenicol (Fig. 1), which has been used as a veterinary antibiotic. Florfenicol is a broad-spectrum antibiotic potentially effective to control a wide variety of bacterial infections in fish^{8,9}.

The purpose of this work is to prepare and characterize new formulations based on HP β CD and chitosan.

Figure 1. Florfenicol Structure.

EXPERIMENTAL

Materials

Florfenicol (Molecular Weight: 358.21) was donated by Centrovet Laboratory, HP β CD (Average Substitution Degree: 0.6; Molecular Weight: 1380), Chitosan (Deacetylation Degree: 97%; Molecular Weight: 103200). All the solvents used were analytical grade. Bidistilled water was used throughout the experiment. The solution of HCl pH 1.2 was prepared as set forth by USP¹⁰.

Florfenicol concentration was quantified by HPLC (Column LiChrospher ® 100 RP-18 5 μ m 4.6 mm x 250 mm; Mobile phase 0.01 N ammonium acetate: acetonitrile, 60/40 v/v; Flow 1 mL / min; Fluorescence Detector with an excitation wavelength of 265 nm and an emission wavelength of 295).

Phase Solubility Study

The phase solubility studies were conducted in water, in triplicate, according to the method described by Higuchi and Connors¹¹. An excess quantity of FF (100 mg) was added to 5 mL of aqueous solution with variable HP β CD concentration (0 – 0.04 M) individually in 10 mL volumetric flasks. Then, these solutions were shaken in a rotary shaker at room temperature for 3 days. Once balance was achieved, an aliquot was filtered through a 0.45 μ m membrane filter and properly diluted. The FF concentration was determined by HPLC. The apparent Stability Constant K_c was calculated based on the phase solubility diagram assuming a 1:1 stoichiometry according to the following equation:

$$K_c = \frac{\text{Slope}}{S_0 (1 - \text{Slope})} \quad (1)$$

Being S_0 the FF solubility in absence of HP β CD.

Preparation of the Inclusion Complexes

The inclusion complexes (CdI) were prepared in a 1:1 ratio by means of 3 different methods: Evaporation (E), Lyophilization (L) and Spray Drying (SD).

For the above, 3 solutions were prepared weighing the required quantities of FF and HP β CD (in 1:1 ratio) to prepare 20 grams of CdI, and they were dissolved in Pure Ethanol. These solutions were kept under constant shaking in a magnetic shaker during 3 days at room temperature.

(a) Evaporation (E): The solution prepared was evaporated under vacuum

at a temperature of 70°C and 100 rpm until total dryness was achieved. The solid obtained was collected and dried at 30°C for 24 hours.

(b) Lyophilization (L): The solution prepared was frozen dipping it in an ethanol bath (-40°C) and then taken to the lyophilization process (-50°C) for 3 days.

(c) Spray Drying (SD): The solution prepared was dried by spraying under the following conditions: Inlet Temperature 130°C; Outlet Temperature 45°C.

Chitosan Microparticle Preparation

To prepare the FF microparticles (MP) using chitosan, 2 solutions were prepared and then dried by SD.

(a) MP of Chitosan-Florfenicol (Q-FF): In this case 500 mg of FF were weighed and then dissolved in 500 mL 1% of acetic acid solution. Once the FF was dissolved, 5 grams of chitosan were added. This solution was kept under constant shaking at room temperature during 3 days. After that, the solution was filtered to eliminate any chitosan remaining and dried by SD under the same conditions described in 2.3 (c).

(b) Chitosan MP-Inclusion complex obtained by spray drying FF (Q-SDFF): A quantity of the inclusion complex obtained by spray drying was weighed, equivalent to 500 mg of FF, processing it in the same way described above.

