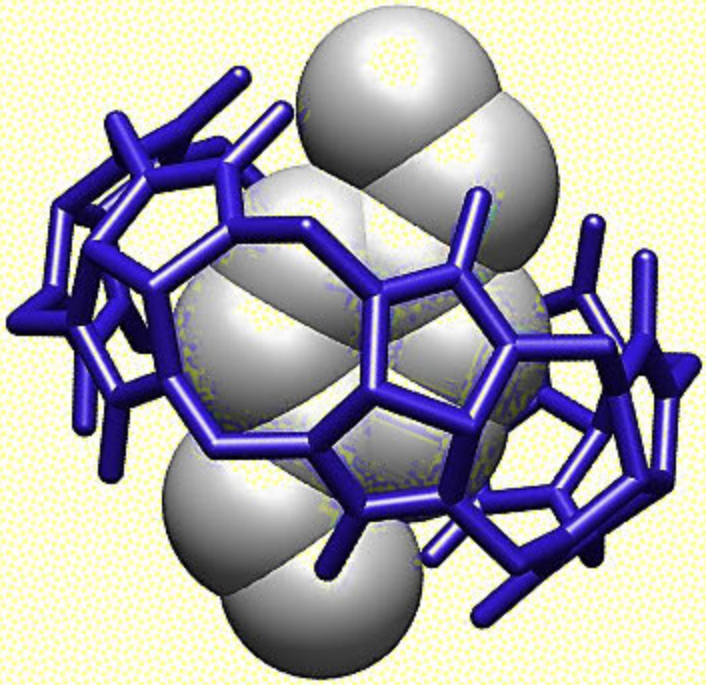
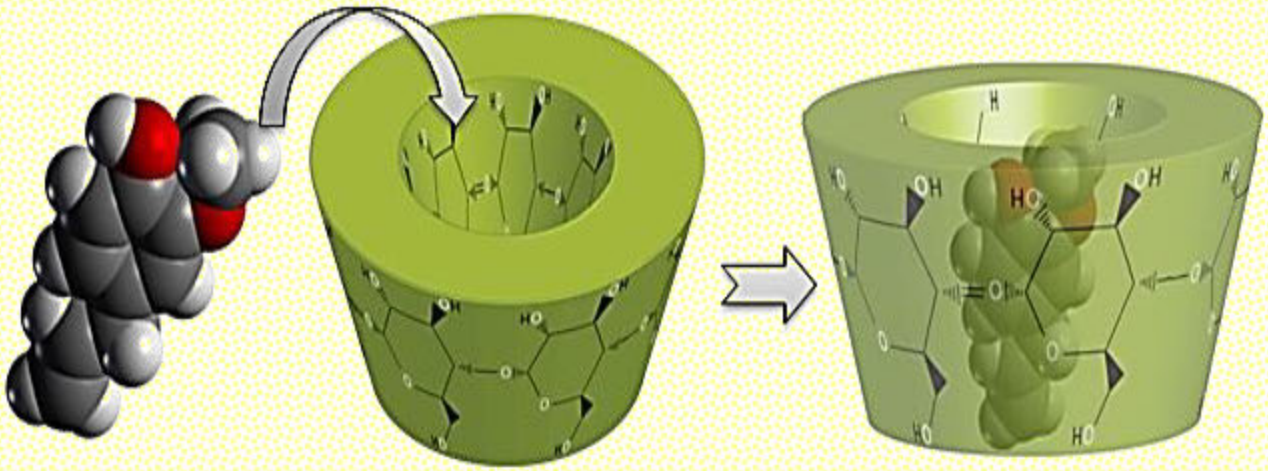
Chapter-4
Preparation and Characterization of Inclusion Complex





4.1 Introduction

In the present decade, optimizing the therapeutic efficiency of drug has gained tremendous focus. This need aroused as obtaining a pharmacological activity by using drugs at therapeutic levels led to their rapid elimination along with their non-specific distribution leading to a suboptimal concentration at the target site. Unless unacceptable toxic amounts of dose get administered the needed therapeutic efficacy was not achieved. The tissue distribution and receptor binding affinity are determined by the lipophilicity of drug, however, inadequate solubility of drug at physiological conditions prevents their successful delivery (1). Among the classification of drug according to BCS system, two classes of drug that present a formulation challenge are those of class II and IV. These classes present solubility issues and together with permeability issues as in case of class IV, the challenge is a surmounting one. Most of the anticancer drugs belong to class IV BCS and obtaining a higher efficacy to side effect ratio is the prime goal of chemotherapy. The limitation of solubility along with a higher toxicity due to non-specific distribution and accumulation of drug in non-target organs are the main constraints faced in achieving therapeutic benefits. Paclitaxel, the drug investigated in the current research, too has solubility issues. It has low aqueous solubility and was formulated in a system consisting of solubilizers and solvent to make intravenous administration feasible. PTX was thus successfully formulated in a vehicle (Taxol®) for intravenous administration and is used after suitably dilution in 5% dextrose solution or saline solution to achieve final solution concentration range of 0.6 – 1.2 mg/ml (0.7-1.4 mM). However, despite achieving increase in solubility, the need of a novel formulation still exists due to the severe hypersensitivity reaction of vehicle employed along with poor solution stability of this marketed formulation (2).

Of the various approaches investigated to improve the solubility of PTX, either chemical modification of the drug by synthesizing prodrugs (3, 4), congeners (5) and analogues (6), (7) or different formulation strategies like use of hydrophilic polymers (8, 9), mixed micellar systems (10-12), emulsions (13-15), solid dispersion (16, 17), liposomes (18, 19) or complex formation with biocompatible polymers (20-22) have been explored. These techniques improve the solubility of PTX by either solubilisation, co-solvency or encapsulation a major drawback that was still

associated was low loading of drug mandating that large dose still has to be administered to achieve therapeutic efficacy.

