 Insights to the phase solubility diagrams of flurbiprofen with inclusion complex

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ABSTRACT: Flurbiprofen (FB) is one of the nonsteroidal anti-inflammatory drugs with poor water solubility. Cyclodextrins have a special structure (hydrophobic inner phase and hydrophilic outer phase), which have been widely used to enhance the solubility and stability of drug substances in pharmaceutical applications. The present study is intended to improve the solubility of FB with the inclusion complexes assembled by using beta-cyclodextrin (β-CD) and hydroxypropyl beta-cyclodextrin (HPβ-CD) and to perform the phase solubility studies to estimate the stability constant and complexation efficiency. The results of phase solubility studies showed that the FB/β-CD complexes have B, type and FB/HPβ-CD complexes have A1 type profiles in both PBS and water media. The interaction forces between FB and HPβ-CD were stronger than FB and β-CD for the formation of inclusion complex in water. Moreover, the complexation efficiency value of FB/HPβ-CD complex in water media was found higher than the FB/HPβ-CD complex in PBS media. These results of phase solubility studies absolutely demonstrated the advantage of HPβ-CD instead of β-CD to obtain the inclusion complex with 1:1 Molar ratio in water and to improve the apparent water solubility of FB. According to these results, the FB/HPβ-CD inclusion complex was prepared with the Freeze-drying method and it was achieved to enhance the water solubility of FB 52.6-fold with using HPβ-CD. In conclusion, the presence of the inclusion complex was successfully confirmed by differential scattering calorimetry, X-ray diffraction, scanning electron microscopy and fourier transform infrared analysis.

KEYWORDS: Flurbiprofen; phase solubility; cyclodextrins; inclusion complex; freeze-drying.

1. INTRODUCTION

Pain and inflammation are among the most frequently reported disorders and the major problems that clinical medicine tries to solve. Especially non-steroidal anti-inflammatory drugs are the most commonly used drug groups in analgesic and anti-inflammatory drugs. Aspirin is the prototype of this group of drugs and the most commonly used subgroup after aspirin are propionic acid derivatives. The flurbiprofen (FB) as an active substance also belongs to this group, and compared to indomethacin, ibuprofen and aspirin, FB has been proven to be more effective in terms of inhibition of prostaglandin. FB is 2-(3-fluoro-4-phenylphenyl) propanoic acid and it is commonly used in treatment of migraine pain, gout, osteoarthritis and rheumatoid arthritis, soft tissue injuries and post-operative ocular inflammation. However, with oral use of these drug groups, some undesirable side effects such as ulceration, abdominal burning, pain, cramping, nausea and gastritis occur. Moreover, since half-life is short (4 hours), there is a need for multiple dosing to achieve and maintain therapeutic concentration (50-75 mg single dose, 3-4 times a day). Therefore, topical application of this drug is highly desired. For the absorption of the drugs with topical applications, solubility and drug permeation through the skin are rate limiting steps. As FB is a low water-soluble drug (BCS class II), problems are observed in obtaining the desired effect. It is planned to solve the solubility problem of FB in this research.

The solubility of drug in the water is a key factor for the solubility of drug particles in the physiological fluids and its contribution to bioavailability [1]. Moreover, lack of efficiency related to the poor aqueous solubility causes failures during drug development [2]. On the market, most drugs have water solubility problem resulting in poor bioavailability, as the dissolution limits the absorption of drug [3,4]. Heimbach et
al. indicated that the 40% of the currently available drugs on the market and approximately 90% of new drugs cannot achieve the therapeutic concentration in physiological fluids because of the low water solubility [5].

