Chapter 5: Experimental Details (Materials and Methods)

5.1 Materials

The following materials were used in the present study and details of the supplier is given in the Table 5.1.

Table 5.1: List of chemicals used in the experiments

S. No.	Materials	Suppliers
1	Acetonitrile (HPLC Grade)	SD Fine Chem. Ltd.
2	Butyl hydroxy toluene	Hi-Media
3	Caco-2 cell line	NCCS, Pune
4	Castor oil	SD Fine Chem. Ltd.
5	Cholecalciferol	Hi-Media
6	Cremophore EL	Fisher scientific
7	Dulbecco's Phosphate Buffered Saline	Hi-Media
8	Eagle's Minimum Essential Medium (EMEM)	Hi-Media
9	Ethanol	SD Fine Chem. Ltd.
10	FBS	Invitrogen Corporation
11	Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich
12	HCl	Rankem
13	HPMC capsules	ACG Capsules, Mumbai, India
14	Hydroxypropylmethyl cellulose acetate succinate	Ashland Industries, Switzerland
15	Lactose monohydrate	Hi-Media
16	MTT dye	Sigma-Aldrich
17	Oleic acid	Himedia

18	Pancreatin	Sigma-Aldrich
19	Phosphate Buffer Saline	Hi-Media
20	Penicillin-streptomycin	Thermo-Scientific
21	Pepsin	Sigma-Aldrich
22	Poloxamer	Sigma-Aldrich
23	Polyethylene glycol	Fisher Scientific
24	Polyvinylpyrrolidone PVP K-30	SD Fine Chem. Ltd.
25	Sodium bicarbonate	Rankem
26	Sodium lauryl sulphate	Hi-Media
27	Tris-buffered saline (TBST)	Hi-Media, India
28	Trypsin-EDTA	Sigma-Aldrich
29	Water (HPLC Grade)	SD Fine Chem. Ltd.

5.2 Equipments

The following equipment were used in the experiments, unless otherwise stated in respective section.

Table 5.2: List of equipment used in the experiments

S.	**Equipments**	**Model**	**Manufacturer**
1.	Centrifuge machine	3-30k	Sigma
2.	Magnetic stirrer	308B	Tarsons
3.	Transmission electron	1500	JEOL
4.	Auto fine coater	JEC-3000FC	JEOL
5.	Dissolution apparatus	DS 8000	Lab India
6.	HPLC	Schimadzu UFLC	Schimadzu
7.	Sonicator	T1-H-15	ELMA Transonic

8.	Particle size analyzer	MPT-2 (Version- 6.02)	Malvern
9.	UV plate reader	Multiskan GO Type 1510	Thermofisher
10.	Electric balance	JB-1603-C	Meter Toledo
11.	Vortexer	Vortex Genie-2	Scientific Industries
14.	FT-IR spectrometer	Spectrum two FT-IR	Perkin Elmer

5.3 Preformulation Studies of Bioactive (Cholecalciferol)

Preformulation studies are preliminary studies that are important to determine those physicochemical properties of drug that could affect the formulation development concomitantly with drug efficacy.

5.3.1 Determination of solubility

The solubility of cholecalciferol in PBS (phosphate buffer saline, pH 7.4) was determined by shake flask technique (Konsoula and Jung, 2008). The saturated solutions of cholecalciferol were prepared in PBS pH 7.4, which were then bath sonicated (Elma/DE-78224, Singen) for 5min and maintained at 37 °C in shaking water bath (New Brunswick Scientific, USA) at 120 RPM for 24 h. Subsequently, samples were centrifuged (Sigma, 3K30, Germany) at 12,000 RPM for 10 min and the absorbance of sample was taken at 265 nm.

5.3.2 Determination of partition co-efficient

The partition coefficient can be represented as the ratio of unionized molecule distributed between the organic and aqueous phase.

$$P\ o/w = \frac{C\ (organic)}{C\ (aqueous)}$$

The partition profile of cholecalciferol was determined in n-octanol: water and n-octanol: PBS (pH 6.8) system. The partition coefficient of cholecalciferol between

octanol and buffers/water was determined at by the shake flask method (Konsoula and Jung, 2008). The weighed amounts of the cholecalciferol were mixed in octanol saturated PBS and were shaken for 4 h in water bath shaker. Subsequently, two phases were separated by centrifugation and the amount of the cholecalciferol in the organic phase and the buffers were estimated by HPLC technique.

5.3.3. Evaluation of melting point

The melting point of cholecalciferol was evaluated by melting point apparatus.