To improve the solubility of drugs, strategies like complexation or molecular encapsulation strategy have gained wide importance. Such complexation strategies provide for better drug solubilisation and stabilization along with controlling the release rate of drug. Cyclodextrins are one such class of molecular complexing agents that have gained tremendous popularity. They are a class of cyclic oligosaccharides that consist of glucopyranose rings covalently linked to each other. Three naturally occurring CDs with 6,7 and 8 rings are α, β and γ respectively. However, of these CDs the maximum aqueous solubility was 232 mg/ml for γ CD or it can also be stated that CDs also have inherently low water solubility due to the strong intermolecular H-bonding in their crystal lattice (table 4.1) (23). As in the mechanism of molecular encapsulation the limiting factor determining the solubility of drug is dependent on the carrier, there was need to synthesize other CDs having higher aqueous solubility so that a concentrated aqueous solution can be obtained in which a greater amount of drug can be molecularly encapsulated. Moreover, these CDs have been reported to have parenteral / renal toxicity by their chronic use. Thus, a range of other CDs were then obtained by chemical or enzymatic modification with improved solubility and modulated toxicity profile. Figure 4.1 represents general structure of CDs along with their structure.

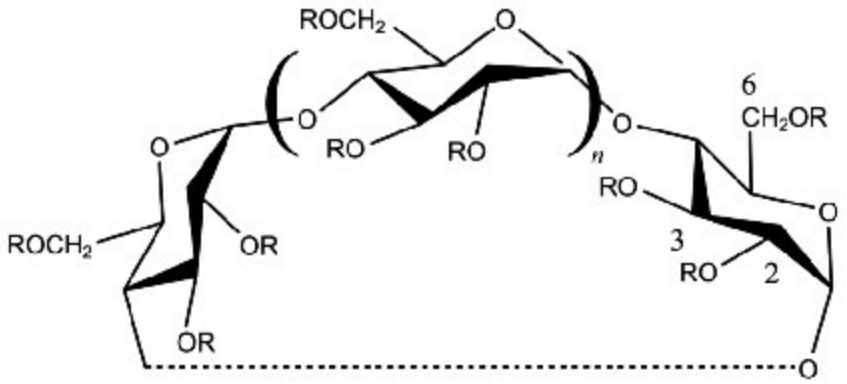


Figure 4.1 General structure of CDs

Table 4.1 Abbreviated names of CDs and their derivatives

Cyclodextrin	Abbreviation	R	n
α cyclodextrin	α CD	H	4
β cyclodextrin	β CD	H	5
γ-cyclodextrin	γ-CD	H	6
Carboxymethyl-β-cyclodextrin	CM-β-CD	CH_2CO_2H or H	5
Carboxymethyl-ethyl-β-cyclodextrin	CME-β-CD	CH_2CO_2H, CH_2CH_3 or H	5
Diethyl β cyclodextrin	DE β CD	CH_2CH_3 or H	5
Dimethyl-β-cyclodextrin	DM-β-CD	CH_3 or H	5
Glucosyl-β-cyclodextrin	G_1-β-CD	Glucosyl or H	5
Hydroxybutenyl-β-cyclodextrin	HBE-β-CD	$CH_2CH(OH)CH_2OH$ or H	5
Hydroxyethyl-β-cyclodextrin	HE-β-CD	CH_2CH_2OH or H	5
Hydroxypropyl-β-cyclodextrin	HP-β-CD	$CH_2CH(OH)CH_3$ or H	5
Hydroxypropyl γ cyclodextrin	HP γ CD	$CH_2CH(OH)CH_3$ or H	6
Maltosyl β cyclodextrin	G_2 β CD	Maltosyl or H	5
Methyl-β-cyclodextrin	M-β-CD	CH_3 or H	5
Random methyl-β-cyclodextrin	RM-β-CD	CH_3 or H	5
Sulfobutylether-β-cyclodextrin	SBE-β-CD	$(CH_2)_4SO_3Na$ or H	5

[a]Derivatives may have differing degrees of substitution on the 2, 3, and 6 positions.

For formation of inclusion complexes, the hydrophobic drug molecules get encapsulated in the inner hollow, cone like structure having apolar core that is electron-rich, whereas the outer region is hydrophilic and facilitates solubilization by interacting with H_20 molecules.

To improve the solubility of PTX, a range of CDs have been investigated and the reported results are presented in table 4.2 below. As evident, CDs mediates a considerable increase in solubility of PTX starting form 12.5 folds for 1.5% β CD to almost a lac fold for 50% DM βCD. Figure 4.2 represent phase solubility diagram for PTX dissolved when solution of different concentration of HPβCD/HE βCD/DMβCD were used.

Table 4.2 PTX solubility enhancement by CDs

Cyclodextrin Concentration [a]	***PTX dissolved*** [b]	***Enhancement factor*** [c]
15% γ	0.020	50
50% HP γ	0.080	200
1.5% β	0.005	12.5
50% Dimaltosyl- β	0.115	288
50% HE β	0.914	2285
60% HE β	2.1	5250

50% HP β	0.856	2140
60 % Hp β	4	10000
50% DM β [d]	39.6	99000

[a]Aqueous concentration of CD (w/v). [b]PTX solubility (mM SD). [C]Enhancement factor = Aqueous solubility of PTX ~ 0.4μM. [d]Limit for solubility not achieved.

Based on the above literature report, DM βCD was selected as it solubilized the PTX to greatest extent. It was also demonstrated that approx. 100% of added drug got molecularly encapsulated in DMβCD employed at the concentration range tested and further showed linear increase in solubilizing PTX as its aqueous concentration was increased (24). Further, the increase in solubility does not change the cytostatic activity of PTX *in vitro*. Thus, DMβCD was selected in the present investigation for preparation of inclusion complexes for PTX.

