AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Inclusion of Diacerein with cyclodextrin carriers: Preparation, physicochemical characterization, cytotoxicity assessment and pharmacological evaluation

Abhishek Pathak[1*], Sadhana J Rajput[1]

1.TIFAC Centre of Relevance and Excellence in NDDS, G H Patel Pharmacy Building, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Fatehgunj, Vadodara-390 002, Gujarat, INDIA.

ABSTRACT

The present investigation aims on the preparation and in-vitro/in-vivo examination of inclusion complex of Diacerein (DAR) an anthraquinone derivative indicated for treatment of osteoarthritis by inhibiting interlukin-1 and hydroxypropyl-β-cyclodextrin (HP-β-CD). In this study, the inclusion complexes of DAR with β-cyclodextrin (β-CD), HP-β-CD, methyl-β-cyclodextrin (M-β-CD) and γ-cyclodextrin (γ-CD) were prepared and characterized for phase solubility study and inclusion efficiency. On the basis of results obtained, HP-β-CD was selected to prepare inclusion complexes with DAR by physical mixing, kneading method and freeze drying method and subjected to solid state characterizations. Differential Scanning Calorimetry (DSC), X-ray Diffraction (XRD) and Infra-red spectroscopy (IR) analysis confirmed the formation of perfect inclusion complex of DAR with HP-β-CD in solids. In-vitro dissolution and % drug content of DAR in freeze dried inclusion complex showed its superiority over plain drug and commercial formulation. . *In-vitro* cell cytotoxicity studies (MTT Assay) using Caco-2 cell line model confirmed the bio-tolerability of DAR-inclusion complex. . The relative oral bioavailability of DAR in Albino rabbits resulted from Freeze dried inclusion complex was found 3.32 fold and 2.03 fold greater than plain DAR and marketed formulation, respectively which ultimately demonstrated the enhancement of oral bioavailability of DAR in freeze dried inclusion complex with HP-β-CD.

Keywords: Inclusion complex, Diacerein, cyclodextrin, cytotoxicity, bioavailability

*Corresponding Author Email: abhipathak2601@gmail.com
Received 18 September 2016, Accepted 04 October 2016

Please cite this article as: Pathak A *et al.*, Inclusion of Diacerein with cyclodextrin carriers: Preparation, physicochemical characterization, cytotoxicity assessment and pharmacological evaluation. American Journal of PharmTech Research 2016.

INTRODUCTION

Cyclodextrins (CDs) were invented more than 100 years before[1]. In the beginning only small amounts of relatively impure CDs could be generated and high production cost prevent their industrial usage. Recent biotechnological advancements have resulted in surprising improvements in the efficiency and purity of various CDs and their derivatives and made them at low cost[2, 3]. Pharmaceutical application of CDs as additive and drug complexing agent is still growing and successful. CDs are cyclic oligosaccharides with a bucket-like structure having a hydrophobic internal cavity and a hydrophilic exterior wall. The interior of the toroid is hydrophobic which can accommodate a variety of lipophilic compounds and form inclusion complexes[4, 5]. This inclusion of CDs with poor water soluble drugs could greatly enhance the solubility of guest molecule with improvement in dissolution rate, bioavailability and stability[6].

In this investigation, the selected drug named Diacerein (DAR) is a Biopharmaceutical classification system class II drug with low solubility and high permeability[7, 8] DAR is a drug used in the treatment of osteoarthritis by inhibiting interleukin-1. It is a slow-acting drug that may slow down the breakdown of cartilage and relieve pain and swelling[9, 10]. There was improvement of OA induced in animal models when treated with DAR [11-13]. Before reaching to blood circulation, DAR is completely metabolized or hydrolyzed by humans and animals to rhein, an anthraquinone derivative and active metabolite of DAR [14]. DAR is a crystalline solid and having low water solubility (about 3-7µg/ml). Systematic name of DAR is 4,5,-diacetyloxy-9,10-dihydro-9,10-dioxo-anthracene-2-carboxylic acid. The chemical formula, molecular mass, Log P, pKa and melting point of DAR are $C_{19}H_{12}O_8$, 368.294 g/mol, 2.47, 2.98 and 242°C-246°C respectively. The oral bioavailability of DAR is about 30-55%[15].

In this research we aimed to report the development and preparation of a stable and efficient inclusion of DAR with CDs to enhance the solubility, dissolution and bioavailability of drug.

The phase solubility study was performed out in order to examine the effect of β-cyclodextrin, 2-hydroxypropyl-β- cyclodextrin, Methyl-β- cyclodextrin and γ- cyclodextrin on the solubility of DAR was studied in buffer solutions (pH-1.2 and pH-6.8) and water. The stability constants between DAR and cyclodextrins were calculated from the obtained phase solubility diagrams. The extent of inclusion of DAR in various cyclodextrins was evaluated by inclusion efficiency study. The Diacerein/cyclodextrin inclusion complexes were prepared by physical mixing, kneading method and freeze drying method. The physicochemical characterization of prepared inclusion complexes was performed by Differential Scanning Calorimetry (DSC), X-ray Diffraction (XRD)

and Fourier Transform Infrared (FTIR) spectroscopy. Dissolution studies were carried out in Distilled water, Phosphate Buffer pH-6.8, Acetate Buffer pH-4.5 and 0.1N HCl. The % content of DAR in DAR inclusion complexes was checked. *In-vitro* Cell Cytotoxicity Studies (MTT Assay) and *in-vitro* permeability assessment of DAR and its inclusion complex were performed using Caco-2 cell line model. Pharmacological evaluation was performed in Albino rabbits to assess the bioavailability and other pharmacokinetic parameters of DAR and its inclusion complex. The developed formulations were found stable at 5°C±3°C (refrigerator) and at room temperature (RT) for a period of 6 months.

MATERIALS AND METHOD

Materials

DAR was obtained as gift sample from Wockhardt Research Centre, Aurangabad, India. Marketed formulation "Dycerin",(Diacerein IP 50 mg, Glenmark Pharmaceuticals Ltd., Mumbai, India) was purchased from local pharmacy. Rhein standard was purchased from Sigma-Aldrich, India. β-Cyclodextrin (β-CD) and Methyl-β-Cyclodextrin (M-β-CD) were purchased from Hi–media Laboratories Pvt. Ltd., Mumbai. Hydroxy propyl -β-Cyclodextrin (HP-β-CD) was obtained as a gift sample from Sun Pharma Advance Research Company, Vadodara, India. γ-Cyclodextrin (γ-CD) was procured as a gift sample from Roquette Pharma, U.S.A. Other chemicals and reagents were of HPLC or analytical grades.

Caco-2 cell lines were purchased from NCCS, Pune, India. Dulbecco's Modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), sodium pyruvate, sodium bicarbonate, penicillin-streptomycin solution, Trypsin-EDTA solution, Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS) were purchased from Himedia, Mumbai, India. Lucifer yellow and MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) dye were purchased from Sigma Aldrich INDIA, Bangluru, India. 12-well Transwell inserts were purchased from Nunc, Denmark. 96-well plates were purchased from Coster, Corning, USA.

Purified HPLC grade water was obtained by filtering double distilled water through nylon filter paper 0.22 µm pore size and 47 mm diameter (Millipore, Bangalore, India).

Preparation of inclusion complex

Physical mixture

The physical mixtures were prepared for reference by mixing DAR and CDs (β-CD, HP-β-CD, M-β-CD and γ-CD) in 1:1, 1:2 and 1:3 drug-CD molar ratios individually. The specified quantities of DAR and CD were accurately weighed individually according to the molar ratio, transferred in a

glass vial, sealed and shaken vigorously to mix the content completely. The mixture then passed through sieve (mesh # 100) and stored in dessicator over activated silica gel till further evaluation.

Kneading Method

Inclusion complexes of DAR with various cyclodextrins (i.e. γ-CD, β-CD, HP-β-CD and M-β-CD) in different molar ratios like 1:1. 1:2 and 1:3 were prepared using Kneading method. A specified and accurately weighed quantity of cyclodextrin as per the pre-decided molar ratio was added to the mortar and small quantity of water was added while triturating to get slurry like consistency. An accurately weighed quantity of DAR was slowly incorporated in the small parts into the slurry with continuous trituration for 1 hour. The increased viscosity of the mixture was indicating the formation of the complex. Finally the mixture was completely dried in an oven at 45^{o}C, ground to get a fine powder, passed through sieve (mesh # 100) and stored in dessicator over activated silica till further evaluation.

Freeze drying Method

Inclusion complexes of DAR with various cyclodextrins (i.e. γ-CD, β-CD, HP-β-CD and M-β-CD) in different molar ratios like 1:1. 1:2 and 1:3 were prepared using freeze drying method. In this method, the specified quantity of cyclodextrin (as per the DAR:CD molar ratio) was transferred in a glass vial containing 10 ml of distilled water and sonicated to dissolve. Then the corresponding quantity of DAR was added and stirred at a high speed magnetic stirrer for 24 hrs at 25^{o}C. Afterwards, the mixture was centrifuged at 5000 rpm for 15mins and clear solution was separated. The obtained solution was freeze-dried immediately after preparation. The acquired solution was filled into glass vials and frozen at -70°C for 24 hr using an ultra cold deep freezer; later the samples were freeze-dried using a Lyophilizer (Heto Dry Winner, Germany) for 24 hr to yield dry powder.