5.4 Methods for the Determination of Cholecalciferol

5.4.1 Preparation of standard curve of cholecalciferol in PBS

One mg of cholecalciferol was dissolved in 50 µL of ethanol and cholecalciferol primary stock solution of 1000 µg/mL was prepared in PBS 7.4 by making up the volume 1ml with addition of 950 µL PBS 7.4. From this stock a secondary stock solution of 20 µg/mL was prepared with PBS 7.4. Aliquots of 100 µL, 200 µL, 300 µL, 400 µL, 5000 µL, 600 µL, 700 µL, 800 µL, 900 µL, 1000 µL were pippeted out into series of 2 mL eppendorfs and volume was made up to 1 mL with PBS 7.4 in order to get concentrations in the range of 2-20µg/ml. The absorbance of the resulting solution was then measured at 265 nm by UV plate reader. The standard curve was obtained by plotting absorbance vs concentration.

5.4.2. Preparation of standard curve of cholecalciferol in ethanol

cholecalciferol primary stock solution (1000 µg/mL) was prepared in ethanol. From this stock a secondary stock solution (100 µg/mL) was prepared with ethanol. Aliquots of 50 µL, 100µL, 150 µL, 200 µL, 250 µL, 300 µL, 350 µL, 400 µL, 450 µL, 500 µL were pippeted out into series of 2ml eppendrofs and volume was made up to 1 mL with ethanol in order to get solution of different known concentrations. The absorbance of the resulting solution was then recorded at 265 nm by UV plate reader. The standard curve was obtained by plotting absorbance Vs concentration.

5.4.3. HPLC method for determination of cholecalciferol

The concentration of cholecalciferol was determined by a previously reported method (Temova and Roskar, 2016) using a HPLC method (Shimadzu, Japan), which comprised of a DGV-20A degasser, LC-20AD pump, SIL-20AHT autosampler, SPD-M20A PDA detector and a computer installed with LabSolutions software. Separation was done by using a HPLC column (RP-18 endcapped 100-4.6 mm) with the mobile phase comprising of acetonitrile and water at a volume ratio of 99:1. The rate of flow of mobile phase was 1 mL/min and all chromatographic separations were performed at 40˚C and the wavelength of detection was set at 265 nm.

5.5 Strategy I: Development and Characterization of Enteric Polymer-based Solid Dispersion for Cholecalciferol Delivery

5.5.1 Preparation of HPMCAS based solid dispersion

HPMCAS based enteric solid dispersion of cholecalciferol was fabricated using solvent evaporation technique. Crystalline cholecalciferol and polymers were dissolved individually in solvents (i.e., cholecalciferol (1.5 mg) and polaxamer 407 (90 mg) in 2mL of ethanol, PVP-K30 (450 mg) in 3mL of ethanol and HPMCAS (450 mg) in 3ml of mixed solvent (1:1 v/v) in ethanol: dichloromethane). Subsequently, a solution of polaxamer 407 with cholecalciferol was mixed with ethanolic PVP-K30 solution and at last HPMCAS solution was added in a beaker with constant stirring at room temperature until complete evaporation of the solvents (Table 5.3). The prepared formulation was placed under desiccator for 48 h to dry completely. After drying, the product was scrapped out carefully from the beaker, grounded in mortal pestle and then passed through sieve mesh no. 30 (Rawat N. *et al.,* 2023b). Similarly, a formulation

comprising cholecalciferol and PVP-K30 was also prepared for comparative studies (Table 5.3).

Table 5.3: Composition of cholecalciferol solid dispersions (CCF-SD-HPMCAS, CCF-SD-PVP).

Constituents	Amount (CCF-SD-HPMCAS)	Amount (CCF-SD-PVP)
Cholecalciferol	1.5 mg	1.5 mg
PVP-K30	450 mg	450 mg
Poloxamer	90 mg	90 mg
HPMC-AS	450 mg	-

5.5.2 Production of physical mixture

The physical mixture of cholecalciferol with carrier polymers was produced in the same proportion as in solid dispersion with porcelain mortar pestle to ensure a uniform product and sieved through sieve mesh 30 and stored in air-tight container.