To determine the Load Capacity (LC) and the Encapsulation Efficiency (EE), MP samples were centrifuged to measure the free FF concentration in the supernatant by HPLC. The Encapsulation Efficiency and Load Capacity were calculated with the following formulas:

$$EE = \frac{\text{Total FF Quantity} - \text{Free FF Quantity}}{\text{Total FF Quantity}}$$

$$LC = \frac{\text{Total FF Quantity} - \text{Free FF Quantity}}{\text{Microparticle Weight}}$$

Characterization of Inclusion Complexes and MPs

Differential Scanning Calorimetry (DSC)

The DSC analysis was conducted with a differential scanning calorimeter. Samples (4.89 – 6.02 mg) were weighed and sealed in aluminium capsule. The analyses were conducted in a temperature range from 0 to 250°C, at a speed of 10°C/min.

Analysis by Scanning Electron Microscopy (SEM)

The surface morphology of pure FF as well as inclusion complexes and MPs were analyzed using a scanning electron microscope (JOEL: Modelo, JSM-6380 LV).

In-Vitro Release Studies

Dissolution profiles of FF, inclusion complexes and MP were analyzed in HCl pH 1.2 (simulated gastric media), phosphate buffer pH 8.0 and bidistilled water, using the paddle method (device II) according to USP¹⁰.

To do the above stated, approx. 12 mg of FF were weighed and an equivalent quantity of inclusion complexes and MP. These quantities were suspended in 500 mL of one of the solutions previously mentioned (table 1). The study was conducted at room temperature and under constant shaking at 50 rpm. During the study, sample aliquots were collected at regular intervals and a quantity equal to the volume removed was added immediately after taking each sample, in order to keep constant the dissolution media volume. FF concentration was determined by HPLC. All the experiments were done in triplicate.

Table 1. Summary of solutions that were performed *in vitro* release studies.

	Bidistilled Water	HCl pH 2	Phosphate Buffer pH 8,0
Florfenicol	X	X	X
CdI E	X		
CdI L	X		
CdI SD	X	X	X
Q-FF		X	
Q-SDFF		X	

RESULTS AND DISCUSSION

Phase Solubility Study

The solubility diagram is useful for explaining inclusion complexation of poorly soluble compounds with cyclodextrins as a host in water because it gives not only the solubilizing ability of host but also the stability constant of the complexes by analysing the solubility curves¹¹.

The phase solubility diagram of the FF-HPβCD system is shown in Fig. 2. Clearly, a linear relation can be observed in this diagram between the quantity of FF solubilized and the HPβCD concentration in the solution, with a curve type A_L¹¹. This linear correlation, as well as the slope lower than 1, suggests the formation of first-order soluble complexes in relation to CD concentration. The apparent stability constant, K_c, was calculated with equation 1, being 181.4 ± 11.1 M⁻¹. In fact, values of obtained stability constant are always within the range of 100 to 1000 M⁻¹, which is believed to indicate an ideal value. Actually, smaller values of K_c indicate a too weak interaction between drug and CD, while a larger value indicates the possibility of limited release of drug from the complex¹².

Figure 2. Phase solubility diagram for the FF-HPβCD system (n=3) (18 ± 1 °C)

Inclusion Complex Yields

The processes yields are summarized in Table 2.

Table 2. Yield from each one of the drying processes used.

Inclusion Complex	Expected g	Obtained g	Yield (%)	FF Inclusion Avg. %
Evaporation	20.0	20.1032	100.5	15.44
Lyophilization	20.0	9.1658	45.8	17.67
Spray Drying	20.0	13.4555	67.3	16.83

The analysis performed with HPLC on the different CdI obtained showed an FF content (based 20.6% as the theoretical composition) of 75%, 86%, and 82% in the complexes treated with Evaporation (E), Lyophilization (L) and Spray Drying (SD), respectively¹³.

Chitosan Microparticle Preparation

Regarding the MP treated with spray drying, results are summarized in Table 3.

Table 3. Summary of results for Chitosan MP.