There are many techniques to increase the water solubility of hydrophobic drug substances. By means of using cosolvents or surfactants, preparing solid dispersions [6,7], complexes, self-emulsifying systems [8], salt formations and decreasing the particle size [9,10] of drug substances; the water solubility of drugs may be increased. The use of surfactants or cosolvents may lead to increased side effects. For example, Willems et al. 2011 reported that the use of Cremophor EL increases the toxicity of Taxol and cyclodextrin, and it causes the nephrotoxicity of itraconazole in Sporanox® [11]. Between these methods, the formation of complex structure by using macromolecules such as cyclodextrin is an often-used formulation strategy to increase the water solubility of hydrophobic drug substances [12,13]. However, this method should be suitable for the molecular structure or chemistry of drug substance as the inclusion complex can be obtained by means of interaction between host cyclodextrin molecule and the guest drug molecule according to their molecular conformation or size. Cyclodextrins are cyclic oligosaccharides consisting of six (α-CD), seven (β-CD), eight (γ-CD) or more (α-1, 4)-linked D-glucopyranose units obtained by degradation of starch with the cyclodextrin glucosyl transferase, and they are known for over 100 years and have been used as pharmaceutical excipients for 20 years [14-16].

As it is shown in Figure 1, CDs are truncated cone or torus shaped molecules with peripheral hydrophilic zone and hydrophobic internal cavity [17]. By means of this molecular structure of CDs, they can act as complexing agents by taking up lipophilic moiety of the drugs into the hydrophobic internal cavity whilst the hydrophilic exterior facilitates high water solubility [18,19] and increase the water solubility of hydrophobic drug substances [20]. Moreover, CDs can take a role as a penetration enhancer for improving drug permeation and absorption through membrane barrier, and by this way they can improve bioavailability of drug substances [21,22].

On the basis of all these beneficial properties and functionalities of cyclodextrins, it was considered that the formation of inclusion complex with cyclodextrin provide the improvement of solubility and bioavailability of FB. For this purpose, the appropriate types of CD were investigated, and it was indicated that natural β-CD and HPβ-CD were suitable and safe for using topical formulations of FB [23]. Also, FB has an affinity for these types of cyclodextrins that form inclusion complexes. To estimate the stability constant and to determine the molar ratio of FB: CD for preparing inclusion complex, phase solubility studies were performed.

The phase solubility studies is a traditional approach described by Higuchi and Connors in 1965 [24] to evaluate the effect of cyclodextrins on the solubility of hydrophobic drug substances according to phase solubility profiles (Figure 2). Moreover, this study provides to determine the stability constant (K_s) complex type and stoichiometry of the equilibrium [25,26]. To obtain the equilibrium, a fixed amount of drug substance (guest molecule) is added to CD solutions of increasing concentration at the constant volume. The results of phase solubility studies, two types (A and B) of inclusion complexes can be obtained. A type profile indicates that a soluble inclusion complex is formed and B type profile indicates that an inclusion complex with limited water solubility is formed [27]. In type A, the equilibrium concentration of drug substances increases linearly with the CD concentration. A type profile can be classified as A_B, A_N and A_L profile (Figure 2). While A_P profile corresponds to a positive deviation from linearity, A_N type profile corresponds to a negative deviation from linearity. In other words, CD is more effective at high concentrations for the A_P type profile while it is less effective for A_N type profile. In the A_L case, the equilibrium concentration of drug substances increases linearly with an increase in CD concentration. The A_L type profile indicates that the complex is of 1:1 stoichiometry, K_s value of the complex can be calculated from the slope of the obtained isotherm and the saturation solubility (S_s) of the drugs in the complexation media. Moreover, the complexation efficiency (CE) which is the concentration ratio between free cyclodextrin and cyclodextrin in a complex can be calculated from the slope of isotherm [28].

According to the results of phase solubility studies, there are different ways to obtain inclusion complex such as freeze drying, supercritical fluid technology, kneading and co-evaporation techniques. Cirri et al. (2005) [29] prepared FB and methyl-β-cyclodextrin (Me-β-CD) complexes in the 1:1 molar ratio using kneading and co-evaporation techniques but they reported that the complexes prepared by co-evaporation and kneading methods required an organic solvent which may cause toxicity. For this reason, in this research freeze drying method was used to obtain inclusion complex.
The aim of the present study was to prepare the inclusion complex of FB with cyclodextrins and to increase the water solubility of FB by preparing an inclusion complex. According to phase solubility studies, the appropriate molar ratio of FB/CD and the types of CD were determined. At the 1:1 molar ratio of FB: HPβ-CD, inclusion complex was prepared by using Freeze-drying method. Then the characterization studies such as Differential Scattering Calorimetry (DSC), X-ray Diffraction (XRD), Scanning electron microscopy (SEM) and Fourier Transform Infrared (FTIR) were performed to confirm the formation of the inclusion complex.