5.5.3 Determination of practical yield

Practical yield (%) is estimated to investigate the efficacy of the preparation method. Prepared enteric solid dispersion-based formulations were collected and weighed to calculate the practical yield by using the formula given below.

$$\% \text{ Practical yield } = \frac{\text{Practical mass (solid dispersion)}}{\text{Theoretical mass}} \times 100$$

5.5.4 Determination of cholecalciferol concentration in solid dispersion

Cholecalciferol concentration was estimated by dissolving 100 mg of formulation, solid dispersion (equivalent to 0.15 mg of cholecalciferol) in 1 mL of mobile phase (acetonitrile: water = 99:1) and the solution was vortexed for 20 min, centrifuged and filtered. The concentration of cholecalciferol was determined at 265 nm using a HPLC

system (Shimadzu Japan). The separation was achieved by using an Chromolith® Performance RP-18 end-capped 100-4.6 mm HPLC column. The rate of flow of the mobile phase was 1ml/min and all chromatographic separations were performed at ambient temperature. The drug content was obtained by using the following equation:

$$\text{Drug content (\%)} = \frac{\text{Actual amount of CCF content present in the solid dispersion}}{\text{Theoretical amount of CCF in solid dispersion}} \times 100$$

5.5.5 Fourier transform–infra red spectroscopy (FT-IR)

FT-IR spectrum of cholecalciferol, polymers, physical mixture, and solid dispersion were recorded by FT-IR instrument (Perkin Elmer Spectrum Two FT-IR Spectrometer version 10.03.06) using KBr pellet method. Briefly, 5 mg of samples were uniformly triturated with 100 mg KBr and KBr discs were fabricated by compacting the mixture under a pressure of 5 tons for 5 min in a manual FTIR KBr pellet press. The FTIR machine was functioned under purging of dry air at scanning range of 4000-600 cm^{-1}.

5.5.6 Scanning electron microscopy (SEM)

Surface morphology of cholecalciferol, polymer and solid dispersion-based formulations was investigated by using a scanning electron microscope (JEOL, Japan). The solid dispersion and other samples were directly fixed on a brass stub with the help of double-sided adhesive tape and subsequently, sputter coated (JEOL JC 3000FC Autofine coater) with platinum-palladium alloy layer (approx. 3-5nm) for 150 seconds at 30W, to make it electrically conductive, and the images are obtained through secondary electron detector at accelerating voltage ranging from 10kV-15 kV.

5.5.7 Differential scanning calorimetry analysis

Thermal behaviour of cholecalciferol and its solid dispersion was investigated by using differential scanning calorimetry. The samples were accurately weighed, placed in

sealed aluminum pans (without pin hole). Subsequently, the samples were heated under nitrogen flow (30 mL/min) with a rate of scanning at 10˚C min^{-1} from 20 to 220˚C.

5.5.8 Characterization of solid dispersion using X-ray diffraction analysis

The investigation of X-ray diffraction profile of cholecalciferol and solid dispersion powder was carried out using a diffractometer. The diffraction patterns were obtained at RT with the help of a D/Max2200 Ultima/PC (Rigaku Miniflex 600) with Ni filtered Cu-Kα radiation powered at 40 kV and 40 mA. The solid dispersion powder was scanned in steps 0.02˚ per sec from 5˚ to 50˚ (diffraction angle 2θ).

5.5.9 Effect of formulation on viability of mimics of intestinal cells (caco-2 cells)

In the present study, the solid dispersion formulation comprises polymers and surfactant and the formulation is intended for oral administration. Therefore, the influence of solid dispersion excipient on the viability of the mimics of intestinal cells (Caco-2 cells) was determined. Caco-2 cells are commonly used as a model system for screening of intestinal absorption and cytotoxicity (Sha *et al.,* 2005). The effect of the formulations on the viability of Caco-2 cells was assessed by using MTT assay. This assay relies on the reduction of a yellow tetrazolium salt (MTT) which is related to cellular metabolic activity as a signal for cellular viability. Caco-2 cells were grown on 75 cm^2 plastic culture flasks (NUNC) in EMEM with Earle's salts, L-glutamine and nonessential amino acids containing HEPES (2.38 g/L), sodium bicarbonate (2 g/L), supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 µg/mL). Caco-2 cell lines were incubated at 37ºC inside the incubator with 5% CO_2 and 95% relative humidity. Caco-2 cells were used between passages 40 and 45 in the experiment. The cells were routinely maintained and trypsinized in T75 tissue culture flasks.

To evaluate the effect of the formulation on viability of Caco-2 cells, the same were seeded in tissue culture plates (density $2x10^4$ cells/well) and subsequently permitted to grow in a monolayer inside the humidified CO_2 incubator for 96 h and the media was changed at an alternate day. Before the treatment, the exhausted media was removed. Once the cells formed a monolayer in a 96 well plates, different concentrations of formulations in HBSS solution were added to each well and kept for 2 h incubation. Thereafter, 20 µL of MTT solution (2.5 mg/mL) prepared in cold PBS was mixed in each well. Subsequently, cells were again incubated for another 4 h to form formazan crystals. The obtained formazan crystals were solubilized by adding 150 µL of DMSO using an orbital shaker and the absorbance was recorded at 570 nm with the help of a microplate reader and percent viability was calculated.