Phase solubility study

The preparation of inclusion complexes in liquid state was performed according to the phase solubility method of Higuchi and Connors[16]. An excess amount of plain DAR (50mg) was suspended in 5 ml aqueous solutions containing successively increased concentrations of CDs (i.e. β-CD, HP-β-CD, M-β-CD and γ-CD) (5-30mM/L) in 15ml capacity stoppered glass tubes, separately. The suspensions were shaken for 48 hours at 80cycles/min at room temperature using Rotospin Test tube Rotator (Tarsons Products Pvt. Ltd. New Delhi, India) until the equilibrium was reached. An excess of DAR was removed by filtration through a 0.45 µm membrane filters (Millipore). Clear solutions were suitably diluted and analyzed by UV spectrophotometer (UV-

1700, Shimadzu, Japan) at 257nm. The phase solubility studies were also carried out using HCl pH-1.2 and Phosphate buffer pH-6.8 in place of water.

Apparent solubility constants (K_s) were calculated from the constructed phase solubility diagrams using the following equation, where S_0 is the intrinsic solubility of DAR in absence of CDs (intercept)[17, 18].

$$K_s = \text{Slope}/S_0(1\text{-Slope})$$

Inclusion efficiency study

All Freeze dried inclusion complexes, kneaded mixtures and physical mixtures of DAR with cyclodextrins (i.e. β-CD, HP-β-CD, M-β-CD and γ-CD) prepared in the selected molar ratios of DAR:CD (1:1, 1:2 and 1:3) were weighed accurately (50 mg) and transferred in 50 ml volumetric flasks individually. Methanol was added (30 mL), mixed thoroughly and sonicated for 10 min to dissolve the content at ambient temperature. The volume was made up to mark with methanol and resulting solution was suitably diluted with methanol for further analysis. Concentration of DAR in solutions was determined using UV spectrophotometer (UV-1700, Shimadzu, Japan) at 257nm. Inclusion efficiency was calculated using the formula[19]:-

$$\%\ \text{Inclusion Efficiency (\%IE)} = \frac{\%\ \text{Drug Content}_{Experimental}}{\%\ \text{Drug Content}_{Theoretical}} \times 100$$

Where;

$$\%\ \text{Drug Content}_{Theoretical} = \frac{\text{Actual amount of drug added to form inclusion complex}}{\text{Total weight of (Drug + carrier)}} \times 100$$

and

$$\%\ \text{Drug Content}_{Experimental} = \frac{\text{Amount of drug extracted from inclusion complex}}{\text{Amount of inclusion complex taken}} \times 100$$

HPLC method for determination of DAR in formulations/Plasma

A HPLC method was developed for determination of DAR. Chromatographic separation was achieved using a Phenomenex Hypersil BDS C18 (150×4.6) mm, 5 µm particle size column attached with Phenomenex Security Guard Standard with C18 cartridge (4.0×3.0) mm. Separation was attained using a mobile phase consisting of acetonitrile and phosphoric acid buffer in the ratio of 50:50 (%v/v), pumped at a flow rate of 1.0 ml min^{-1}. The eluent was monitored using UV detector at a wavelength of 257 nm. The column was maintained at ambient temperature and an injection volume of 20 µL was used. The mobile phase was vacuum filtered through 0.22 µm nylon membrane filter followed by degassing in an ultrasonic bath prior to use.

CHARACTERIZATION

FTIR Spectroscopy

IR spectra were recorded using a Bruker ALPHA FT-IR spectrometer equipped with DTGS detector and OPUS/Mentor software (Bruker Optics, Germany). Data were collected over a spectral region from 4000 cm^{-1} to 600 cm^{-1} with resolution 4 cm^{-1} and 100 scans.

DSC analysis

Examination of thermal properties and physical state of prepared samples were carried out using Differential Scanning Calorimeter (DSC 60-A, Shimadzu, Japan). For this purpose, accurately weighed samples (4-7 mg) were placed in hermatically sealed aluminium pans and thermograms were obtained at a heating rate of 10 °C min^{-1} over the scan range of 30 °C to 300 °C.

XRD study

The XRD spectra of plain DAR, pure HP-β-CD, physical mixture, Kneaded mixture and freeze dried inclusion complexes of DAR:HP-β-CD in selected molar ratio were obtained using X-Ray Diffractometer (X-Pert-PRO, PANalytical, Netherland). The samples were mounted on a sample holder and XRD patterns were recorded in the range of $3^{o} < 2\theta < 50^{o}$ at the speed of 5^{o} min^{-1}.

Assay of freeze dried inclusion complex

Accurately weighed sample (equivalent to 25 mg of DAR) was transferred in a 25 ml volumetric flask and 5 ml DMSO was added. Content was sonicated to dissolve and volume was made up to the mark with mobile phase. The sample solution was filtered with 0.22 µm disposable membrane filter (Millipore India, Bengaluru) and filtrate was suitably diluted with mobile phase to get the sample concentration at 10 µg/ml. Standard solution of DAR (10 µg/ml) was also prepared and both the solutions were injected into the HPLC system (*section 2.5*) in triplicate

In vitro dissolution study

In vitro dissolution studies were carried out using clear hard gelatin capsules (Size 0) filled with an accurately weighed quantity of lyophilized DAR:HP-β-CD inclusion complex (DAR-IC) or plain DAR (DAR-P) (equivalent to 50 mg of DAR) and marketed formulation (Dycerin, Label claim-50mg: DAR-M) in different dissolution mediums (i.e. Distilled water, Phosphate Buffer pH-6.8, Acetate Buffer pH-4.5 and 0.1N HCl) using USP dissolution apparatus II (paddle method).

The experiments were performed on 900mL media at 37°C±0.5°C at a rotation speed of 75 rpm. At preselected time intervals, 5 mL samples were withdrawn, filtered immediately and replaced with 5 mL of pre-thermostated fresh dissolution medium. Quantitative determination was performed by UV spectrophotometer at 257 nm. Dissolution tests were performed in triplicate and graph of percent cumulative drug release vs time was plotted. Dissolution profiles were further evaluated on the basis of Dissolution efficiency (DE), Dissolution percentage at 5 min and 60 min

(DP_5 and DP_{60}), time required to release 50% and 90% of drug (t_{50} and t_{90}), Correlation coefficient (r^2), Mean dissolution time (MDT) and Area under curve (AUC). The DDSolver, an Excel add-in software package, which is designed to analyze data obtained from dissolution experiments was used to calculate different dissolution parameters[20].

Stability studies

Stability studies of lyophilized DAR-IC in selected molar ratio was carried out at 5°C±3°C (refrigerator) and at room temperature (RT) for a period of 6 months. Periodically, samples were withdrawn at 1st, 3rd and 6th month and subjected to examine for chemical stability. Chemical stability was checked by assessing the percentage content of DAR in stored formulations using HPLC (*Section 2.5*).

CELL LINE STUDIES OF DAR AND ITS INCLUSION COMPLEX WITH HP-B-CD USING CACO-2 CELL LINE MODEL

Cell culture

Caco-2 (Human, Epithelial colorectal adenocarcinoma) cells of passages in between 40-45 were used for *in-vitro* cytotoxicity study and *in-vitro* permeability study experiments. Caco-2 cells were cultured in 25cm^2 tissue culture flasks. Dulbecco's MEM medium with 1.5mM/Liter glutamine, supplemented with 20% FBS, 1mM sodium pyruvate, 1.5gm/Liter of sodium bicarbonate and 1%penicillin-streptomycin solution was used as culture medium. Cells were cultured as a monolayer in an incubator which was set at 37°C in a humidified atmosphere of ~85% relative humidity and ~5% CO_2. Medium was replenished every alternate day [21, 22].

In vitro cell cytotoxicity study (MTT Assay)

MTT stock solution (1 mg/ml) was prepared by dissolving accurately weighed 10 mg of MTT reagent powder with 10 ml phosphate buffered saline (PBS) in an amber colored 10 ml volumetric flask. The stock solution was stored in dark place at 4°C till the further use.

The *in vitro* cytotoxicity of lyophilized DAR-IC and DAR-P was evaluated for Caco-2 cells using MTT assay. The cells were cultured in 96-well plates (prelabelled as 4 hour, 24 hour and 48 hour) at a seeding density of 1.0×10^4 cells/well for 48 hours. Samples were dissolved in DMSO and different dilutions were made with DMEM culture medium so that the concentration of DMSO did not exceed more than 1% v/v in any diluted sample. Experiments were initiated by replacing the culture medium in each of 96 well of each plate with 100µl of sample solutions (0.1, 1, 10, 100, 250, 500 & 1000 µg/ml) and incubated at 37°C in ~85% relative humidity and ~5% CO_2 environment. After 4 hour of incubation, prelabelled 4 hour-96 well plate was removed from incubator into laminar flow hood area, sample solution was discarded and 100µl of MTT reagent

(1 mg/ml) in phosphate buffered saline (PBS) was added aseptically. The plate was again incubated at 37°C in ~5% CO_2 environment for another 4 hours. At the end of incubation period, medium was removed carefully and intracellular formazan was solubilized with 100µl DMSO by agitating cells on orbital shaker for 15 mins. Absorbance was measured at 590 nm with a reference filter of 620 nm using Micro plate multi detection instrument (680-XR, Bio-Rad Laboratories, France). The medium treated cells were used as controls. Same procedure was followed for 24 hour and 48 hour plates.

Percentage of cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the negative control. To compare the sensitivity of cells to the DAR and its formulation, IC_{50} values (concentration of the drug that leads to 50% inhibition in cell proliferation) were calculated.

In-vitro permeability assessment using Caco-2 cell line model

Caco-2 cell model has been the most extensively characterized and useful cell model in the field of drug permeability study [21-27]. It has been suggested that Caco-2 cells can be wholly used to predict the oral absorption of drugs in humans because the permeation characteristics of drugs across Caco-2 cell monolayers correlates with the human intestinal mucosa permeation charateristics.