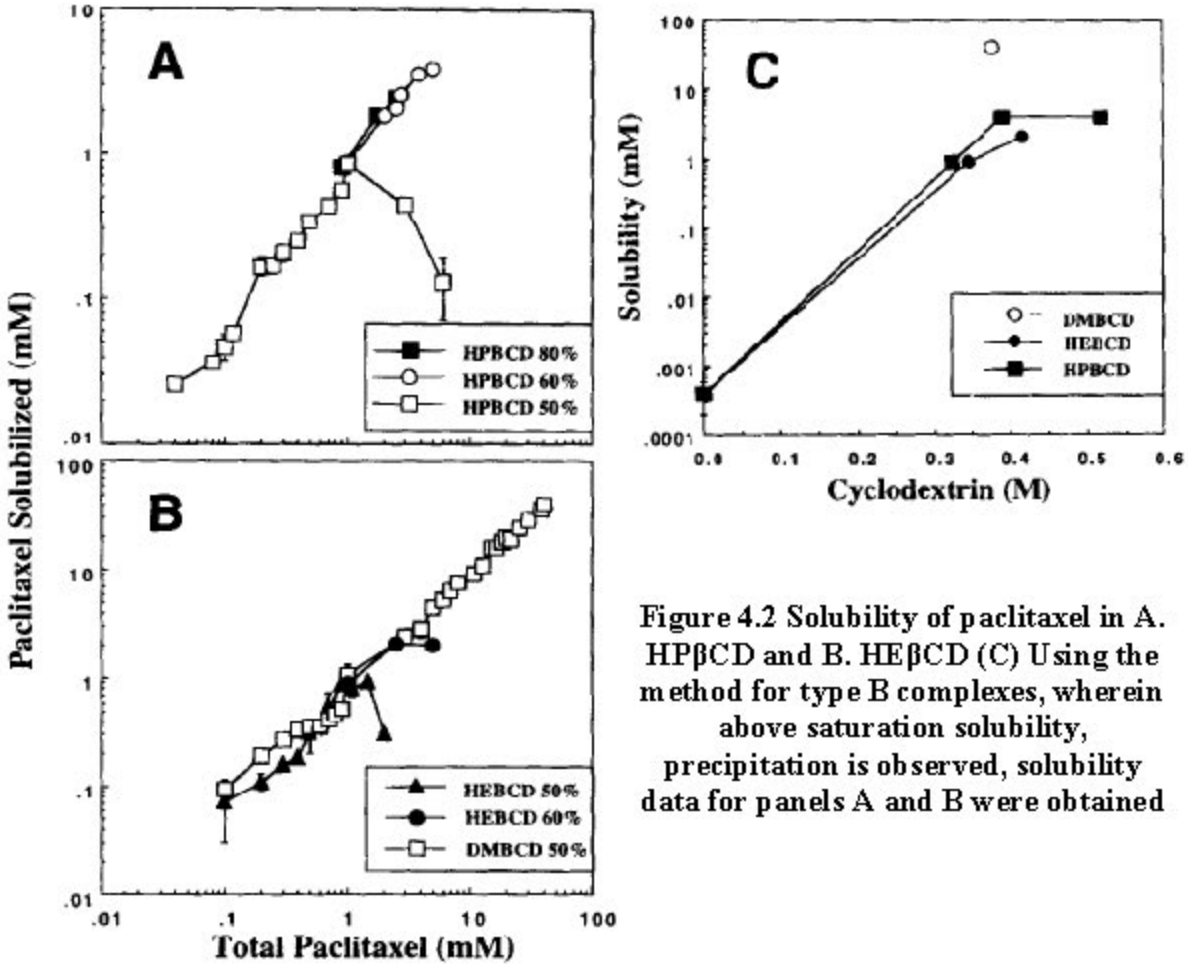


Figure 4.2 Solubility of paclitaxel in A. HPβCD and B. HEβCD (C) Using the method for type B complexes, wherein above saturation solubility, precipitation is observed, solubility data for panels A and B were obtained

Various approaches are available for preparation of inclusion complexes and are briefly described herein:

A. Physical blending: Physical mixture of drug and CDs are blended homogenously and passed through sieve of definite size to obtain product.

B. Kneading: In this method, drug and CD are triturated intensely to completely grind the mixture followed by kneading with hydroalcoholic solution till a paste like consistency is obtained. The kneaded mass is dried to remove excess of solvent followed by storing in a desiccator, sieved and stored (23).

C. Solvent evaporation or solid dispersion method: Separately an alcoholic drug solution and an aqueous solution of CD are prepared. The drug solution is then slowly added drop wise to the aqueous solution under stirring to get molecular dispersion of drug in CD. The solvent is then evaporated at room temperature or under vacuum to remove all solvent obtain dried powder which is sieved to get free flowing powder (25).

D. Spray drying: In a hydroalcoholic biphasic solvent system consisting of ethanol and water at 50:50 v/v ratio, monophasic solution of drug and CD is prepared. The solution is stirred and allowed to reach equilibrium by stirring at room temperature for 24 hr. The equilibrated solution is then spray dried to obtain dry powder (26).

E. Freeze drying or lyophilization: Stoichiometric amount of drug is dissolved in hydroalcoholic solution of CD under stirring. The solution is then frozen and lyophilized in freeze dryer under reduced pressure to obtain porous, amorphous powder. This method is useful for thermolabile drugs (27).

F. Microwave irradiation: It is an industrially feasible method requiring shorter process time and higher product yield. Irradiation reaction of drug with CD is done in a microwave oven, wherein microwave provide the energy for complexation (28).

G. Melting: CD is heated to obtain a melt and drug is then dispersed in this melt. The dispersion is then allowed to cool and washed with solvent to remove excess amount of melted CD or under vacuum by sublimation (29).