5.5.10 Dissolution study

The *in vitro* dissolution of the developed solid dispersion formulations was conducted using two-stage dissolution (Li *et al.*, 2021) using a dissolution apparatus (Lab India DS 8000). The preparation of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were done as reported previously (Marques *et al.*, 2011; Vertzoni *et al.*, 2004). The experiment was performed using a paddle apparatus operated at 100 rpm and the dissolution media was kept at 37±0.5˚C. In the first stage, dissolution was conducted in SGF medium (250 mL) and subsequently double strength SIF media (250 mL) was added for the second stage of experiment. The dissolution studies were performed for cholecalciferol, physical mixture and solid dispersion. The cholecalciferol release was estimated by removing 2 mL samples at different time intervals, followed by filtration through 0.45 µm PVDF membrane filter and replacing with an equal volume of dissolution medium. The collected samples were analyzed by HPLC at 265 nm.

5.5.11 Determination of relative bioavailability of cholecalciferol

The relative bioavailability of cholecalciferol and its developed formulations were determined in Wistar rat using a previously described method (Ibrahim *et al.*, 2013). Wistar rats (160–200 g) were divided into three groups (8 animals per group). Prior to the administration of experimental samples, the blood samples from each rat were collected to get the serum. The determination of the cholecalciferol concentration in these samples represent control level ("0 day") of animal. The first group received plain cholecalciferol (dispersed in 2% NaCMC), the second received cholecalciferol solid dispersion (CCF-SD-PVP) and third group received cholecalciferol enteric solid dispersion (CCF-SD-HPMCAs). In the given test samples 40,000 IU/kg dose of cholecalciferol was administered in animal. After the dosing, the blood samples were collected after 1, 2, 4, 6, 8, 24, 34, and 48 h. After collection of blood, the serum was separated by centrifugation of sample at 6000 rpm for 15 min and used for the determination of cholecalciferol concentration using HPLC method.

5.5.12 Stability study

The solid dispersion formulations were placed in sealed containers and stability study was performed by storing the samples at 4˚C in refrigerator, 25˚C/60%RH, and 30˚C/65%RH for three months. The samples were analysed every month for drug content.

5.5.13 Statistical analysis

The data are represented as mean ± standard deviation. The statistical analysis of data was performed by using one-way analysis of variance (ANOVA) and Student's *t*-test.

A *p*-value <0.05 was designated as statistically significant and level of significance is shown as *** for P<0.001, ** for P<0.01, and * for P<0.05.

5.6 Strategy II: Development and Characterization of Delayed Release HPMC Capsules (DRHCap) for Efficient Delivery of Cholecalciferol Solid Dispersion

5.6.1 Preparation of DRHCap-SD of cholecalciferol

The solid dispersion of cholecalciferol in PVP-K30 comprising various weight ratios (1:200, 1:400, 1:600, 1:800 of CCF: PVP K-30, designated as SD1, SD2, SD3 and SD4, respectively) were prepared by solvent evaporation method (Table 5.4). Required quantity of cholecalciferol and PVP K-30 was separately dissolved in the minimum amount of ethanol. The solution containing cholecalciferol was poured in solution containing PVP K-30 and the system was mixed using magnetic stirrer. After 20 min of mixing, poloxamer (PVP: poloxamer = 10:1) was added slowly to the solution mixture and solvent was evaporated and prepared formulation was placed under desiccators for 48 h to dry completely. After drying the hardened mixtures was pulverized with porcelain pestle and sieved through mesh #18. The solid dispersion (SD-PVP) was filled in delayed release HPMC capsule and subsequently the product (DRHCap-SD) was placed in air tight container (Rawat N. *et al.*, 2023a).

Table 5.4: Formulation composition of solid dispersion formulation for DRHCap-SD

S. No.	SD code	Cholecalciferol: PVP	PVP: Poloxamer
1	SD1	SD1 (1:200 w/w)	10:1
2	SD2	SD2 (1:400 w/w)	10:1
3	SD3	SD3 (1:600 w/w)	10:1
4	SD4	SD4 (1:800 w/w)	10:1

5.6.2 Physical mixture preparation

The preparation of physical mixture of cholecalciferol with carrier polymers was carried out in same proportion as in solid dispersion with porcelain mortar pestle to ensure uniform product and sieved through mesh #18 and stored in air tight container.