Caco-2 cell passages in between 40-45, cultured in 12 well cell culture inserts (pore size-0.4µm, diameter-12/18 mm, area-1.13 cm^2, Product code 12565009, NUNC™, Rosklide, Denmark), were used for *in-vitro* permeability assessment of DAR-IC, DAR-P and DAR-M after 21 days post seeding. Prior to the experiment, the inserts were washed twice and equilibrated for 30 min with pre-warmed transport medium (Hank's balanced salt solution-HBSS containing 25 mM of HEPES, pH-7.4). Accurate quantities of samples were dispersed in transport medium and sonicated to prepare the solutions having DAR concentration at 250µg/ml. The integrity of the monolayer was checked by monitoring the permeability of para-cellular leakage marker (Lucifer Yellow) across the monolayer. The cell monolayers were considered tight enough for the transport experiment enough when the apparent permeability coefficient (P_{app}) for Lucifer Yellow was less than $0.5x10^{-6}$ cm/s. All Transport studies were conducted aseptically at 37°C in an atmosphere of ~85% relative humidity and ~5% CO_2. The 150 µl of transport buffer containing 250 µg/ml test compounds was added to the apical side while the basolateral side of the inserts contained 1.5 ml of transport medium. After the 30, 60, 120, 180, 240 and 480 min of incubation, aliquot of 100 µl was withdrawn from the receiver chamber and was immediately replenished with an equal volume of pre-warmed transport medium. The samples were stored at -20°C until analyzed. The concentration of the test compounds in the transport medium were analyzed using developed RP-

HPLC method as described in *Section 2.5*. The apical to basolateral permeability coefficient (P_{app} in cm/sec) was calculated according to following equation:

$$P_{app} = \frac{dQ/dt}{A \times C_0 \times 60}$$

Where, dQ/dt (flux) is the amount of drug transported across the monolayer from apical to basolateral compartment as a function of time (mg/min), A is the monolayer membrane surface area (cm^2) and C_0 is the initial concentration of drug on the apical compartment (mg/ml).

Pharmacological evalation of freeze dried DAR:HP-β-CD inclusion complex using Albino rabbit animal model

Animals

The pharmacokinetic study was performed in Albino rabbits (NewZealand variety) (weight-1.7 to 2.0 kg, age-09 to 12 months and either sex). Animals were housed into two groups and standard diet was supplied to animals with free access of water. Animals were kept at general environment conditions (i.e. 25°C±2°C temperature and 65%±5% RH) under natural light/dark conditions. The protocol for the animal study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Govt. of India.

Experimental: Dosing and sampling

Relative bioavailability of DAR-IC was determined with respect to DAR-P and DAR-M. Animals were divided in three treatment groups and each group contained 6 rabbits. The animals were fasted over night prior to the experiment with free access of water. The DAR-IC, DAR-P and DAR-M (equivalent to 9.25 mg of DAR) were filled in hard gelatin capsule (Capsugel®#size 5) and administered orally. Blood samples (1.0 ml) were collected through marginal ear vein using fresh sterilized disposable needles and syringes in heparinized tubes at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 8, 12, 24 and 48 hours after administration. Collected blood samples were vortexd for 1 min and centrifuged at 20,000 rpm for 10 mins at 4°C (Ultra-centrifuge, 3K 30 Sigma Laboratory Centrifuge, Osterode, Germany). Separated plasma samples were withdrawn and stored at -20°C till further processing.

Plasma sample preparation and analysis

Liquid-liquid extraction method was adopted for the extraction of rhein from plasma. 250 µL of each plasma sample was transferred in separate pre-labeled ria vials. 25 µl of Fenofibrate solution (100 µg/mL) and 3.5% perchloric acid solution were added to all samples as internal standard and

buffering agent, respectively. Then 2 ml of ethyl acetate was transferred to all samples individually, vortexed for about 5 mins and centrifuged at 4000 rpm at 4°C for 10 mins. Organic layer was carefully transferred to prelabelled ria vials after freezing the aqueous layer at -20°C and the samples were evaporated to dryness at 40°C under the gentle stream of nitrogen. The dried residues were further reconstituted with 50 µl of mobile phase and vortexed for 30 second. Samples of each time point were prepared in triplicate and 20 µL volume of each sample solutions were injected in the HPLC system under the chromatographic conditions described earlier in *Section 2.5.*

Non-compartmental trapezoidal method was employed to calculate the various pharmacokinetic parameters using the computer based statistical package PKsolver add-in for microsoft excel[28-30]. All data were reported as mean ± SD. The statistical significance of the differences between the groups was tested by one-way ANOVA followed by Bonferroni multiple comparison test.

RESULTS AND DISCUSSION

Selection of inclusion complex

Phase solubility study

Phase solubility analysis has been the very important and initial requirement for optimizing the development process of an inclusion complex of a drug as it allows the assessment of affinity between CD and drug molecule in aqueous phase. Phase solubility study provides the stability constant for drug-cyclodextrin inclusion complex as well as it also present the insight into stoichiometry of the complex at equilibrium[31].

The phase solubility diagrams were constructed between the apparent equilibrium concentrations of DAR (at Y-axis) and defined concentrations of CDs (i.e. β-CD, HP-β-CD, M-β-CD and γ-CD) at X-axis in water, phosphate buffer pH-6.8 and HCl pH-1.2 as shown in the Figure 1(A), 1(B) and 1(C) respectively. The slopes, intercepts, R^2 and apparent stability constants (K_s) were tabulated (Table 1).

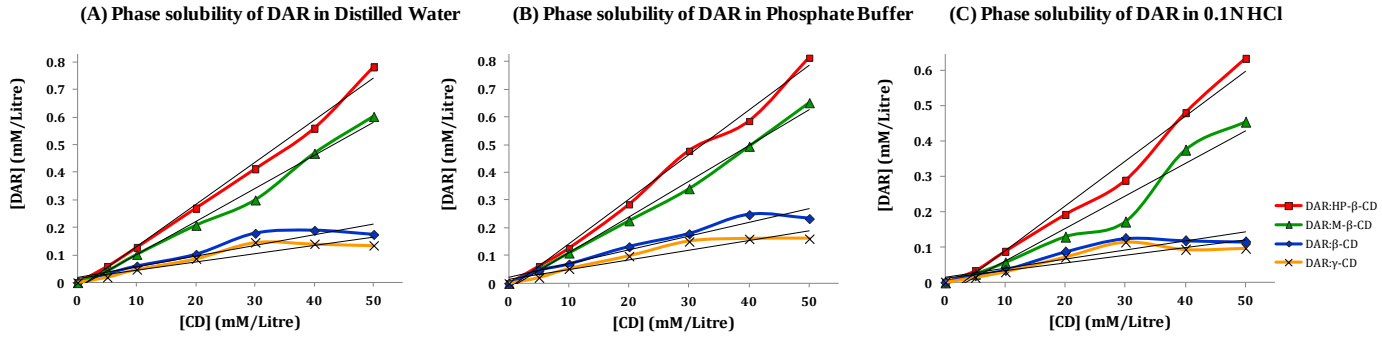

Figure 1 Phase solubility diagrams of DAR with CDs in (A) Water, (B) Phosphate buffer and (C) 0.1N HCl.

Table 1 Comparison of slopes, intercepts, R^2 and K_S of phase solubility studies in water, phosphate buffer and 0.1N HCl for DAR with CDs.

Drug:CD	Slope	Intercept	R^2	K_S (M^{-1})	Type of Graph
In Distilled Water*					
DAR: HP-β-CD	0.015±0.001	0.022±0.002	0.992±0.002	**711.5±6.9**	A_L-Type
DAR: M-β-CD	0.012±0.002	0.019±0.003	0.989±0.009	629.3±9.2	A_L-Type
DAR: β-CD	0.004±0.001	0.019±0.002	0.887±0.005	207.2±8.4	A_N-Type
DAR: γ-CD	0.003±0.001	0.015±0.004	0.860±0.005	196.7±3.7	A_N-Type
In Phosphate Buffer, pH-6.8*					
DAR: HP-β-CD	0.016±0.002	0.021±0.001	0.994±0.002	**806.1±11.7**	A_L-Type
DAR: M-β-CD	0.013±0.003	0.018±0.002	0.984±0.003	726.0±8.6	A_L-Type
DAR: β-CD	0.005±0.001	0.020±0.002	0.950±0.008	255.01±6.5	A_N-Type
DAR: γ-CD	0.004±0.002	0.015±0.001	0.915±0.006	242.2±6.1	A_N-Type
In HCl pH-1.2*					
DAR: HP-β-CD	0.013±0.001	0.035±0.003	0.986±0.005	**366.7±7.4**	A_L-Type
DAR: M-β-CD	0.009±0.001	0.032±0.002	0.952±0.003	294.8±5.8	A_L-Type
DAR: β-CD	0.003±0.002	0.015±0.002	0.840±0.008	173.8±8.9	A_N-Type
DAR: γ-CD	0.002±0.001	0.013±0.003	0.794±0.004	168.4±9.7	A_N-Type

* Data are shown as Mean±SD, n=3

The results indicated that the low solubility of DAR was increased linearly with all the CDs in all the mediums and the value of K_S for inclusion complex increased in the order of (DAR:HP-β-CD)>(DAR:M-β-CD)>(DAR:β-CD)>(DAR:γ-CD). The smaller values of K_S (less than 200 M^{-1}) indicate a week interaction between drug and CD, while larger values of K_S (more than 1000 M^{-1}) are symptomatic of an incompatible drug release from the inclusion complex[32]. The resultant values of K_s predicted that HP-β-CD and M-β-CD formed sufficiently stable inclusion complex with DAR where as the stability of DAR:β-CD and DAR:γ-CD were not found good, comparatively. The linear increase in solubility of DAR with increase in CDs concentration, giving rise to A_L-type phase solubility diagram for DAR:HP-β-CD and DAR:M-β-CD while DAR:β-CD and DAR:γ-CD showed A_N-type of solubility curves at different pH values. The R^2 values were also increased in the order of (DAR:HP-β-CD)>(DAR:M-β-CD)>(DAR:β-CD)>(DAR:γ-CD). It can be seen that DAR:HP-β-CD and DAR:M-β-CD possess good stability but K_S for DAR:HP-β-CD was greater in all the mediums and found highest in phosphate buffer pH-6.8. This may be due to acidic nature of DAR which was completely unionized at this pH and lead to formation of a stable complex with HP-β-CD. The pH value has significant influence on the interaction mode between drug and CDs, indicating the different affinity of acidic, neutral and basic drugs for the

inclusion complex formation and additionally the increase in drug ionization at particular pH resulted in decrease of the complex stability constant[33, 34]. On the basis of phase solubility study, it can be concluded that DAR:HP-β-CD formed most stable inclusion complex with highest solubility, among the four.