H. Supercritical antisolvent technique: Solution of drug and CD in solvent is fed through a nozzle in expansion chamber containing supercritical CO_2. Upon contact, the solvent loses its solubilization property and supersaturation occurs

thus precipitating the drug in CD in form for fine particles. The solvent is removed by flow of supercritical fluid (30).

I. High pressure homogenization: Solution of drug and CD in solvent is passed through homogenizer to cause particle dispersion and disintegration. The solution containing dispersed particles is then evaporated to dryness to obtain solid complex.

In the present research work, we have used modified solvent evaporation method to prepare complex of PTX with CD as the method is simple and easily reproducible at lab scale. Further, the modification involved lyophilizing the complex instead of evaporating the solvent which was done to impart longer stability to the complex.

4.2 Materials and Instruments

Materials

Sr No	Chemicals/Materials	Source/Manufacturer
1.	Paclitaxel	Sun Pharmaceutical Industries. Ltd. (Vadodara, India) – Gift sample
2.	2,6-di-O-methyl beta cyclodextrin (DMβCD)	Cyclolab Ltd. (Budapest, Hungary) – Gift sample

All other chemicals used were of analytical reagent grade and were used without any further purification.

Instruments

Sr No	Instruments	Company
1.	Rotary evaporator	IKA Rotavapor RV-10,
2.	Magnetic Stirrer	Remi, India
3.	Lyophilizer	Virtis Advantage Plus
4.	RP-HPLC	Shimadzu LC-20AT, Japan
5.	Differential Scanning Calorimetry	DSC-41, Shimadzu, Japan
6.	Fourier transform infrared spectrophotometer/FTIR	Bruker, Germany
7.	x-ray diffractometer	X'pert Pro, PANalytical,
8.	Scanning electron microscopy/SEM	JEOL, JSM-5610LV,
9.	Circular Dichroism Spectrometer	Jasco J-815 CD

4.3 Methods

4.3.1 Preparation of PTX ICs

PTX-DMβCD ICs were formulated using a modified co-solvent lyophilization method (24), (31). Briefly, PTX was dissolved in methanol and required quantity of DMβCD was solubilized in acetate buffer pH 5.0 separately at room temperature (RT). ICs were prepared at varying molar ratio of PTX: DMβCD from 1:1 to 1:25. Both phases were allowed to stir individually to ensure formation of clear solution, after which PTX solution was added to the DMβCD solution in a drop-wise manner under stirring on magnetic stirrer. The resultant hydro-alcoholic solution was stirred for 6 hr in stoppered glass vial at RT. After 6 hr, it was centrifuged at 7000 rpm for 10 min, filtered through 0.2 µm syringe filter and solvent was allowed to evaporate under reduced pressure using rotary evaporator (IKA Rotavapor RV-10, IKA® India Private Limited). The ICs were then lyophilized using Virtis Advantage Plus Lyophilizer to yield ICs as white amorphous dry powder. The ICs were then subjected to solid state physiochemical characterization. The ICs were reconstituted with acetate buffer pH 5.0 to the original aqueous volume. After reconstitution, solution was centrifuged at 5000 rpm to the separate supernatant which was filtered through 0.2 µm syringe filters and analyzed by chromatography to back calculate PTX concentration in ICs.

4.3.2 Characterization of PTX ICs

4.3.2.1 Entrapment efficiency and solubility enhancement

Estimation of PTX content in inclusion complexes was performed by reverse-phase HPLC method/RP-HPLC (Shimadzu LC-20AT, Japan) using C18 ODS (octa decyl silane) column (250 mm *4.6 mm* 5 µm, Thermo scientific) at ambient temperature. The mobile phase Acetonitrile: Methanol: Water (45:30:25), pH adjusted to ~3 with 1% v/v of glacial acetic acid was run at a flow rate of 1 ml/min. Samples were prepared by appropriate dilutions of PTX in methanol and final dilution was done with mobile phase. Concentrations from 1 to 50 µg/ml were estimated to prepare calibration curve. 20 µl of each solution was injected into HPLC and chromatogram was run for 10 minutes. Estimation of PTX was done using UV-visible detector at wavelength of 227 nm.

4.3.2.2 Physicochemical evaluation

Solid state physicochemical evaluation of PTX, DMβCD and PTX-DMβCD lyophilized ICs were performed by DSC, FTIR, XRD and SEM analysis. CD was done in a solution state.

Differential Scanning Calorimetry

PTX, DMβCD and PTX-DMβCD ICs were subjected to thermal analysis using Differential Scanning Calorimetry (DSC-41, Shimadzu, Japan) to confirm complexation. Accurately weighed samples were placed in hermetically sealed aluminum pans and empty aluminum pan was used as a reference. Heating scans by heat runs for each sample was set from 30 °C to 300 °C at 10 °C/ min in a nitrogen atmosphere.

Fourier transform infrared spectroscopy

Infrared spectroscopy was used to confirm complexation and to determine the interaction between cyclodextrin and the guest molecules in the solid state. PTX, DMβCD, and PTX-DMβCD ICs were investigated using Fourier transform infrared spectrophotometer/FTIR (Bruker, Germany) range between 4000 and 400 cm−1, with a resolution of 2 cm^{-1}. All powder samples were compressed into KBr disks for the FTIR measurement.

X-ray diffraction studies

The crystal structure of drug and changes in crystallinity after complexation were observed using powdered X-ray diffraction studies (XRD). Samples were observed using a powder x-ray diffractometer (X'pert Pro, PANalytical, Singapore) with Ni-filtered Cu-Kα radiation, and voltage diffraction. Scan was performed at a range 2Θ of 5^0-50^0 with a scan rate of 3^0/min. The XRD patterns of PTX alone, DMβCD, physical mixtures of both and inclusion complexes were recorded.