5.6.3 Percentage practical yield of SD-PVP

The practical yield (%) is estimated to investigate efficacy of method. Prepared solid dispersions-based formulation was collected and weighed to estimate practical yield by employing the formula given below.

$$\text{Practical yield (\%)} = \frac{\text{practical mass of solid dispersion}}{theoretical\ mass} \times 100$$

5.6.4 Determination of cholecalciferol content in SD-PVP

The cholecalciferol content was estimated by solubilizing 100 mg of formulation in 1mL of mobile phase (acetonitrile: water = 99:1) and the solution was vortexed for 20 min and then centrifuged, filtered and concentration of cholecalciferol was determined at 265 nm using a HPLC method (Temova and Roskar, 2016). Separation was achieved by using an Chromolith® Performance RP-18 endcapped 100-4.6 mm HPLC column. The mobile phase flow rate was 1 mL/min and all chromatographic separations were performed at ambient temperature. The drug content was obtained by using following equation:

$$\text{Drug content (\%)} = \frac{\text{Actual amount of CCF content present in the solid dispersion}}{\text{Theoretical amount of CCF in solid dispersion}} \times 100$$

5.6.5 Solubility determination of cholecalciferol in SD-PVP

Excess amount of cholecalciferol and its solid dispersion were dissolved in 1 mL of PBS buffer 7.4 with continuous vertexing and incubated for 24 h in water bath (37±0.5

°C). Thereafter, samples were subjected to centrifugation at 10,000 g for 10 min and supernatant were analyzed using HPLC at 265 nm.

5.6.6 FT-IR spectroscopy of cholecalciferol, SD-PVP and excipients

FT-IR spectra of cholecalciferol, polymers and solid dispersion were obtained using FT-IR (Perkin Elmer) using KBr pellet method. Briefly, 5 mg of samples were uniformly triturated with 100 mg KBr and KBr discs were obtained using concentional method. The instrument performed under dry air purge at 4000-400 cm^{-1} scanning range.

5.6.7 Morphological examination using scanning electron microscopy

Surface characteristics of cholecalciferol, PVP-K30 and solid dispersion was examined by using SEM (JEOL, Japan). Formulations and excipients were separately fixed on a brass stub and were sputter coated (JEOL JC 3000FC Autofine coater) with platinum-palladium alloy layer (approx. 3-5nm) for 150 sec at 30W, to made it electrically conductive, and images are obtained through secondary electron detector at accelerating voltage ranging from 10KV-15 KV.

5.6.8 Differential scanning calorimetry analysis

Thermal behaviour of cholecalciferol and its solid dispersion was investigated by using differential scanning calorimetry. Weighed quantity of samples were kept in aluminum pans and heating was done under the influence of nitrogen flow (30ml\min) with a rate of scanning at 10 °C min^{-1} from 20 to 220 °C.

5.6.9 X-ray diffraction analysis

XRD analysis of powder samples was conducted using a diffractometer. The diffraction patterns were recorded using a Bruker D8 venture diffractometer system (XPERT-

PRO) with INCOATEC microfocus X-ray source (Mo Kα) at 45 kV and 40 mA. Various samples were scanned with scan step time 10.16 sec from 5° to 80° (diffraction angle 2θ) at measurement temperature of 25 °C.

5.6.10 Effect of formulation of viability of Caco-2 cells

In this study, the solid dispersion of cholecalciferol comprises surfactant in addition to other excipients and the formulation is intended for oral administration. Therefore, influence of formulation on the activity of the Caco-2 cells was determined. Caco-2 cells are widely explored as a model system for screening of intestinal absorption and cytotoxicity (Sha *et al.,* 2005). The effect of the formulations on the activity of Caco-2 cells was investigated by MTT test. Caco-2 cells were grown on 75 cm^2 plastic culture flasks (NUNC) in EMEM with Earle's salts, L-Glutamine and nonessential amino acids containing HEPES (2.38 g/L), sodium bicarbonate (2 g/L), FBS (10%), penicillin (100 IU/mL) and streptomycin (100 µg/ml). Caco-2 cell lines were incubated at 37 °C inside the incubator with CO_2 (5%) and relative humidity (95%). Caco-2 cells were used between passages 40 and 45 in the experiment. The cells were routinely maintained and trypsinized in T75 tissue culture flasks.

In order to estimate the effect of the formulation on viability of Caco-2 cells, the same were seeded ($2x10^4$ cells/well) inside the humidified CO_2 incubator for 96 hr and the media was changed at an alternate day. Before the treatment, the exhausted media was removed. Once the cells formed a monolayer in a 96 well plates, different concentrations of formulations in HBSS solution were added to each well and kept for 2 hr incubation. Thereafter, 20 µL of MTT solution (2.5 mg/mL) prepared in cold PBS was mixed in each well and the cells were again incubated for another 4 h to form formazan crystals. The formazan crystals were dissolved by adding 150 µL of DMSO

under mild agitation and the absorbance was recorded at 570 nm and percent viability was calculated.