Inclusion efficiency study

Inclusion efficiencies (%IE) of all Freeze dried inclusion complexes, kneaded mixtures and physical mixtures of DAR:β-CD, DAR:HP-β-CD, DAR:M-β-CD and DAR:γ-CD in the selected molar ratios of DAR:CD (1:1, 1:2 and 1:3) were determined and results were presented in Table 2. The results clearly showed that the %IE of DAR:HP-β-CD inclusion complex in molar ratio of 1:2 was found higher for physical mixture (72.39%±2.87%), kneaded mixture (84.61%±1.28%) and freeze dried inclusion complex (99.32%±1.41%) than the other inclusion complexes prepared by respective mode of preparation. It indicated that DAR was uniformly distributed in DAR:HP-β-CD inclusion complex in molar ratio of 1:2 and others did not show satisfactory drug incorporation.

Table 2 Inclusion efficiency values of all Freeze dried inclusion complexes, kneaded mixtures and physical mixtures of DAR:β-CD, DAR:HP-β-CD, DAR:M-β-CD and DAR:γ-CD in molar ratios of 1:1, 1:2 and 1:3.

DAR:CD	% Inclusion Efficiency (% IE)*		
	For molar ratio (1:1)	For molar ratio (1:2)	For molar ratio (1:3)
Physical Mixtures			
DAR: HP-β-CD	40.18±1.52	**46.39±2.87**	32.44±1.63
DAR: M-β-CD	31.98±3.66	35.21±2.54	29.21±1.92
DAR: β-CD	22.27±4.12	28.36±2.96	29.15±2.03
DAR: γ-CD	25.68±0.98	27.57±2.74	31.62±1.28
Kneaded Mixtures			
DAR: HP-β-CD	57.63±2.51	**64.61±1.28**	53.29±1.92
DAR: M-β-CD	51.64±1.97	56.23±3.02	49.89±2.14
DAR: β-CD	40.14±1.75	45.35±1.84	50.98±1.98
DAR: γ-CD	43.56±1.58	48.87±1.76	50.25±2.31
Freeze dried inclusion complex			
DAR: HP-β-CD	81.37±1.03	**99.32±1.41**	71.18±3.22
DAR: M-β-CD	73.74±4.08	91.59±1.67	62.31±1.53
DAR: β-CD	46.28±1.49	53.36±2.28	57.96±1.64
DAR: γ-CD	51.63±2.37	58.93±2.16	60.19±1.78

* Data are shown as Mean±SD, n=3

Based on the results obtained from phase solubility studies and inclusion efficiency estimation, DAR:HP-β-CD in the molar ratio of 1:2 was selected as best fitted inclusion complex for further studies due to its superior solubilizing capacity and greater inclusion efficiency. Moreover, earlier reports suggest that the modified β-cyclodextrins (HP-β-CD) have enormous applicability in

development of solid oral dosage forms due to their higher complexation efficiency and lower cytotoxicity than the β-cyclodextrin[35-38].

Characterization

FTIR Spectroscopy

The FTIR Spectroscopy of Plain DAR, pure HP-β-CD, physical mixture, kneaded mixture and freeze dried inclusion complex for DAR: HP-β-CD::(1:2)M was carried out and result had been represented in Figure 2.

The IR studies of DAR exhibited peaks at 3452.33 cm^{-1} and 3071.08 cm^{-1} were due to O-H and aromatic stretching. Peaks at 1768.81 cm^{-1}, 1694.88 cm^{-1} and 1211.30 cm^{-1} were due to C=O stretching of carbonyl group, C=O stretching of keto group and C-O stretching of ester group, respectively. Aromatic bending was observed from 760.1 cm^{-1} to 705.65 cm^{-1}. These bands confirmed the structure of DAR. However, the FTIR spectra of HP-β-CD showed a large and broad band at 3381.51 cm^{-1} corresponding to absorption by hydrogen bonded O-H groups.

The IR spectrum of physical mixture of DAR:HP-β-CD::1:2M had shown peaks at 1771.16 cm^{-1}, 1676.74 cm^{-1} and 1209.98 cm^{-1} were due to C=O stretching of carbonyl group, C=O stretching of keto-group and C-O stretching of ester group, respectively. The intense appearance and little shifting of these peaks indicate week interaction between drug and excipients.

The IR spectrum of kneaded mixture of DAR:HP-β-CD::1:2M had shown peaks at 1770.86 cm^{-1}, 1678.26 cm^{-1} and 1210.95 cm^{-1} were due to C=O stretching of carbonyl group, C=O stretching of keto-group and C-O stretching of ester group, respectively. The intense appearance and little shifting of these peaks indicate week interaction between drug and excipients but more than the physical mixture. Whereas in the IR spectrum of freeze dried inclusion complex of DAR:HP-β-CD::1:2M, all the characteristic peaks of DAR disappeared which indicate a good inclusion and interaction of DAR with HP-β-CD at the selected molar ratio. Moreover this study also proved the efficiency of selected method of preparation.

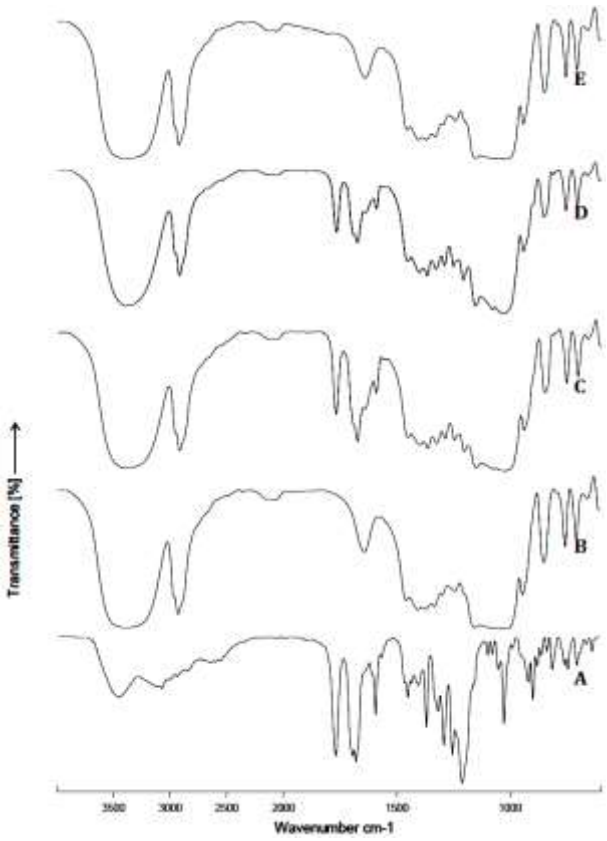

Figure 2 IR spectrums of (A) Plain DAR, (B) HP-β-CD , (C) Physical Mixture for DAR: HP-β-CD::(1:2)M, (D) Kneaded Mixture for DAR: HP-β-CD::(1:2)M and (E) Freeze dried inclusion complex of DAR: HP-β-CD::(1:2)M.

DSC analysis

The thermal behavior of plain DAR, pure HP-β-CD, physical mixture, kneaded mixture and freeze dried inclusion complex for DAR: HP-β-CD::(1:2)M were studied using Differential Scanning Calorimetry (DSC) in order to confirm the formation of solid inclusion complexes (Figure 3).

The DSC thermogram of DAR showed a sharp endothermic peak for at 251.8°C corresponding to its melting point. The DSC thermogram of HP-β-CD exhibited a broad endothermic peak at 88.7°C which corresponded to the loss of hydration water of the material. The HP-β-CD decomposed at the temperature of 300°C hence not showing any melting peak of HP-β-CD in between 30°C to 300°C.

In the DSC thermogram of physical mixture in DAR:HP-β-CD::1:2 molar ratio showed two sharp endothermic peaks, corresponding to HP-β-CD and DAR indicated that inclusion of drug within CD was not achieved. The DSC thermogram of kneaded mixture for DAR:HP-β-CD::1:2M also showed two endothermic peaks corresponding HP-β-CD and DAR but the height of DAR endothermic peak was reduced considerably in comparison with pure DAR and physical mixture

indicating the interaction between DAR and HP-β-CD but true complex had not been formed. The occurrence of DAR peak also reflected the existence of few DAR crystals in the preparation.

The DSC thermogram of freeze dried inclusion complex of DAR:HP-β-CD::1:2M had shown an endothermic peak for HP-β-CD but the disappearance of characteristic endothermic peak due to DAR with this system, clearly indicated the formation of true inclusion complex. The absence of DAR peak might also be attributed to the amorphous form of the drug in the complex formation. After this study, it can be concluded that preparation of inclusion complex followed by freeze drying was the best suitable method for formation of inclusion complex of DAR.