2. RESULTS AND DISCUSSION

2.1. Determination of maximum wavelength and calibration curve of FB

The absorbance of 5 µg/ml solution of FB was measured at 200-400 nm and the maximum wavelength of FB was determined to be 247 nm. At this wavelength, the method was found linear in the range between 2 and 10 µg mL\(^{-1}\) presenting a good correlation coefficient (\(r = 0.9999, n = 8\)). Accuracy and precision analysis showed good percentage recoveries (95-105%) and low relative standard deviation (RSD< 2.00%). Results of validation studies of UV spectrophotometric quantification method for FB in PBS (pH 7.4) were to be linear, accurate, precise and robust.

The calibration curve of FB was obtained by measuring the absorbance in phosphate saline buffer (pH 7.4). The graph of the absorbance versus the concentration (µg/mL) of FB showed the linearity (Figure 3). The \(R^2\) value of this equation is calculated and this value is found 0.9999 which indicates linearity.
2.2. Evaluation of phase solubility studies

Flurbiprofen is a low water-soluble active substance and to overcome the solubility problem of FB, β-CD and HPβ-CD were used in this research. The interaction of β-CD and HPβ-CD with FB was evaluated using phase solubility studies in water and PBS at 37 °C. The phase solubilities were carried out by shaking the vials containing an excess amount of FB and solutions of β-CD and HPβ-CD in which the concentration ranged from 0 to 20 mM according to the method described by Higuchi and Conner's. After that, the phase solubility diagrams were obtained by plotting the total amount of dissolved FB versus the increasing amount of cyclodextrin. (Figure 4 and 5). Figure 4 shows that the phase solubility plots obtained by using β-CD both in the water and PBS media at 37 °C. The FB/β-CD solubility curve was a typical B-type phase solubility diagram which is indicative of the formation of inclusion complexes with limited water solubility. The similar diagram was observed for the inclusion complex of FB/β-CD in PBS media (Figure 4).
linearly increasing FB solubility (Table 1). Moreover, it was determined that the results of increasing β-CD concentration from 0 to 0.008 mol, and solubility of FB in the PBS media was increased linearly from 1.552±0.0395 mg/mL to 1.7749±0.0392 mg/mL. But after increasing the concentration of β-CD from 0.008 to 0.020 mol, a negative deviation on the plot and the limited solubility of FB was observed (approximately 1.6 mg/mL) (Table 1).

<table>
<thead>
<tr>
<th>Concentration of β-cyclodextrin (mol)</th>
<th>Solubility of Flurbiprofen (mg/mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>PBS (pH 7.4)</td>
</tr>
<tr>
<td>0</td>
<td>0.0301±0.0003</td>
<td>1.552±0.0395</td>
</tr>
<tr>
<td>0.004</td>
<td>0.0751±0.0308</td>
<td>1.5885±0.0565</td>
</tr>
<tr>
<td>0.008</td>
<td>0.0764±0.0264</td>
<td>1.7749±0.0392</td>
</tr>
<tr>
<td>0.012</td>
<td>0.0894±0.0184</td>
<td>1.6144±0.0794</td>
</tr>
<tr>
<td>0.016</td>
<td>0.0912±0.0295</td>
<td>1.6145±0.0658</td>
</tr>
<tr>
<td>0.020</td>
<td>0.0949±0.0163</td>
<td>1.4953±0.0942</td>
</tr>
</tbody>
</table>

In Figure 5, the phase-solubility diagram was represented as the amount of total dissolved FB against the increasing concentration of HPβ-CD. The A1 type profiles which indicate a linear increase in the solubility of FB as a function of HPβ-CD concentration in both water and PBS media were observed at the end of phase solubility studies. R² values of plots were calculated as 0.9694 and 0.9898 for PBS and water media, respectively.