5.6.11 Dissolution study

The *in vitro* dissolution study of the solid dispersion encapsulated in delayed release HPMC capsule was conducted using two-stage dissolution (Li *et al.,* 2021) using a dissolution apparatus (Lab India DS 8000) and dissolution profile was compared with native solid dispersion, physical mixture and cholecalciferol. The preparation of biorelevant media (SGF and SIF) were performed as described earlier (Marques *et al.,* 2011; Vertzoni *et al.,* 2004). The experiment was performed using a paddle apparatus operated at 100 rpm and the dissolution media was kept at 37±0.5°C. In the first stage, dissolution was conducted in simulated gastric fluid (SGF) medium (250 mL) and subsequently double strength simulated intestinal fluid (SIF) media (250 mL) was added for the second stage of experiment. Dissolution profile was estimated by removing 2 mL samples at different time intervals, followed by filtration using 0.45 µm PVDF filter and equal volume of dissolution medium was replaced. The collected samples were estimated for cholecalciferol by HPLC at 265 nm.

5.6.12 Flow properties

Cholecalciferol SDs and its physical mixture were evaluated for flow properties. Angle of repose was evaluated by passing the samples to flow through funnels and the angle between horizontal surface and slope of the heap of samples was noted. Bulk density and tapped density (after 50 times tapping) were determined using conventional method and Carr's compressibility Index (CI), angle of repose (Ɵ) and Hausner's Ratio were calculated according to following formulas:

$$\Theta = tan^{-1}\frac{h}{r}$$

Where, h represents height and r represent radius of the powder pile.

$$\text{Compressibility index} = \frac{(tapped\ density - bulk\ density)}{tapped\ density} \times 100$$

$$\text{Hausner's ratio} = \frac{tapped\ density}{bulk\ density}$$

5.6.13 Stability study

The DRHCap-SD of cholecalciferol and solid dispersion formulations were placed in sealed containers and stability study was performed by storing the samples at 4 °C (refrigerator), 25°C (60%RH), and 30°C (65%RH) for three months. Formulations were removed from stability chamber after every month and analyzed for drug content.

5.6.14 Statistical analysis

The data are shown as mean ± S.D. The statistical analysis of data was performed by using ANOVA and Student's *t*-test. A *p*-value <0.05 was designated as statistically significant and level of significance is shown as *** for P<0.001, ** for P<0.01, and * for P<0.05.

5.7 Strategy III: Development and Characterization of Self-emulsifying Drug Delivery Systems (SEDDS) for Cholecalciferol

5.7.1 Selection of oil, surfactant and co-surfactant

The solubility profile of cholecalciferol in a number of oils, surfactants, and co-surfactants was evaluated by mixing an excess quantity of cholecalciferol in 0.5 ml of each vehicle. After capping the tubes, the mixtures were shaken at 25˚C for 24 hrs on water bather shaker. Subsequently, samples were subjected to centrifugation at 3000 rpm for 15 min and then the supernatant was obtained which was filtered through

0.45µm Teflon syringe filter and diluted with ethanol. The solubility of cholecalciferol in supernatant was estimated by UV spectrophotometer at 265 nm.

5.7.2 Preparation of pseudo-ternary phase diagram

Ternary phase diagrams were generated to select an appropriate composition of excipient on the basis of micro emulsion region. Based on the solubility studies the selected oil, surfactant and co-surfactant were oleic acid, Cremophor EL and polyethylene glycol 400, respectively. These excipients were further used at different concentration (Table 5.5) to obtain pseudo-ternary phase diagram to identify good emulsification region and various composition were evaluated for tendency to form emulsion (self-emulsification), phase separation, density of globules and uniformity in globules. The emulsion forming tendency of composition was determined by dispersing 100 µL of formulation into 40 mL of purified water. Further, visual inspection of phase separation in emulsion with selected composition was determined by observing the emulsion at 5 min and 60 min end points.

5.7.3 Development of self-emulsifying drug delivery system (SEDDS)

On the basis of phase diagram, three compositions have been selected and evaluated for self-emulsification (Table 5.6). The formulations were prepared by incorporating cholecalciferol (1.5 mg) in the specified mixture of oleic acid, Cremophor EL and polyethylene glycol 400 with continued stirring to get homogenous phase. The cholecalciferol loaded SEEDS formulations were characterized on the basis of different variables including emulsification tendency, precipitation of drug, phase separation, density of globules and globules uniformity.