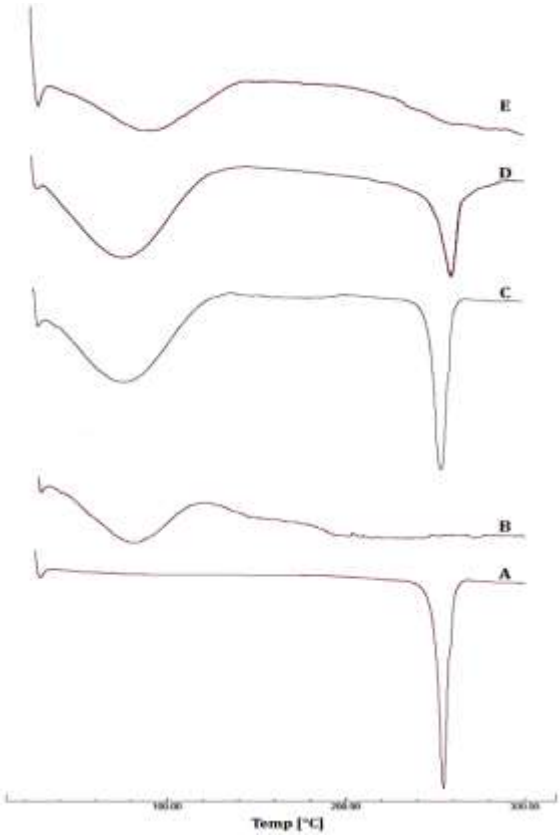

Figure 3 DSC thermograms of (A) Plain DAR, (B) HP-β-CD , (C) Physical Mixture for DAR: HP-β-CD::(1:2)M, (D) Kneaded Mixture for DAR: HP-β-CD::(1:2)M and (E) Freeze dried inclusion complex of DAR: HP-β-CD::(1:2)M.

XRD study

The Powder X-Ray Diffraction (XRD) Study of Plain DAR, pure HP-β-CD, physical mixture, kneaded mixture and freeze dried inclusion complex for DAR: HP-β-CD::(1:2)M was carried out and result had been represented in Figure 4.

XRD study was carried out to investigate the crystalline state of drug which is influencing the dissolution and stability behaviour of compound. The preservation of the crystal structure of the

drug in the formulation is crucial for the sustained stability of the drug during its shelf-life. The peak position (diffraction angle) is an identification tool of a crystal structure, where as the number of peaks is a measure of sample crystallinity in a diffractogram[39]. The development of an amorphous form confirmed that the drug was dispersed completely in a molecular state with cyclodextrin. It had been investigated by several researchers that the occurrence of a diffused diffraction pattern, appearance of new peaks and elimination of characteristic peaks of the drug molecule, evident for the formation of an inclusion complex of drug with cyclodextrins[40-43].

The XRD pattern of pure DAR exhibited various diffraction peaks at 10.3, 17.2, 21.3, 24.9 and 27.6 °2θ indicating the crystalline nature of drug. No diffraction peaks were observed in the diffractogram of HP-β-CD, showed the amorphous form of HP-β-CD. The XRD patterns of physical mixture and kneaded mixture showed sufficiently visible characteristic peaks of DAR, pointing toward the insufficient inclusion or lack of inclusion of DAR in HP-β-CD. The XRD of freeze dried inclusion complex of DAR: HP-β-CD::(1:2)M showed a halo pattern, with the disappearance of all characteristic peaks of DAR which indicated the complete incorporation of DAR in HP-β-CD cavity and formation of complete and stable inclusion complex. The results obtained from XRD analysis were in good agreement with DSC observations.

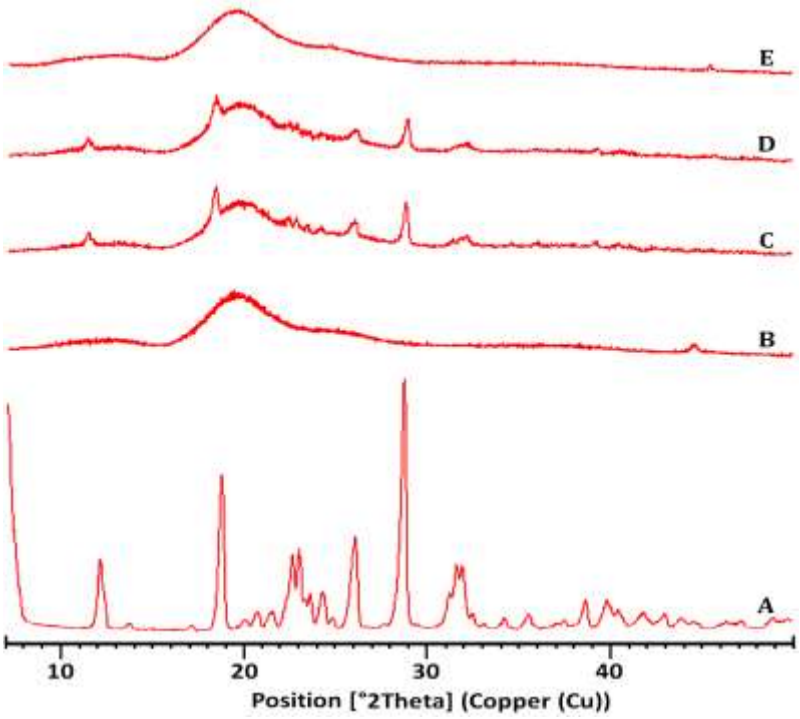

Figure 4 XRD patterns of (A) Plain DAR, (B) HP-β-CD , (C) Physical Mixture for DAR: HP-β-CD::(1:2)M, (D) Kneaded Mixture for DAR: HP-β-CD::(1:2)M and (E) Freeze dried inclusion complex of DAR: HP-β-CD::(1:2)M.

Assay of freeze dried inclusion complex

Percentage assay of DAR in lyophilized inclusion complex of DAR:HP-β-CD in (1:2) molar ratio was found to be 100.02±1.62% which indicate the suitability of freeze drying method for production of inclusion complex.

In vitro dissolution study

The disolution profiles of DAR-P, DAR-M and DAR-IC in phosphate buffer pH-6.8 (PB), acetate buffer pH-4.5 (AB), 0.1N HCl and water are graphically presented in Figure 5. It was evident from the graphs that optimized inclusion complex of DAR-IC served better dissolution profile and drug release than the DAR-P and DAR-M in all the dissolution mediums. The DAR-IC, DAR-M and DAR-P showed better dissolution profile in phosphate buffer pH-6.8 and water as compared to acetate buffer pH-4.5 and 0.1N HCl which may be due to low solubility of drug in acidic medium[8]. The significant improvement in dissolution characteristics of inclusion complexes may be due to the formation of readily soluble inclusion complex in the dissolution medium, increased drug particle wettability and reduction of the crystallinity of the drug product.

Various dissolution parameters were calculated using DDsolver, an excel add-in program and reported in Table 3. The Dissolution efficiency (DE), Dissolution percentage at 5 min and 60 min (DP_5 and DP_{60}) and Area under curve (AUC) values were increased in the following order: DAR-P<DAR-M<DAR-IC; while time required to release 50% and 90% of drug (t_{50} and t_{90}) and mean dissolution time (MDT) were increased in vice versa i.e. DAR-P>DAR-M>DAR-IC. The t_{50} and t_{90} for DAR-IC were significantly reduced to 7.2-14.8 mins and 23.9-48.3 mins respectively as compared to DAR-M and DAR-P in all the dissolution mediums but these were found least in phosphate buffer pH-6.8. This may be due to acidic nature of DAR which was completely unionized at this pH.

All the results indicated that the DAR-IC prepared by freeze drying technique was having superior characteristics to plain drug and marketed formulation, indicating a major prospect to enhance the bioavailability of such drugs by inclusion complexation for oral administration where solubility and dissolution are rate limiting factors in bioavailability in the body. Thus inclusion complexation of poor soluble drug with hydrophilic cyclodextrin is an effective and successful technique in order to improve their biopharmaceutical properties.

Table 3 Comparision of various dissolution parameters of plain DAR (DAR-P) marketed formulation (DAR-M) and Freeze dried inclusion complex of DAR: HP-β-CD::(1:2)M (DAR-IC) in phosphate buffer pH-6.8 (PB), acetate buffer pH-4.5 (AB), 0.1N HCl and water.

	DE	DP_5	DP_{60}	t_{50}	t_{90}	MDT	AUC
In Phosphate Buffer, pH-6.8*							
DAR-IC	0.90±0.06	38.9±0.9	99.2±0.5	7.2±0.1	23.9±0.7	11.0±0.4	10847±61
DAR-M	0.84±0.04	36.4±0.5	93.5±0.5	9.7±0.4	48.3±0.7	14.8±0.2	10036±35
DAR-P	0.42±0.01	8.1±0.2	49.8±0.3	>60	>60	32.0±0.4	5009±28
In Acetate Buffer, pH-4.2*							
DAR-IC	0.48±0.02	13.8±0.6	54.2±0.4	48.4±0.4	>60	19.4±0.7	5743±42
DAR-M	0.36±0.02	14.4±0.3	40.1±0.7	>60	>60	18.9±0.7	4264±19
DAR-P	0.11±0.02	1.9±0.4	12.8±0.2	>60	>60	39.7±0.5	1324±25
In 0.1N HCl*							
DAR-IC	0.40±0.05	10.2±0.6	45.9±0.5	>60	>60	18.2±0.4	4753±41
DAR-M	0.31±0.01	8.3±0.8	35.6±0.3	>60	>60	20.8±0.3	3761±29
DAR-P	0.09±0.05	1.2±0.1	11.2±0.2	>60	>60	37.5±0.6	1106±17
In water*							
DAR-IC	0.88±0.03	30.1±0.5	98.3±0.6	8.9±0.6	30.4±0.1	13.2±0.3	10609±52
DAR-M	0.76±0.03	25.8±0.2	85.6±0.2	14.8±0.7	>60	20.5±0.8	9078±31
DAR-P	0.37±0.04	7.2±0.5	44.1±0.5	>60	>60	26.8±0.3	4397±15

* Data are shown as Mean±SD, n=3. DE: Dissolution efficiency, DP_5: Dissolution percentage at 5 min, DP_{60}: Dissolution percentage at 60 min, t_{50}: time required to release 50% of drug (min), t_{90}: time required to release 90% of drug (min), MDT: Mean dissolution time (min), AUC: Area under curve.