Scanning electron microscopy

Scanning electron microscopy/SEM (JEOL, JSM-5610LV, Japan) was performed to observe morphology of samples. Samples fixed on SEM-stub were made electrically conductive using very thin coating of gold. The acceleration voltage used for analysis was of the order of 10-kV.

Circular Dichroism spectroscopy

Circular Dichroism spectroscopy is an important method which detects conformational changes in molecule and sensitive to small variations in structure of the molecule as well as polarity of the solvent in which it is solubilized. As PTX possesses chiral centers linked with several chromophores, it is circular dichroism active. Therefore, this technique has been used to confirm interaction and complexation of PTX with DMβCD. Circular dichroism spectra of free PTX, DMβCD and PTX-DMβCD ICs in a liquid state in methanol/water solution (30:70 v/v) were carried out using a Jasco J-815 CD spectrometer CD-01 with scanning speed of 20 nm/min and 6 accumulations. The Circular dichroism spectra were scanned for 200 nm to 340 nm wavelength. The path length of the quartz cuvette used was 1 mm.

4.3.2.3 Stability study of ICs

Stability study was performed for lyophilized PTX-DMβCD inclusion complex stored at 25° ± 2 °C/60% ± 5 %RH and at 2-8 °C for three months. At particular time interval PTX-DMβCD complex was reconstituted to original volume by acetate buffer pH 5.0 and evaluated for physical and chemical stability using RP-HPLC analysis. PTX-DMβCD was diluted with dextrose and kept for 24 hr at 25 °C. The samples were examined for presence of any precipitates and concentration of PTX retained was determined by RP-HPLC analysis (24).

4.4 Result and Discussion

4.4.1 Preparation of PTX ICs

Varieties of cyclodextrins such as Beta-cyclodextrin (β-CD), 2-Hydroxypropyl-beta- cyclodextrin (HP-β-CD), (2,6-di-O-methyl)-beta-cyclodextrin

(DMβCD) and captisol® were screened for improvement of water solubility of PTX at various concentrations. Of the various CDs, DMβCD was selected for the plausible advantage of highest aqueous solubility (32). Based on the earlier reports, the saturation aqueous solubility of DMβCD has not been determined and 50% w/v solution was employed for determining the solubility of PTX (24). ICs were prepared at varying molar ratios of PTX: DMβCD from 1:1 to 1:25. With the increasing molar ratio of DMβCD, solubility of PTX was found to linearly increase, and at 1:25, 11 mg/ml of aqueous solubility was achieved for PTX. Based on the highest entrapment of PTX of almost 100% in ICs and physical stability of ICs, the 1:20 ratio was optimized and selected for further use in this work. Stirring time of 1, 3, 6 and 12 hr were compared, and the highest entrapment efficiency of PTX was found at 1:20 molar ratio after 6 hr of stirring. This may be due to sufficient time for interaction of PTX with DMβCD till a stable equilibrium to achieve highest entrapment. Stirring time of 3 hour or less resulted in low entrapment efficiency of PTX in ICs. Our results are in agreement with those obtained by Jing et al. wherein molar ratio of PTX to CD had pronounced influence on the entrapment efficiency. But, we have essentially screened higher ratio for complexation and have obtained higher loading and entrapment of PTX which reaches a plateau above 1:25 molar ratio. Further, the complex formation was carried out at RT only thus minimizing the negating impact of elevated temperature on complex stability.

Figure 4.3 shows PTX entrapment efficiency in ICs and its resultant aqueous solubility at varying PTX to DMβCD molar ratios.

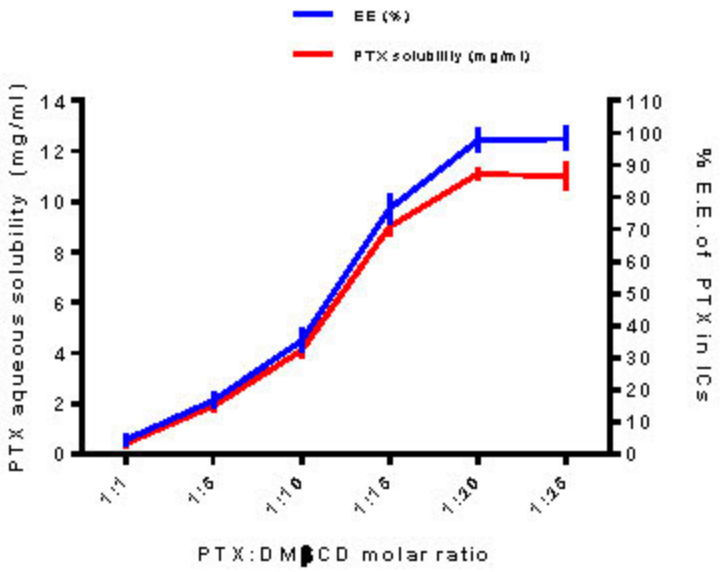


Figure 4.3 Entrapment efficiency and solubility of PTX ICs at various molar ratio.

For ratio, less then 1:15 complexes were found unstable as precipitation of PTX in solution was observed. The results are consistent with the report by Jing Ye et al. which demonstrates the lower encapsulation efficiency at low molar ratios as one CD cavity may not completely encapsulate PTX molecule having high molecular weight and due to its complex structure that has many functional sites for interaction with CD (33).

4.4.2 Characterization of PTX ICs

4.4.2.1 Entrapment efficiency and solubility enhancement

Solubility of pure PTX in water was found to be 0.38±0.05 µg/mL at 25 ^{0}C while after DMβCD complexation, its solubility increased to 11.1±0.22 mg/ml at PTX: DMβCD molar ratio of 1:20. Acetate buffer pH 5.0 was used to reconstitute the drug and complex for stability reasons. This higher amount of entrapment efficiency observed at higher molar ratio for PTX is anticipated due to the larger cavity of the DM-β-CD molecule available for encapsulation, in comparison to the other non-substituted CDs (24).