Table 2 shows the effect of concentration of HP-β-CD on the solubility of FB (mg/mL) in PBS (pH 7.4) and distilled water. In the presence of HP-β-CD, a continuous linear increase of FB solubility (from 0.0301±0.0003 mg/mL to 1.5818±0.1012 mg/mL) was observed in water media, when the HP-β-CD concentration was increased from 0 to 0.020 mol. The similar results are indicated for the PBS media, the apparent solubility of FB increased linearly from 1.5483±0.0366 mg/mL to 2.9943±0.0392 mg/mL, by increasing HP-β-CD concentration in the 0 – 0.020 mol range (Table 2).
Table 2. Results of phase solubility studies of flurbiprofen with Hydroxypropyl-β-Cyclodextrin (Mean±SD, n=3).

<table>
<thead>
<tr>
<th>Concentration of Hydroxypropyl-β-Cyclodextrin (mol)</th>
<th>Solubility of Flurbiprofen (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>0</td>
<td>0.0301±0.0003</td>
</tr>
<tr>
<td>0.004</td>
<td>0.3878±0.0197</td>
</tr>
<tr>
<td>0.008</td>
<td>0.6932±0.0520</td>
</tr>
<tr>
<td>0.012</td>
<td>0.8597±0.1765</td>
</tr>
<tr>
<td>0.016</td>
<td>1.3226±0.1625</td>
</tr>
<tr>
<td>0.020</td>
<td>1.5818±0.1012</td>
</tr>
</tbody>
</table>

2.3. Evaluation of stability constant (Kc) and complexation efficiency (CE) of complex

The effects of the concentration of β-CD and HPβ-CD, in the range of 0 to 20 mM on the apparent solubility of FB in water and PBS media were investigated by means of performing phase solubility studies (Figure 4 and 5). The most fundamental parameters in the quantitative analysis of interaction force between the CD and drug molecule and the stability of complex, are the apparent stability constant (Kc) and the complexation efficiency (CE). The Kc and CE of the FB/β-CD and FB/HPβ-CD complexes were calculated from the linear part of solubility diagrams according to Eq. (1) and Eq. (2), respectively. The saturation solubility (Sa), the slopes of the phase solubility diagram (m), the linear correlation coefficient (R), the association constant (Kc1:1) and the complexation efficiency (CE) in water and PBS media were given in the Table 3. While the stability constants (Kc) of FB/ β-CD inclusion complex in water and PBS were found 308.670 M−1 and 1.706 M−1, stability constants (Kc) of FB/ HPβ-CD inclusion complex in water and PBS were found 3764.697 M−1 and 72.015 M−1, respectively (Table 3). It means the interaction forces between FB and HPβ-CD was stronger than the interaction forces between FB and β-CD for the formation of inclusion complex in water and PBS media.

The use of HPβ-CD provides to obtain A1 type profile. In this type of profile, the stability constant was Kc1:1 and it indicates that one molecule of FB forms an inclusion complex with one molecule of HPβ-CD [32]. Thus it was considered that the formation of stable inclusion complexes between FB and HPβ-CD should be in a 1:1 molar ratio. Moreover, the Kc1:1 value of FB/HPβ-CD complex in water media was found higher than the Kc1:1 value of FB/HPβ-CD complex in PBS media. This result can be related to the low water solubility of FB compared to its solubility in PBS media. The water solubility of FB was increased from 0.0301±0.0003 mg/mL to 1.5818±0.1012 mg/mL by means of preparing inclusion complex with HPβ-CD. As it is shown in Table 3, the CE values which indicate the concentration ratio between CD in complex and free CD were also calculated from the slopes of phase solubility plots [33]. The CE values of FB/HPβ-CD complex were found higher than the FB/β-CD complex in both media, especially in water media.

Table 3. Phase solubility datasets of FB in presence of β-CD and HPβ-CD in water and PBS (pH 7.4).

<table>
<thead>
<tr>
<th>Media</th>
<th>Solubility of FB with β-CD</th>
<th>Solubility of FB with HPβ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S0 (mM) a</td>
<td>Slope (m)b</td>
</tr>
<tr>
<td>Water</td>
<td>0.123</td>
<td>0.011</td>
</tr>
<tr>
<td>PBS</td>
<td>6.356</td>
<td>0.011</td>
</tr>
</tbody>
</table>

aSolubility of FB in absence of cyclodextrin.
bSlope of the phase solubility plot (Figure 4 and 5).
cR = linear correlation coefficient.
edCalculated association constant for the 1:1 drug/CD complex.