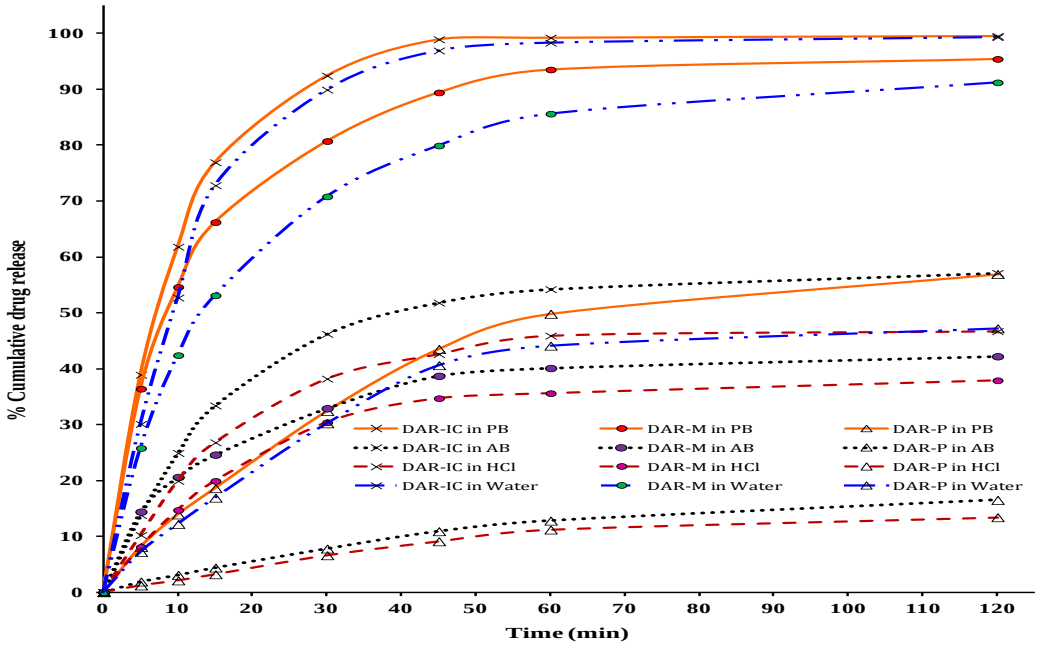

Figure 5 Graphical representation of % Cumulative drug release versus sampling time of plain DAR (DAR-P) marketed formulation (DAR-M) and Freeze dried inclusion complex of

DAR: HP-β-CD::(1:2)M (DAR-IC) in phosphate buffer pH-6.8 (PB), acetate buffer pH-4.5 (AB), 0.1N HCl and water.

Stability studies

The stored formulations were found physically and chemically stable at 5°C±3°C and at room temperature over the period of 6 months. No change in physical appearance of formulation was observed during the 6 months at both the storage conditions. The % DAR content in stability samples of DAR-IC were matched with initial value (100.02%) and found more than 99.45% at both the conditions over the period of 6 months

Cell line studies of DAR and its inclusion complex with HP-β-CD using Caco-2 cell line model

In vitro cell cytotoxicity study (MTT Assay)

Cytotoxicity study of Freeze dried inclusion complex of DAR: HP-β-CD::(1:2)M (DAR-IC)and DAR-P was accomplished in Caco2 cells by mitochondrial activity (MTT assay) to assess the safety/tolerability of prepared formulation on viability of cells. As Caco2 cells were used as absorption model, biocompatibility and tolerability of DAR-P and DAR-IC on absorption barrier was necessary. Cytotoxicity graphs at 4 hours, 24 hours and 48 hours were constructed and presented in Figure 6(A), 6(B) and 6(C). At initial 4 hr and 24 hr, the % cell viability is more than 80% at the 250 µg/ml concentration of DAR-P and DAR-IC. Hence for permeability studies, the drug and formulation concentration was fixed at 250 µg/ml. It can be observed that the DAR-IC showed very less cytotoxicity than the plain DAR upto 48 hours at all the concentrations. This confirms the biocomatibility of DAR-IC and explains that composition of inclusion complex did not contribute to toxicity of Caco2 cells[36, 44]. At initial 4 hours, 24 hours and 48 hours, DAR-IC was found to have less cytotoxicity with more than 80% cell viability as compared to DAR-P at all the concentrations except at 1000µg/ml in 48 hours condition. This could be attributed to protective action of HP-β-CD due to cavitization of drug molecule in CD. IC_{50} values were calculated for DAR-P and DAR-IC and displayed in Table 4. The higher IC_{50} values for DAR-IC than DAR-P at all the incubation time conditions concluded to lack of cytotoxicity due to formulation of a bio-tolerable inclusion complex.

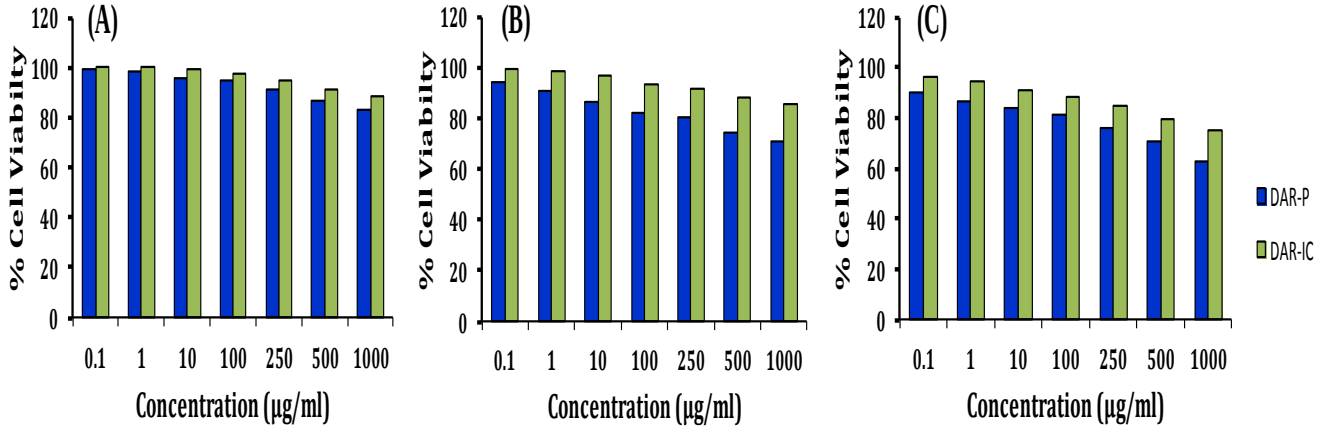

Figure 6 In vitro cytotoxicity studies of DAR-P and DAR-IC in Caco2 cell lines at (A) 4 hours, (B) 24 hours and (C) 48 hours.

Table 4 IC_{50} values of DAR-P and DAR-IC in Caco2 cell lines at 4 hours, 24 hours and 48 hours.

Conditions	IC_{50} Values (µg/ml)*	
	DAR-P	DAR-IC
At 4 hours	3010.96±19.62	4129.75±29.74
At 24 hours	1878.42±14.11	3646.95±35.17
At 48 hours	1408.16±15.18	2316.70±19.67

*****Data are shown as Mean±SD, n=3.

In-vitro permeability assessment using Caco-2 cell line model

In this study, *in vitro* permeability assessment of DAR-IC, DAR-P and DAR-M was done by calulating apparent permeability coefficient (P_{app}) from apical to basolateral (Table 5). Transepithelial permeability of DAR was measured at concentration of 250µg/ml, as negligible toxicity towards Caco-2 cells was found at this concentration during MTT assay of the same. The average P_{app} for Lucifer yellow with Caco-2 cells was found (0.87±0.07) x 10^{-6} cm/sec, confirmed the integrity of monlayers and suitability of monolayers for further experiment. The P_{app} for DAR-P and DAR-M were calculated and found to be (5.95±0.24) x 10^{-6} cm/sec and (8.73±0.82) x 10^{-6} cm/sec respectively while the P_{app} for DAR-IC was observed at (30.26±0.38) x 10^{-6} cm/sec which is about 5.09 fold and 3.47 fold higher than the DAR-P and DAR-M, respectively. The found results were very much satisfacory and matching with the aim of the project. It can be concluded that the higher P_{app} for DAR-IC was because of molecular state of drug and presence of HP-β-CD in the formulation[45, 46]. Whereas the lower permeability coefficient of DAR-P can be attributed to hydrophobicity and low permeation (log P 2.47) of drug. If the P_{app} value of a compound is less than 1 x 10^{-6} cm/sec, in between 1-10 x 10^{-6} cm/ sec, and more than 10 x 10^{-6} cm/sec can be

classified as poorly (0-20%), moderately (20-70%) and well (70-100%) absorbed compounds, respectively[24, 47].