4.4.2.2 Physicochemical evaluation

Differential Scanning Calorimetry

Figure 4.4 shows thermal analysis of PTX, DMβCD and PTX-DMβCD ICs using DSC technique. PTX exhibit sharp endothermic peak at around 218^{0}C which shows its characteristic melting point. That peak was absent in DSC spectrum of PTX-DMβCD ICs confirming the complete complexation and amorphization of PTX.

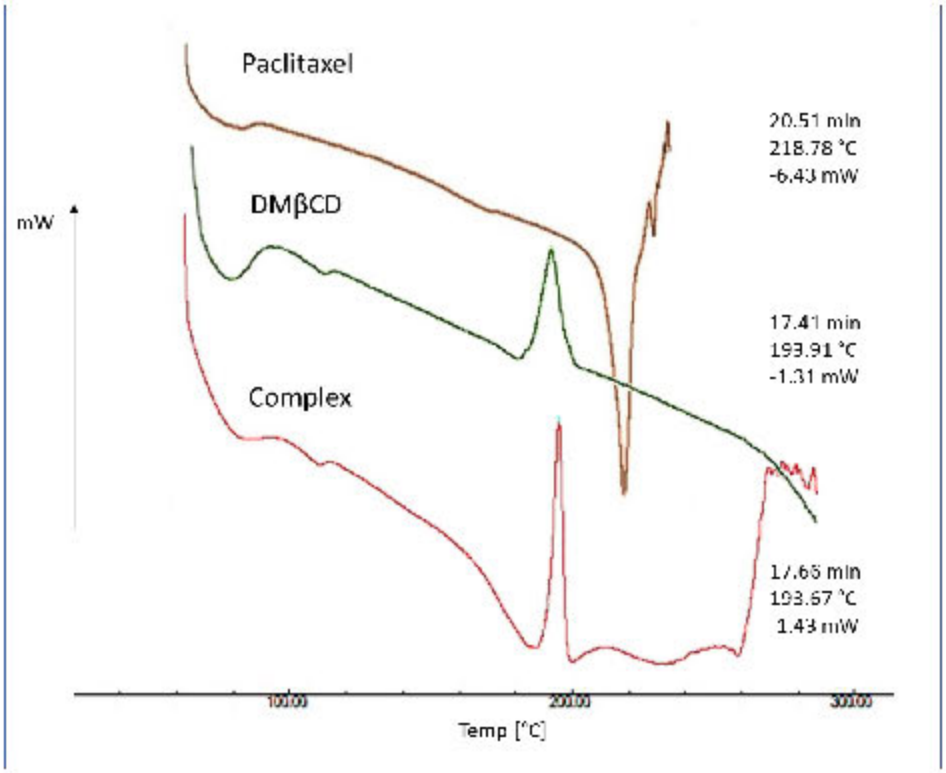


Figure 4.4 DSC thermograms of PTX, DMβCD and PTX-DMβCD inclusion complex.

Fourier transform infrared spectroscopy

For confirmation of complex formation between PTX and DMβCD, FTIR analysis was performed (Figure 4.5). Spectra of PTX drug pellets exhibit strong absorption bands in the range of 1730 cm^{-1}–1600 cm^{-1}, 1380 cm^{-1}–1180 cm^{-1} and 900 cm^{-1}-710 cm^{-1}. Further the IR spectra of DMβCD can be characterized by the intense bands at 3430 cm^{-1}- 3420 cm^{-1} due to absorption by hydrogen-bonded OH groups as

well as the bands at 3000 cm^{-1}–2830 cm^{-1} corresponding to vibration of the –CH and $–CH_2$ groups. Herein, as the numbers of guest molecules are less in number or the mass ratio of CD to PTX is high, most of the characteristic stretching bands of PTX may get masked by the host species. Such observation is consistent with the change in FTIR spectra for PTX after complexation with DMβCD. For PTX molecule, stretching band for carbonyl ester at 1724 cm^{-1} was altered leading to increase in intensity. The typical carbonyl bond stretching at 1650 cm^{-1}-1600 cm^{-1} was masked due to complex formation. Shift of absorbance bands to higher frequencies due to cleavage of hydrogen bonding during complex formation was confirmed by change in aromatic carbon stretching from 1372 cm^{-1} to 1369 cm^{-1}. Further, a decrease in stretching vibration to 1106 cm^{-1} to 1113 cm^{-1} was observed.