Table 5.5: Various composition for the selection of best micro-emulsion region

Oil+S+CS*	**Oil+S+CS**	**Oil+S+CS**
70+30+0	65+35+0	60+40+0
70+25+05	65+30+05	60+35+05
70+20+10	65+25+10	60+30+10
70+15+15	65+20+15	60+25+15
70+10+20	65+15+20	60+20+20
70+05+25	65+10+25	60+15+25
	65+05+30	60+10+30
		60+05+35
*Oleic acid (oil), Cremophor (S: surfactant) and polyethylene glycol (CS: co-surfactant)		

Table 5.6: Composition of selected SEDDS formulations

Formulations	**Oil (%)**	**Cr EL (%)**	**PEG (%)**	**Cholecalciferol (mg)**
SF-1	70	20	10	1.5
SF-2	65	15	20	1.5
SF-3	60	20	20	1.5

5.7.4 Characterization of self-emulsifying drug delivery system

5.7.4.1 Evaluation of self-emulsification

SEDDS result in formation of fine oil-in-water (O/W) microemulsions when mixed with aqueous media and subjected to mild agitation. It is well established that as the free energy requirement for the formation of a microemulsion is very low, therefore self-emulsifying drug delivery system formation is thermodynamically spontaneous (Kim *et al.*, 2000). The spontaneity of microemulsion formation can be evaluated simply by visual examination. Evaluation of the self-emulsifying characteristics of self-emulsifying drug delivery system was conducted by visual examination. SEDDS formulation (50 µL) was mixed with distilled water (50 mL) with gentle magnetic stirrer at 120 rpm and 37˚C temperature. The self-emulsification time and appearance of globule under the microscopic was observed.

5.7.4.2 Morphological examination by transmission electron microscopy

Morphological examination of SEEDS was performed by transmission electron microscopy (TECNAI G^2 20 Twin, FEI, Netherland). SEEDS formulation was suitably diluted with aqueous media (1:20) followed by mild agitation. Subsequently, the diluted SEDDS formulation sample was kept on the copper grids, stained in 2% phosphotungstic acid solution and microscopic image was captured.

5.7.4.3 Determination of average particle diameter and zeta potential of SEDDS

The average size of the SEDDS droplet obtained by the mixing of SEDDS concentrate with aqueous media (1:20 *v/v*) was determined by Zetasizer (Malvern Instruments) with dynamic light scattering at a scattering angle of 90^oat 25 ^oC. Zeta potential of the emulsion formed after dilution (1:20 *v/v*) was measured using Zetasizer Nano ZS (Malvern Instruments).

5.7.4.4 Drug content

The drug content was determined by dispersing 100 µL of SEDDS in 1mL of mobile phase comprising acetonitrile and water (99:01). The solution was then sonicated for 10 min to release the encapsulated drug in mobile phase. Centrifugation at 10000 rpm for 10 min was allowed and supernatant was analyzed for drug concentration using HPLC (Shimadzu UFLC) at 265 nm.

5.7.5 *In-vitro* stability assessment in SGF and SIF

Effect of pH and robustness to dilution was determined by subjecting SEDDS formulation to various dilution such as 50, 100 and 1000 times with SGF (pH 1.2) and SIF (pH 6.8). The obtained diluted microemulsions were examined for changes in physical parameters including coalescence of droplets, precipitation or phase separation after 2h incubation.

5.7.6 *In vitro* dissolution study

The dissolution profile of cholecalciferol was investigated in SEDDS formulation containing 1.5 mg of cholecalciferol. This experiment was conducted using a USP II paddle type dissolution apparatus (Lab India DS 8000). The SEDDS formulations and equivalent amount of cholecalciferol were filled separately in a capsule and kept in 250 mL of SGF (pH 1.2) kept at 37±0.5˚C. The dissolution paddle speed was maintained at 50 rpm. For the analysis of dissolution profile, a sample of 5 mL was taken out from the dissolution medium at a scheduled time period. An equal volume of fresh medium kept as similar condition was added to the dissolution medium. The samples (5 mL) obtained were filtered (0.45 µm membrane filter) and estimated for cholecalciferol content using UV spectrophotometer at 265 nm.

5.7.7 *In-vitro* bio-accessibility

The bioaccessibility is an important characteristic of the SEDDS formulation and for hydrophobic material it is represented as the amount that is solubilized within the mixed micelle phase following digestion of lipid. The bioassessibility of cholecalciferol using SEDDS in gatro-intestinal tract (GIT) or more specifically, the amount of cholecalciferol available for absorption after the lipid digestion from the formulation was determined using a two-stage GIT model consisting of a gastric and small intestinal phase (Ozturk *et al.*, 2015).