Table 5 Apparent permeability coefficient (P_{app}) from apical to basolateral for DAR-P, DAR-M and DAR-IC using Caco-2 cells model.

Drug/Formulation	Apparent permeability coefficient (P_{app})±SD (10^{-6} cm/sec)*
DAR-P	5.95±0.24
DAR-M	8.73±0.82
DAR-IC	30.26±0.38

*Data are shown as Mean±SD, n=3.

Pharmacological evaluation of freeze dried DAR:HP-β-CD inclusion complex using Albino rabbit animal model

The pharmacological characteristics and *in-vivo* availability of DAR from DAR-IC, DAR-M and DAR-P was studied in Albino rabbits. The DAR is completely metabolized in rhein before entering in the systemic blood circulation, after oral dosing. The mean plasma concentration of rhein versus time course graph was constructed and presented in Figure. 7 whereas the pharacokinetic parameters for DAR calculated by PKsolver add-in in microsoft excel, after *in-vivo* study of DAR-IC, DAR-M and DAR-P were tabulated in Table 6. It was found that the prepared inclusion complex DAR-IC generated higher maximum rhein plasma concentration (C_{max}) compared to DAR-M and DAR-IC with numerical values of (7.81±0.42)µg/ml, (3.44±0.31)µg/ml and (2.91±0.26)µg/ml, respectively. The time to reach C_{max} (T_{max}) for the DAR-IC was shorter which was 2.50±0.04 h compared to 3.50±0.23 h and 3.00±0.17 h for DAR-P and DAR-M, respectively. The shortest T_{max} for DAR-IC may be due to fastest dissolution rate and amorphization of drug due to formation of inclusion complex and the highest T_{max} of DAR-P could be attributed to crystalline nature of drug[48]. The extent of absorption of DAR from the DAR-IC, as represented by the total area under curve (AUC_{0-t}) was also higher than the AUC produced by DAR-P and DAR-M. AUC_{0-t} of rhein was found 25.96±1.25 µg*h/ml for DAR-IC which was 3.32 fold and 2.03 fold higher with that of DAR-P (7.83±0.19 µg*h/ml) and DAR-M (12.81±0.62 µg*h/ml), respectively. The area under momentum curve ($AUMC_{total}$) showed significantly higher value for DAR-IC (194.75±7.83 µg*h^2/ml), compared to DAR-P (50.47±2.31 µg*h^2/ml) and DAR-M (89.95±2.59 µg*h^2/ml). The enhancement in AUC and C_{max} of DAR-IC compared to DAR-P and DAR-M could be due to the quick absorption of drug molecule by gastrointestinal wall due to the tremendous increase in solubility and improved dissolution rate of DAR present in form of inclusion complex with HP-β-CD[49-51]. Mean residence time (MRT) for DAR-IC was found almost same as for DAR-P and DAR-M. The relative bioavailability (F) of DAR in DAR-IC was

increased by 3.32 fold and 2.03 fold compared to DAR-P and DAR-M, respectively. These results could be explained by greater dissolution rate, increased wettability, increased hydrophilicity and reduced crystallinity of DAR in DAR-IC when compared to DAR-P and DAR-M. These findings indicated that a correlation between *in-vitro* dissolution of the DAR from DAR-IC and the results obtained from *in-vivo* study.

Table 6 Pharmacokinetic parameters after oral administration of DAR-P, DAR-M and DAR-IC in Albino rabbits.

Pharmacokinetic parameters*	DAR-P	DAR-M	DAR-IC
C_{max} (µg/ml)	2.91±0.26	3.44±0.31†	7.81±0.42$^{\dagger\#}$
T_{max} (h)	3.50±0.23	3.00±0.17†	2.5±0.04$^{\dagger\#}$
AUC_{0-t} (µg*h/ml)	7.83±0.19	12.81±0.62†	25.96±1.25$^{\dagger\#}$
$AUC_{0-\infty}$ (µg*h/ml)	8.09±0.36	13.19±0.91†	27.02±1.74$^{\dagger\#}$
$AUMC_{total}$ ((µg*h^2/ml)	50.47±2.31	89.95±2.59†	194.75±7.83$^{\dagger\#}$
MRT (h)	6.16±0.14	7.12±0.09†	7.50±0.09$^{\dagger\#}$
$T_{1/2}$ (h)	7.94±0.42	10.65±0.57†	10.21±0.26$^{\dagger\#}$
$K_{elimination}$ (h^{-1})	0.12±0.02	0.07±0.01†	0.07±0.01$^{\dagger\#}$
F (%) w.r.t DAR-P	100	163.60†	331.55$^{\dagger\#}$

***** Data are shown as Mean±SD, n=3, †P<0.05 compared with DAR-P, $^{\#}$P<0.05 compared with DAR-M.

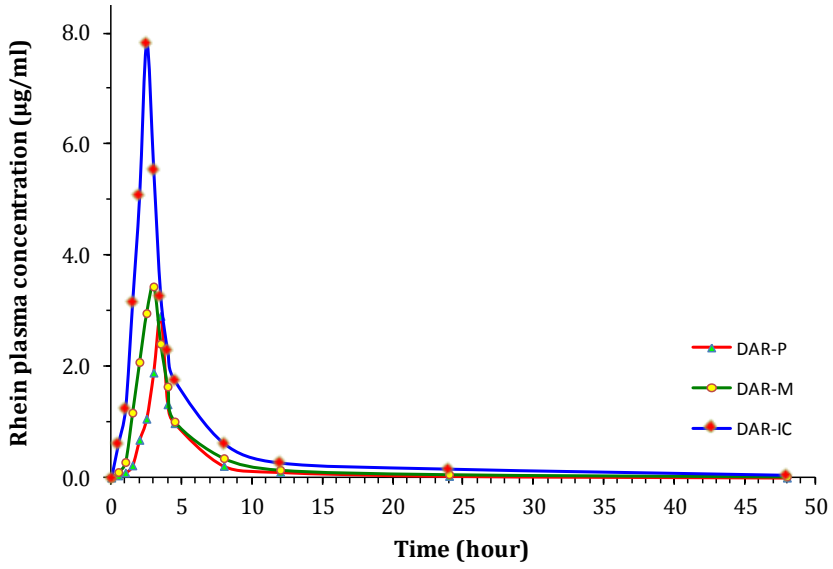

Figure 7 Graphical representation of rhein plasma profile for DAR-P, DAR-M and DAR-IC in Albino rabbits following oral administration.

4. Conclusion

The results of phase solubility studies demonstrated that DAR formed more stable inclusion complex with HP-β-CD than those formed with β-CD, M-β-CD and γ-CD. Additionally the results of inclusion efficiency also stated that DAR:HP-β-CD inclusion complex in molar ratio of 1:2 was

having superior solubilizing capacity and greater inclusion efficiency while others did not show satisfactory drug incorporation.

The results obtained by FTIR, DSC and XRD studies were in excellent agreement and confirmed the formation of true inclusion complex of DAR with HP-β-CD in 1:2 molar ratio by freeze drying method. Percentage assay of DAR in lyophilized inclusion complex of DAR:HP-β-CD in (1:2) molar ratio predicted the suitability of freeze drying method for peoduction of inclusion complex. The prepared formulation was found physically and chemically stable at 5°C±3°C and at room temperature over the time period of 6 months. The freeze dried inclusion complex DAR:HP-β-CD in (1:2) molar showed a good performance in dissolution profile. The MTT assay of prepared formulation indicated the formaton of a bio-tolerable inclusion complex. The P_{app} for Freeze dried inclusion complex was found 5.09 fold and 3.47 fold higher than the plain DAR and marketed formulation, respectively. The relative oral bioavailability of DAR in Albino rabbits resulted from Freeze dried inclusion complex was found 3.32 and 2.03 fold greater than plain DAR and marketed formulation, respectively.

The obtained reults justified the selection of cyclodextrin, molar ratio and method of preparation for the formulation of efficient and stable inclusion complex of DAR with cyclodextrin. The outcome was supported by FTIR, DSC and XRD studies which further lead to enhanced dissolution properties, low cytotoxicity and improved bioavailability of DAR in inclusion complex with HP-β-CD.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support provided by All India Council of Technical Education (AICTE), New Delhi in form of National Doctoral Fellowship to Abhishek Pathak. We are also thankful to Wockhardt Research Centre, Aurangabad, India, for providing gift sample of Diacerein, Sun Pharma Advance Research Company (SPARC), Vadodara, India for supplying gift sample of Hydroxy propyl -β-Cyclodextrin and Roquette Pharma, U.S.A for giving gift sample of γ-Cyclodextrin.

REFERENCES

1. Villiers A. Sur la fermentation de la fecule par l'action du ferment butyrique. Compt Rend Acad Sci. 1891; 112: 536-9.
2. Cramer F. Inclusion Compounds. Berlin: Springer-Verlag, 1954, p.11-30.
3. French D. The Schardinger Dextrins. Adv Carbohydr Chem. 1957; 12: 189-260.
4. Szejtli J. Cyclodextrins. Drug Investigation. 1990; 2: 11-21.