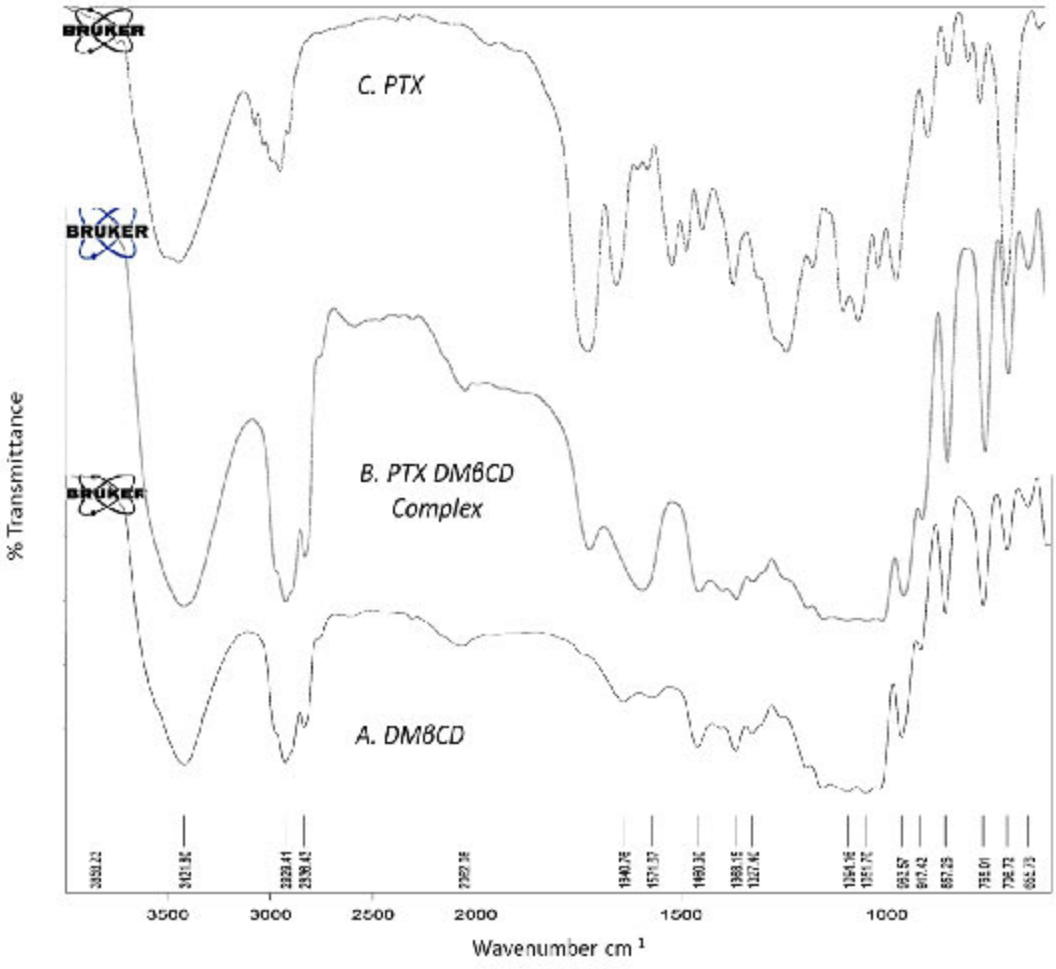


Figure 4.5 FTIR spectra of (A) DMβCD, (B) PTXD-β-CD ICs and (C) PTX.

X-ray diffraction studies

The X-ray diffraction patterns of PTX, DMβCD, as well as ICs are provided in figure 4.6. Diffractogram of PTX confirmed the showed a crystalline nature of the drug, DMβCD is amorphous in nature. ICs displayed a diffused pattern at 10^0-15^0 2Θ area where the peaks corresponding to the PTX almost disappear thus confirming that PTX is probably present in amorphous state upon complexation.

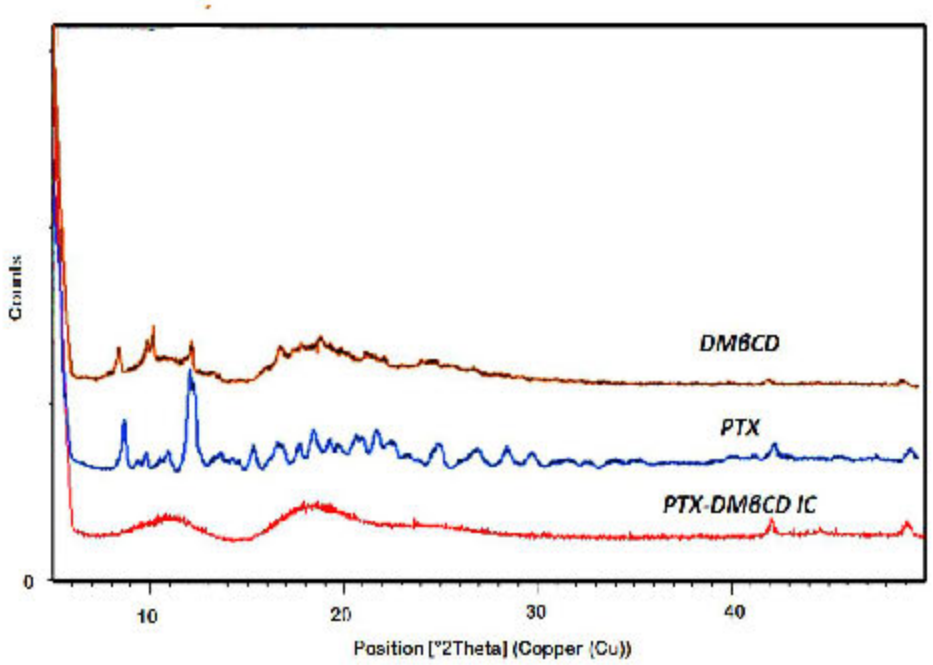


Figure 4.6 X-ray diffraction patterns of PTX, DMβCD and PTXD-β-CD ICs

Scanning electron microscopy

Figure 4.7 shows SEM micrographs of PTX and PTX-DMβCD ICs. PTX were observed as long needle-shaped crystals with a wide particle size distribution, whereas PTX-DMβCD ICs consisted of irregular amorphous particles devoid of any needle shaped crystal typically observed in case of PTX thus confirming complexation.