Gastric phase: SGF was prepared by mixing 200 mg of NaCl and 700 µL of HCl and 379 mg of pepsin in water (100 mL) and the pH was maintained to 2.5 using 1 M HCl. The formulation was exposed to 30 mL of SGF for 2 h (37^{o}C, 100 rpm).

Intestinal phase: Sample from gastric phase was initially incubated for 10 min at room temperature and pH was adjusted to 7.0. Bile solution (187.5 mg of sodium

deoxycholate in 4ml phosphate buffer, pH 7) and calcium chloride solution (110 mg $CaCl_2$ in 1mL phosphate buffer, pH 7) was subsequently added into the sample. Finally, the enzyme solution (60 mg of lipase in 2.5 mL phosphate buffer pH 7.0) was added for complete digestion of lipids. The incubation period for intestinal phase was 2 h.

The bioaccessibility was determined by comparing the amount of drug present in the raw digest and micelle phase which was obtained previously by exposing formulation to GIT model. Micelle phase was obtained by centrifugation of sample from the intestinal stage (4000 rpm, Sigma K-30 centrifuge) at 25˚C for 40 min. On visual inspection, the solution was observed to be separated in three distinct phases including an opaque sediment phase at the bottom, thereafter a clear micelle phase in middle and there was a thin creamed phase at top or along the centrifuge tube wall. Subsequently, a portion (1 ml) of the micelle phase was then withdrawn and samples were then mixed with a solution of ethanol and water (9:1) for extraction of cholecalciferol from the micelle phase. Similar, extraction procedure was applied in case of raw digesta (raw digesta was the pure SEDDS formulation which was not treated by any of stomach or intestinal stage media). The extraction procedure was then preceded with centrifugation at 1750 rpm for another 10 min. The supernatant was collected and concentration of cholecalciferol was estimated using HPLC. The biaccessibility of cholecalciferol was calculated using formula given below:

$$\text{Bioaccessibility} = \frac{\mathbf{C}\ \text{micelle}}{\mathbf{C}\ \text{raw digesta}} \times 100$$

Where C micelle and C raw *digesta* represent the concentration of cholecalciferol in micelle fraction and raw digesta determined after digestion experiment.

5.7.8 Evaluation of compatibility of the formulation with the caco-2 cells

Caco-2 cell line were grown on 75 cm^2 plastic culture flasks (NUNC) in EMEM containing 25 mM d-glucose, 25 mM HEPES, 3.7g/L sodium bicarbonate, supplemented with fetal bovine serum (10% v/v), non-essential amino acid solution (1% v/v), l-glutamine (1% v/v), 100 IU/mL penicillin (100 IU/mL) and streptomycin (100 µg/ml). The cells were incubated at 37^0C inside the humidified carbondioxide incubator with CO_2 (5%) and relative humidity (95%). Caco-2 cells were used between passage 30 and 40 in the experiment. The cells were routinely maintained and trypsinized in T75 tissue culture flasks.

Cell viability was determined by using MTT assay in Caco-2 cells. Caco-2 cells were seeded separately in 96-well flat bottom tissue culture plates (NUNC) at a density of $2x10^4$ cells/well and allowed to grow in monolayer inside the humidified CO_2 incubator for 96 h. Before starting the treatment, again exhausted media were removed. Once the cells formed monolayer, treatment was given in 96 well plate with 100µL of SEDDS formulation diluted in HBSS solution and kept for 2 h incubation. After 2 h treatment, 20 µL of MTT solution (2.5mg/mL) prepared in cold PBS was added in each well and the cells were again incubated for another 4 h to form formazan crystals (Sha et al., 2005). The formazon crystals were dissolved by adding 150 µL of DMSO using an orbital shaker and absorbance was recorded at 570 nm by a microplate reader and percent viability was determined using the following formula (Kushwah *et al.*, 2017).

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100$$

5.7.9 Stability study

The SEDDS formulation capsule were placed in sealed containers and stability study was performed by storing the samples at 4˚C in refrigerator, 25˚C/60% RH, and 30˚C/65% RH for 3 months. The samples were analysed every month for drug content.

5.7.10 Statistical analysis

The data are presented as mean ± standard deviation. The statistical analysis of data was performed by using one-way analysis of variance (ANOVA) and Student's t-test. A *p*-value <0.05 was designated as statistically significant and level of significance is shown as *** for P<0.001, ** for P<0.01, and * for P<0.05.