MP	Yield (%)	Load Capacity (LC) (%)	Encapsulation Efficiency (EE) (%)
Q-FF	46	16.89	14.60
Q-SDFF	67	90.91	93.52

The LC and EE increase observed in Q-SDFF MP is due to a higher FF availability in the solution as a result of the solubility increase produced by CD.

Differential Scanning Calorimetry (DSC)

The thermal analysis has been reported to be a method to characterize CD complexes^{14,15}. When guest molecules are embedded in CD cavities or in the crystal lattice, their melting, boiling or sublimation point generally shift to a different temperature or disappear within the temperature range where CD is decomposed¹⁶.

Fig. 3 to 9 shows thermograms of FF complexes obtained by E, L, SD, Chitosan alone, and MPs, respectively. In the pure FF thermogram there is a typical endothermic peak at 154°C, corresponding to the melting point. The same peak is found in the thermograms of complexes obtained by evaporation and lyophilization (as well as an endothermic peak at 100°C approx. corresponding to water molecules present in the product or in the CD) but with lower intensity and drifted from the typical value of FF melting point. This sharp reduction in the intensity and/or expansion and change to lower temperatures in the FF endotherm in these systems is a signal of partial inclusion of the active principle in the HP β CD cavity, but it does not seem to be indicating a real inclusion¹⁶. However, in the thermogram of the complex prepared by spray drying, the typical FF peak is not observed. In fact, when the active-principle molecule is lodged in the CD cavity, its melting point disappears or changes to lower temperatures¹⁷. Besides, this phenomenon indicates a higher interaction between FF and HP β CD in solid state¹⁸.

On the other hand, in the thermogram of chitosan MP prepared by spray drying, it is possible to observe a very similar trend to that showed by the inclusion complexes, indicating a possible partial inclusion in the HP β CD.

Figure 3. DSC Thermograms of FF.

Figure 4. DSC of CdI obtained by Evaporation.

Figure 5. DSC of CdI obtained by Liophilization.

Figure 6. DSC of CdI obtained by Spray-Drying.

Figure 7. DSC of Chitosan.

Figure 8. DSC of MP of Q-FF.

Figure 9. DSC of MP of Q-SDFF.

Scanning Electron Microscopy (SEM) Analysis

The Scanning Electron Microscopy (SEM) is a qualitative method to study the structural aspects, in this case, of the products obtained by different preparation methods^{19,20}.

The FF micrographs showed irregular-shaped crystals forming aggregates (Fig. 10 a). As to the product prepared by Evaporation (Fig. 10 b), the presence of crystals can be observed together with structures that seem to be HPβCD adhered to its surface.

Figure 10: SEM (a) Florfenicol; (b) Evaporated Inclusion Complex; (c) Lyophilized Inclusion Complex (at different magnifications).

On the other hand, in the micrographs of the product prepared by lyophilization (Fig. 10 c), a typical solid of this process is observed forming clusters where the FF presence can be hardly differentiated.

Regarding the product prepared by SD (Fig. 11), it is observed as spherical and homogeneous particles with a very significant reduction in particle size. Besides, like in the case of the product prepared by lyophilization, it is not possible to distinguish the presence of FF particles.

Figure 11: SEM of Inclusion Complex prepared by SD at different magnifications.

Analyzing micrographs of chitosan microparticles obtained by spray drying, spherical particles are observed between FF and chitosan (Fig. 12), as well as between Cdl from HPβCD spray drying and chitosan (Fig. 13). However, in a closer examination of MP Q-FF, there seems to be FF adhered to the surface (which could explain the endothermic peak in the DSC thermogram).

Figure 12: SEM of Q – FF Microparticles obtained by SD at different magnifications.

Figure 13: SEM of Q – SDFF Microparticles obtained by SD at different magnifications

Studies of Cdl *in vitro* Release

Fig. 14 shows the dissolution profiles for FF and Cdl prepared with the 3 drying methods (E, L and SD). Under the trial conditions, the dissolved quantity of free FF was less than 10% after 15 minutes. The hydrophobic property of the drug prevented its contact with the dissolution medium causing it to float on the surface, and consequently hindering its dissolution.