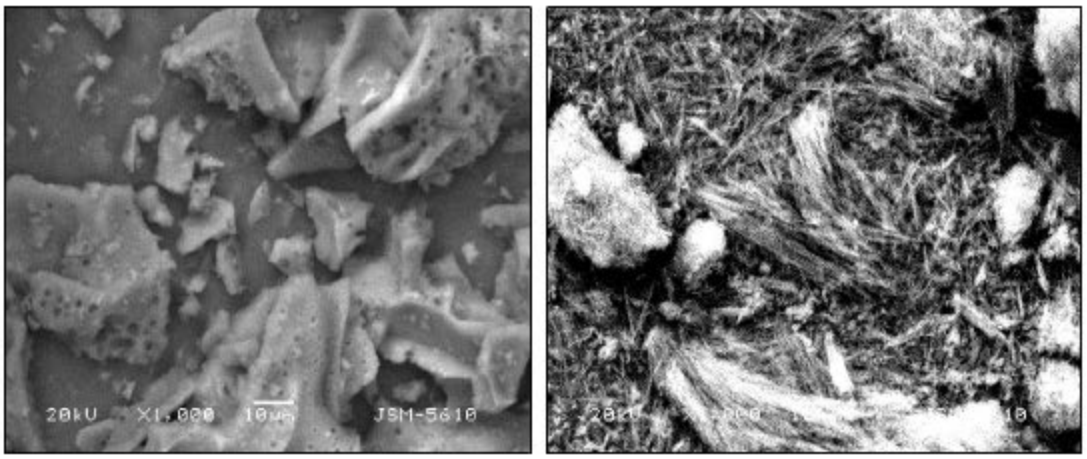


Figure 4.7 SEM images PTX-DMβCD ICs (left) and PTX (right)

Circular Dichroism spectroscopy

Circular dichroism spectroscopy was performed to confirm the interaction between PTX and DMβCD. PTX, DMβCD and PTX-DMβCD ICs were studied in hydro-alcoholic solution to evaluate influence of complexation on spectroscopic characteristic in circular dichroism (Figure 4.8). DMβCD did not show any bands as it UV light transparent in the specified wavelength. On the other hand, PTX showed two circular dichroism bands at 230 nm and at 290 nm. Interaction between PTX and DMβCD was confirmed by relatively large increase in signal intensity. The prominent negative band at 295 nm which may be due to π- π* transition of the aromatic ring in PTX and positive band at 230 nm is due to n- π* transition involving carbonyl bond as the sample is dissolved in methanol-water mixture, both these transitions represents interaction of PTX with CD. A 2-3 nm shift towards lower wavelengths was also observed for both bands after complexation. The increase in intensity of negative band was due to change in environment of aromatic group due to inclusion complex formation between PTX by DMβCD. Disaggregation of PTX into monomers could have led to increase in positive band (34).

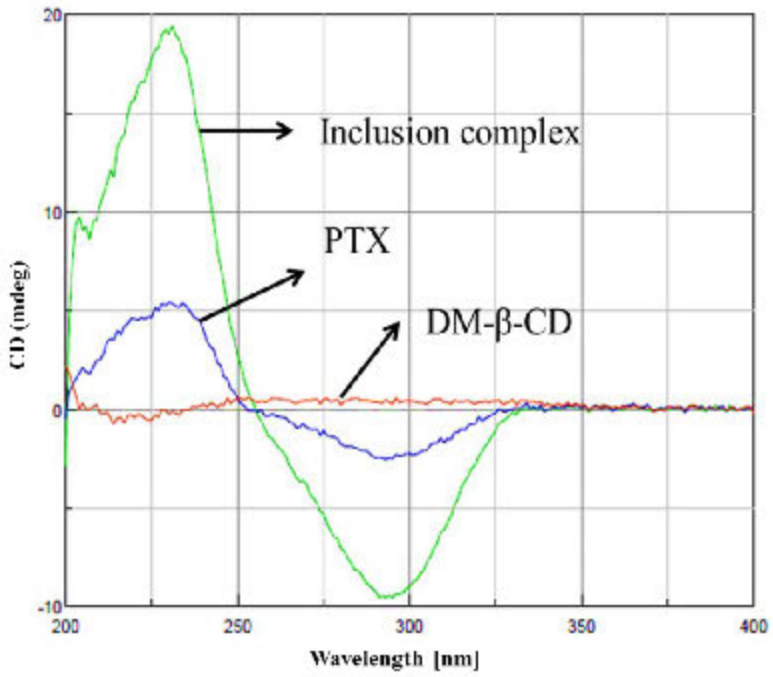


Figure 4.8 Circular dichroism spectrum of PTX, DMβCD and PTXD-β-CD ICs.

4.4.2.3 Stability study of ICs

PTX-DMβCD ICs in lyophilized form was found to be stable at 2-8 °C for three months. A common pharmaceutical problem encountered for the ICs is precipitation upon dilution. Thus, to determine dilution stability of PTX-DMβCD ICs, dilutions were made and stability of ICs was determined. The stability was stated as the concentration of PTX retained in complex at individual time interval compared to its concentration instantly after reconstitution. PTX-DMβCD ICs with molar ratios 1: 20 and 1: 25 showed no precipitation upon 10 times dilution in dextrose for the specified period of time. PTX-DMβCD ICs with molar ratios 1:10 and 1:15 showed precipitation after 4-folds dilution or more, with 69-83% of initial PTX remaining in the complex. The PTX was precipitated out from lower molar ratios of ICs even upon 2-folds dilution. Based on the obtained results of dilution stability study and entrapment efficiency of PTX in ICs, PTX-DMβCD ICs with a molar ratio of 1:20 was selected because at lower ratios, the stability of complex was found to be significantly reduced, while a higher ratio did not result in a considerable enhancement of stability and solubility of PTX. The reason for precipitation after dilution might be due to the higher inter-molecular affinity interaction of PTX (35).

Based on concentration of PTX and solvent polarity, PTX molecules interact via intermolecular H-bonds which results in molecular stacking of PTX in solution. These stacks of PTX impact the physical stability of complex as they could act as main nuclei supporting accumulation of other PTX molecules and finally leads to precipitation. PTX-DMβCD ICs were found to be stable at 2-8 °C for three months and up to 10 times dilution no precipitation was seen even after 24 hr.

4.5 Reference

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