fComplexation efficiency.
These results of phase solubility studies absolutely demonstrated the advantage of using HPβ-CD instead of β-CD to obtain the inclusion complex with FB and improve the apparent water solubility of FB. Moreover it was determined that for the formation of inclusion complexes, the strong interactions between FB and HPβ-CD were occurred at 1:1 molar ratio in water. These results can be related to the molecular interaction between FB and CD hydroxyl groups. The hydroxyalkyl derivatives of CD such as HPβ-CD have higher water solubility compared to β-CD and they provide the conversion of the drug from a crystalline state into an amorphous state. In accordance with the results, the inclusion complex was prepared with HPβ-CD and FB (1:1 molar ratio) in water media by using Freeze drying method and after that the presence of inclusion complex was confirmed by solid state characterization studies.

2.4. Evaluation of solid state characterization studies

2.4.1. Scanning electron microscopy (SEM)

SEM analysis were performed on coarse powder of FB, HPβ-CD, their physical mixture and the inclusion complex to investigate the possible morphological changes caused by different treatments during the process. The scanning electron micrographs of coarse powder FB, HPβ-CD, their physical mixture and the inclusion complex were shown in Figure 6, respectively. FB particles existed in irregular shaped crystals at micro meter size with broad size distribution while HPβ-CD was observed as homogenous amorphous spheres. In the physical mixture both the irregular shape of FB and the spherical shape of HPβ-CD could be observed. The inclusion complex differed in morphology from FB and HPβ-CD and appeared in the form of irregular shaped particles. When the inclusion complex is prepared, the cavities in the HPβ-CD are filled by FB molecules. This result is an evidence of interaction between FB and HPβ-CD in complex structure [31].

2.4.2. Differential scattering calorimetry (DSC)

The DSC curves of FB, HPβ-CD, their physical mixture and the inclusion complex were shown in Figure 7. FB displayed one sharp endothermic peak at 114 °C, which means the melting point of the crystalline form. The broad endothermic peak of HPβ-CD was observed at approximately 80 °C because of the loss of water and this result was similar to previously published studies [34-36]. The DSC thermogram of the physical mixture mainly showed the characteristic peaks of FB and HPβ-CD. In contrast, the disappearance of sharp endothermic peak of FB could be found for the inclusion complex. This situation can be explained in the way that the inclusion complex was successfully prepared by means of freeze drying process and the FB particles are completely incorporated in the cavity of HPβ-CD ring molecule.

2.4.3. Fourier transform infrared (FT-IR)

The FT-IR spectrum of FB, HPβ-CD, their physical mixture and the inclusion complex in the 4000-600 cm⁻¹ regions were shown in Figure 8. As seen in the Figure 8, the characteristic sharp peaks of FB at 1694.7, 1414.7 and 1216.1 cm⁻¹ were due to C=O stretching, O-H bending and C-F stretching, respectively. The characteristic band of FB due to the hydrogen bonds of the carboxyl group appeared in the range of the 3400-2400 cm⁻¹ [37]. On the other hand, the spectrum of HPβ-CD is characterized by intense bands at 3300-3500 cm⁻¹ due to O-H stretching vibration, while the vibration of the –CH and –CH₂ groups appears in the 2800-3000 cm⁻¹ region [13]. For the physical mixture, both of the fingerprints of FB and CD were observed but it presented the reduction in intensity of the characteristic bands of FB for the low content of FB in the mixture. The FT-IR spectrum of the inclusion complex was found similar to be to the pure HPβ-CD spectrum since FB was contained within the hydrophobic cavity of HPβ-CD molecule.

2.4.4. X-ray diffraction (XRD)

X-ray diffractometry is a useful method to confirm the formation of inclusion complex. The XRD patterns of inclusion complex should be clearly different from the patterns of each component. When the true complex has been formed, the reduction in the crystalline state of powders was observed. This can be explained with the increase in the amorphous state during the formation process of the inclusion complex. The XRD patterns of FB, HPβ-CD, their physical mixture and the inclusion complex are shown in Figure 9. The XRD patterns of FB showed the crystallinity, while amorphous state was observed for HPβ-CD. The patterns of physical mixture consisted of superimposed figures of pure FB and HPβ-CD with the peaks having lower intensity. These patterns showed most of the HPβ-CD character but some of the FB characteristics remained. It can be related to the dilution of FB in the mixture. On the basis of the inclusion complex patterns, just the amorphous characteristic of HPβ-CD was observed. The absence of specific crystallinity peak of FB provided the evidence of obtaining inclusion complex successfully.
Figure 6. Scanning electron micrographs (magnitude 1000x) of (a) flurbiprofen, (b) HPβ-CD, (c) their physical mixture, (d) inclusion complex.