5. Szejtli J. Introduction and General Overview of Cyclodextrin Chemistry. Chem Rev. 1998; 98: 1743-54.
6. Challa R, Ahuja A, Ali J and Khar R. Cyclodextrins in drug delivery: an updated review. Aaps Pharmscitech. 2005; 6: E329-E57.
7. Elsayed I, Abdelbary AA and Elshafeey AH. Nanosizing of a poorly soluble drug: technique optimization, factorial analysis, and pharmacokinetic study in healthy human volunteers. Int J Nanomedicine. 2014; 9: 2943-53.
8. Jain A, Singh SK, Singh Y and Singh S. Development of lipid nanoparticles of diacerein, an antiosteoarthritic drug for enhancement in bioavailability and reduction in its side effects. J Biomed Nanotechnol. 2013; 9: 891-900.
9. Nguyen M, Dougados M, Berdah L and Amor B. Diacerhein in the treatment of osteoarthritis of the hip. Arthritis Rheum. 1994; 37: 529-36.
10. Pelletier JP, Yaron M, Haraoui B, et al. Efficacy and safety of diacerein in osteoarthritis of the knee: a double-blind, placebo-controlled trial. The Diacerein Study Group. Arthritis Rheum. 2000; 43: 2339-48.
11. Mazieres B, Berdah L, Thiechart M and Viguier G. [Diacetylrhein on a postcontusion model of experimental osteoarthritis in the rabbit]. Rev Rhum Ed Fr. 1993; 60: 77S-81S.
12. Mazieres B, Blanckaert A, Thiechart M and Viguier G. [Diacetylrhein administrated "curatively" in an experimental model of post-contusion osteoarthritis in rabbits]. Rev Prat. 1996; 46: S42-5.
13. Moore AR, Greenslade KJ, Alam CA and Willoughby DA. Effects of diacerhein on granuloma induced cartilage breakdown in the mouse. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 1998; 6: 19-23.
14. Debord P, Louchahi K, Tod M, Cournot A, Perret G and Petitjean O. Influence of renal function on the pharmacokinetics of diacerein after a single oral dose. Eur J Drug Metab Pharmacokinet. 1994; 19: 13-9.
15. https://en.wikipedia.org/wiki/Diacerein. 2016.
16. Higuchi T and Connors KA. Phase-solubility techniques. In: Reilly CN, (ed.). Advances in Analytical Chemistry and Instrumentation. New York: Wiley-Interscience, 1965, p. 117-217.
17. Almandoz MC, Sancho MI, Duchowicz PR and Blanco SE. UV–Vis spectroscopic study and DFT calculation on the solvent effect of trimethoprim in neat solvents and aqueous mixtures. Spectrochim Acta Mol Biomol Spectrosc. 2014; 129: 52-60.

18. Loftsson T, Hreinsdóttir D and Másson M. Evaluation of cyclodextrin solubilization of drugs. Int J Pharm. 2005; 302: 18-28.
19. Patel SG and Rajput SJ. Enhancement of Oral Bioavailability of Cilostazol by Forming its Inclusion Complexes. AAPS PharmSciTech. 2009; 10: 660-9.
20. Zhang Y, Huo M, Zhou J, et al. DDSolver: An Add-In Program for Modeling and Comparison of Drug Dissolution Profiles. The AAPS Journal. 2010; 12: 263-71.
21. Zhou S, Feng X, Kestell P, Paxton JW, Baguley BC and Chan E. Transport of the investigational anti-cancer drug 5, 6-dimethylxanthenone-4-acetic acid and its acyl glucuronide by human intestinal Caco-2 cells. Eur J Pharm Sci. 2005; 24: 513-24.
22. Wang Z, Hop CE, Leung KH and Pang J. Determination of in vitro permeability of drug candidates through a Caco-2 cell monolayer by liquid chromatography/tandem mass spectrometry. J Mass Spectrom. 2000; 35: 71-6.
23. Rubas W, Cromwell M, Shahrokh Z, et al. Flux measurements across Caco-2 monolayers may predict transport in human large intestinal tissue. J Pharm Sci. 1996; 85: 165-9.
24. Artursson P and Karlsson J. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem Biophys Res Commun. 1991; 175: 880-5.
25. Artursson P. Cell cultures as models for drug absorption across the intestinal mucosa. Crit Rev Ther Drug Carrier Syst. 1990; 8: 305-30.
26. Wang X-d, Meng M-x, Gao L-b, Liu T, Xu Q and Zeng S. Permeation of astilbin and taxifolin in Caco-2 cell and their effects on the P-gp. Int J Pharm. 2009; 378: 1-8.
27. Li AP. Screening for human ADME/Tox drug properties in drug discovery. Drug Discov Today. 2001; 6: 357-66.
28. Shin S-C, Bum J-P and Choi J-S. Enhanced bioavailability by buccal administration of triamcinolone acetonide from the bioadhesive gels in rabbits. Int J Pharm. 2000; 209: 37-43.
29. Zhang Y, Huo M, Zhou J and Xie S. PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. Comput Meth Prog Biomed. 2010; 99: 306-14.
30. Liversidge GG and Cundy KC. Particle size reduction for improvement of oral bioavailability of hydrophobic drugs: I. Absolute oral bioavailability of nanocrystalline danazol in beagle dogs. Int J Pharm. 1995; 125: 91-7.

31. Sathigari S, Chadha G, Lee YHP, et al. Physicochemical Characterization of Efavirenz–Cyclodextrin Inclusion Complexes. AAPS PharmSciTech. 2009; 10: 81-7.
32. Mukne AP and Nagarsenker MS. Triamterene-beta-cyclodextrin systems: preparation, characterization and in vivo evaluation. AAPS PharmSciTech. 2004; 5: E19.
33. Jug M, Kos I and Bećirević-Laćan M. The pH-dependent complexation between risperidone and hydroxypropyl-β-cyclodextrin. J Incl Phenom Macrocycl Chem. 2009; 64: 163-71.
34. Omari MMA, Zughul MB, Davies JED and Badwan AA. Effect of Buffer Species on the Inclusion Complexation of Acidic Drug Celecoxib with Cyclodextrin in Solution. J Incl Phenom Macrocycl Chem. 2006; 55: 247-54.
35. Zeng J, Ren Y, Zhou C, Yu S and Chen W-H. Preparation and physicochemical characteristics of the complex of edaravone with hydroxypropyl-β-cyclodextrin. Carbohydr Polym. 2011; 83: 1101-5.
36. Kiss T, Fenyvesi F, Kovacsne BI, et al. [Cytotoxic examinations of various cyclodextrin derivatives on Caco-2 cells]. Acta Pharm Hung. 2007; 77: 150-4.
37. Kiss T, Fenyvesi F, Bacskay I, et al. Evaluation of the cytotoxicity of beta-cyclodextrin derivatives: evidence for the role of cholesterol extraction. Eur J Pharm Sci. 2010; 40: 376-80.
38. Kiss T, Fenyvesi F, Pasztor N, et al. Cytotoxicity of different types of methylated beta-cyclodextrins and ionic derivatives. Pharmazie. 2007; 62: 557-8.
39. Veiga MD, Diaz PJ and Ahsan F. Interactions of griseofulvin with cyclodextrins in solid binary systems. J Pharm Sci. 1998; 87: 891-900.
40. Nakai Y, el-Said Aboutaleb A, Yamamoto K, Saleh SI and Ahmed MO. Study of the interaction of clobazam with cyclodextrins in solution and in the solid state. Chem Pharm Bull (Tokyo). 1990; 38: 728-32.
41. Okawara M, Tokudome Y, Todo H, Sugibayashi K and Hashimoto F. Enhancement of diosgenin distribution in the skin by cyclodextrin complexation following oral administration. Bio Pharml Bull. 2013; 36: 36-40.
42. Taupitz T, Dressman JB and Klein S. New formulation approaches to improve solubility and drug release from fixed dose combinations: case examples pioglitazone/glimepiride and ezetimibe/simvastatin. Eur J Pharm Biopharm. 2013; 84: 208-18.

43. Taupitz T, Dressman JB, Buchanan CM and Klein S. Cyclodextrin-water soluble polymer ternary complexes enhance the solubility and dissolution behaviour of poorly soluble drugs. Case example: itraconazole. Eur J Pharm Biopharm. 2013; 83: 378-87.
44. Codruţa M. Şoica1 CIP, Sorina Ciurlea, Rita Ambrus, Cristina Dehelean Physico-chemical and toxicological evaluations of betulin and betulinic acid interactions with hydrophilic cyclodextrins. FARMACIA. 2010; 58(5).
45. Shen Y, Wang M, Zhang L, et al. Effects of hydroxypropyl-beta-cyclodextrin on cell growth, activity, and integrity of steroid-transforming Arthrobacter simplex and Mycobacterium sp. Appl Micro Biotech. 2011; 90: 1995-2003.
46. Shen Y, Liang J, Li H and Wang M. Hydroxypropyl-beta-cyclodextrin-mediated alterations in cell permeability, lipid and protein profiles of steroid-transforming Arthrobacter simplex. Appl Micro Biotech. 2015; 99: 387-97.
47. Yee S. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man--fact or myth. Pharm Res. 1997; 14: 763-6.
48. Sigfridsson K, Forssén S, Holländer P, Skantze U and de Verdier J. A formulation comparison, using a solution and different nanosuspensions of a poorly soluble compound. Eur J Pharm Biopharm. 2007; 67: 540-7.
49. Lin S-Z, Wouessidjewe D, Poelman M-C and Duchêne D. Indomethacin and cyclodextrin complexes. Int J Pharm. 1991; 69: 211-9.
50. Guyot M, Fawaz F, Bildet J, Bonini F and Lagueny AM. Physicochemical characterization and dissolution of norfloxacin/cyclodextrin inclusion compounds and PEG solid dispersions. Int J Pharm. 1995; 123: 53-63.
51. Soliman OAE, Kimura K, Hirayama F, et al. Amorphous spironolactone-hydroxypropylated cyclodextrin complexes with superior dissolution and oral bioavailability. Int J Pharm. 1997; 149: 73-83.