On the other hand, it was obvious that the 3 products showed a marked increase in FF dissolution compared with FF alone, showing a burst effect of more than 60% during the first 2 min and after 15 min the quantity of FF dissolved from the 3 products was higher than 90%. This FF dissolution increase in Cdl with HPβCD can be explained based on its high solubility in water, as well as its higher power for moisturizing, solubilizing and forming complexes in solid state. This action resulted in an *in situ* inclusion process causing a fast increase in the quantity of drug dissolved²¹.

Figure 14. Dissolution profile of FF and inclusion complexes prepared by Evaporation (E), Lyophilization (L) and Spray-Drying (SD), in bidistilled water (18 ± 1 °C).

Although no significant differences among the 3 products (data not shown), we decided to continue with the dissolution profile studies using the Cdl prepared with SD.

Regarding the dissolution profiles of Chitosan Microparticles, they were conducted in HCl pH 1.2.

In acid media (Fig. 15), FF dissolution was not complete (less than 10%), whereas dissolution of chitosan MPs was not higher than 50%. However, the Cdl prepared by SD had a better profile with dissolution above 80% after 15 minutes of analysis. Although the dissolved quantity of FF was less in the MPs compared with Cdl, release data from MPs showed an improvement of the dissolution rate for FF, which mean that the resulting MPs exhibit better solubilisation properties compared with FF alone. Furthermore, in the Q-FF, as well as, Q-SDFF MPs the FF was released in a sustained manner compared with Cdl. According to Filipovic-Grcic et al.⁴ this difference could be attributed to the difference in the degree of swelling and subsequent uptake of water that could result in a local solubilisation action of the MPs. On the other hand, Cerchiara et al.⁶ attributed that the retardant effect of chitosan can be explained by the slow diffusion of the drug through the more hydrophilic chitosan/cyclodextrin matrix layer around the lypophilic drug.

As for the dissolution profiles in buffer pH 8.0, the observed behavior was very similar to that obtained in the dissolution profiles made in bidistilled water, where the FF dissolved amount was less than 10% after 15 minutes while the amount of FF dissolved from Cdl obtained by SD was greater than 90% (Fig. 16), with a burst effect of more than 80% during the first 2 min.

Figure 15. Dissolution profiles of FF, FF-SD, Q-FF, Q-SDFF in HCl pH 1.2 (18 ± 1 °C).

Figure 16. Dissolution Profiles of FF, FF-SD in buffer pH 8.0 (18 ± 1 °C)

CONCLUSION

In summary, it could be inferred that FF forms an inclusion complex with the product obtained by spray drying (evidenced by the absence of the FF endothermic peak in the DSC thermogram, as well as the absence of FF traces in micrographies by SEM).

Regarding the drying method, its selection must be assessed considering not only the yield of product obtained, but other factors such as simplicity, low cost, yield, and how fast and easy it could be to implement it at industrial scale. Based on the results achieved, the drying methods by evaporation and spray drying shall be suitable for industrial production at large scale. The drying method by evaporation generates a better yield, but comparing it to the drying method by spray drying, the latter has the advantage of being a one-step process, thus reducing the preparation steps, saving time and cost. Therefore, this method was selected to prepare the inclusion complexes, and the chitosan microparticles as well.

Regarding the dissolution profiles of the complexes, they were higher compared to pure FF. In general, the evaporation and spray-drying methods showed excellent dissolution properties compared to the lyophilized and chitosan methods.

Finally, the best result was obtained by the formation of inclusion complex with HP β CD by spray-drying. This complex was the most suitable for increasing the solubility of FF in both distilled water and in acid pH or basic pH. As for the MPs, despite having obtained a smaller increase in the solubility of FF compared to the complex, the retardant effect allow to foresee the potential use of this product, as a system for controlled-release drug administration.

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