Figure 7. DSC thermograms of FB, HPβ-CD, their physical mixture, inclusion complex.
As a result, the interaction between FB and HPβ-CD resulted in formation of inclusion complex and this inclusion complex increased the water solubility of FB. According to the phase solubility results the inclusion complex were prepared using 1:1 molar ratio of FB and HPβ-CD in water by using freeze drying method. After that, the presence of inclusion complex was investigated by DSC, XRD, FTIR and SEM analysis. The complex showed different physicochemical characteristics from the coarse powder of FB and HPβ-CD and the physical mixture of them. Thus the presence of inclusion complex was successfully confirmed and it was considered that the complexation with HPβ-CD is a promising approach to improve the solubility of low water soluble drug substances in pharmaceutical industry.
3. CONCLUSION

In conclusion, hydrophobic molecules such as flurbiprofen have greater affinity into the cyclodextrin cavity. By means of specific structure of cyclodextrins; the physical and chemical properties of the hydrophobic molecule, such as solubility and stability can be improved. Thus, cyclodextrins have become important for pharmaceutical products. In this study, to solve the solubility problem of FB, the inclusion complex of FB and HPβ-CD was successfully prepared. All the characterization studies also showed that the freeze-drying technique is suitable to produce the inclusion complex of FB and HPβ-CD. Compared to the other techniques, the freeze-drying technique is possible to scale up and seems to be more applicable to the pharmaceutical industry. Moreover, this inclusion complex can be used to prepare novel nanotechnological formulations of several poorly soluble substances in order to increase solubility, stability and to improve bioavailability by allowing a dose reduction of the drug molecules applied.

4. MATERIALS AND METHODS

4.1. Materials

Flurbiprofen was kindly provided by Sanovel Pharma® (Istanbul, Turkey). Beta cyclodextrin and hydroxypropyl beta cyclodextrin were purchased from Sigma- Aldrich®(USA). Other chemicals were of analytical grade.

4.2. Methods

4.2.1. Determination of maximum wavelength and calibration curve of FB

UV spectrophotometer (Cary 60 Uv-Vis Agilent®, Malaysia) was used to determine the amount of FB in the samples which are taken during the phase solubility studies. First of all, a stock solution (100 μg/mL) of FB in pH 7.4 phosphate saline buffer was prepared. Accurately weighed 10 mg of FB was transferred to a 100 ml volumetric flask, dissolved in 100 ml phosphate saline buffer (pH 7.4) by shaking manually for 10 min. After that, appropriate volume (0.5 ml) of standard stock solution of FB was transferred into a 10 ml volumetric flask, diluted to get concentration of 5 μg/ml. The resulting solution was scanned in the UV range (200–400 nm). In spectrum FB showed maximum absorbance at 247 nm. At this wavelength, the linearity, accuracy, precision, sensitivity, repeatability, ruggedness was determined to obtain calibration curve of FB and to validate the UV method. The prepared stock solution of FB was diluted to get 2.5; 3; 4; 5; 6; 8; 9, 10 μg/mL. The absorbance values corresponding to these samples were measured in the spectrophotometer at 247 nm as maximum wavelength. When the concentration values are plotted against the absorbance values, the standard equation was obtained.

4.2.2. Phase solubility studies

The phase solubility studies were performed according to the method described by Higuchi and Connors [24]. Different concentrations of β-CD and HPβ-CD were utilized to prepare solutions in PBS (pH 7.4) and in distilled water. An excess amount of FB was added to 10 mL of buffer solution and distilled water containing different amounts (0-4-8-12-16-20 mM) of β-CD and HPβ-CD. The volumetric flasks were placed and shaken at the 37 °C in shaking water bath (Nüve ST 30, Turkey) until equilibrium is established (72 hours). The temperature was selected as 37 °C to increase the complexation coefficient [38] and to mimic the physiological temperature [39]. After 72 hours, the excess amount of FB was ultracentrifugated at 25 °C for 45 min at 12000 xg (relative centrifugal force) (Hitachi CS 150GXL, Japan) and the solutions were filtered using membrane filter (pore size 0.45 μm) and analysed by UV spectrophotometry at 247 nm. The phase solubility curves were obtained by plotting the total concentration of dissolved FB against the respective concentration of HPβ-CD and β-CD. By means of calculating the concentration of FB in 10 mL of distilled water and buffer solution at the absence of cyclodextrins (0 mM), saturation solubility (S0) of FB was determined.

4.2.3. Calculation of stability constant (Kc) and complexation efficiency (CE)

The apparent stability constant (Kc) was calculated from the slope of the phase solubility curves according to Equation (1), where the S0 was the saturation solubility of FB in absence of CD at 37 °C [24,30,31]. According to Equation (2), complexation efficiency can be calculated from slope of the curves.
\[ K_c = \text{Slope} / [\text{Sox}(1 - \text{slope})] \quad \text{(Eq. 1)} \]
\[ CE = \text{Slope} / (1 - \text{slope}) \quad \text{(Eq. 2)} \]

4.2.4. Preparation of physical mixture

Flurbiprofen and HPβ-CD according to 1:1 molar ratio were mixed in ceramic mortars. The obtained powder mixture was utilized as physical mixture of FB and HPβ-CD.

4.2.5. Preparation and lyophilization of the complex of flurbiprofen and HPβ-CD

Flurbiprofen and HPβ-CD according to 1:1 molar ratio were dissolved in 10 mL of distilled water and stirred at 37 °C for 48 h. After that, the mixture was filtered through 0.45 μm membrane filter, the filtrate was put inside of the vials and then they were frozen at -80 °C for 3 hours. The freeze-drying process (lyophilization) was applied at -50 °C, 0.021 mbar for 72 hours using Christ Alpha 1-2 LD® Freeze Dryer, Germany.

4.2.6. Solid state characterization studies

To confirm the presence of inclusion complex, the solid state studies such as differential scattering calorimetry (DSC), X-ray diffraction (XRD), scanning electron microscopy (SEM) and fourier transform infrared (FTIR) were performed after the lyophilization process.

4.2.7. Scanning electron microscopy (SEM)

The morphological properties of FB, HPβ-CD, their physical mixture and the inclusion complex were analyzed with scanning electron microscopy (SEM) (Quanta 400F Field Emission, USA). Prior to examination, powder samples were mounted onto a metal stub using double adhesive tape. Before scanning, the samples were coated with gold palladium in vacuum and observed at a voltage of 5-20 kV by SEM. All samples were magnified 1000x.

4.2.8. Differential scanning calorimetry (DSC)

DSC analysis was carried out for FB, HPβ-CD and their physical mixture and the inclusion complex with Shimadzu DSC 60, Japan. Each powder weighed (2 mg) and heated in the aluminum pans between 10 °C to 300 °C temperature with heating rate of 10 °C/min. DSC thermograms were obtained under nitrogen flow of 100 mL/min using Indium as temperature calibrator. In the same way, the empty pan was utilized as reference. Three different measurements were carried out for each sample, then extrapolated onset temperature and the average of maximum peak were determined.

4.2.9. Fourier transform infrared (FT-IR)

The FT-IR spectrum of FB, HPβ-CD and their physical mixture and the inclusion complex were obtained with (Perkin-Elmer Spectrum 400 FT-IR, USA). All samples were scanned for absorbance over the range from 4000-600 cm⁻¹ at the resolution of 4 cm⁻¹.

4.2.10. X-ray diffraction (XRD)

The X-ray powder diffraction patterns of FB, HPβ-CD, their physical mixture and the inclusion complex were collected with X-ray diffractometer (RigakuUltima-IV Powder Diffractometer, USA). The patterns were recorded by sample scan from 5° to 120° at 20 at a scan rate of 1°/min and a voltage of 40 kV.

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