7. Summary and Conclusion

7.1 Summary

The deficiency of Vitamin D is gaining immense recognition as a serious health issue leading to a variety of health issues (Holick MF, 2012a; Hossein and Holick 2012). This vitamin is synthesized in the skin following the exposure of sunlight or this micronutrient can also be obtained from food sources (Hickey and Gordon, 2004). Food based options for this vitamin is scanty and deficiency of vitamin D has been recognized in many countries (Gessner et al., 2003; Rucker et al., 2002). Limited exposure to the sunlight and sedentary lifestyle is the main factors associated with the deficiency of vitamin D. The deficiency of vitamin D is linked with an increased susceptibility towards many ailments such as diabetes, cancers, cognitive decline and depression (Smit et al., 2012; Holick MF 2012b). Vitamin D is an essential micronutrient owing to its ability to maintain calcium and phosphorus concentrations at the desired level by improving the ability of the intestine to absorb calcium and phosphorus from the food sources. The vitamin D deficiency leads to rickets, osteomalacia, hyperparathyroidism, and osteoporosis (Thacher et al., 2011; Kennel et al., 2010). Cardiovascular mortality has also been reported in type 2 diabetes mellitus patients with vitamin D deficiency (Joergensen et al., 2010). The regular dose of vitamin D (about 2000 IU/d) was reported to reduce the risk of developing type 1 diabetes as its low levels are associated with insulinemia and glucose intolerance (Schwalfenberg G. 2008). The potential of vitamin D derivatives as an antitumor molecule has been documented owing to their property of hampering angiogenesis (Deeb et al., 2007). Keeping all these aspects into considerations, the intake of vitamin D has become essential.

Vitamin D is a hydrophobic micronutrient and it is a necessary component in the human diet for the good health of the individual. Two main chemical versions of vitamin D include vitamin D2, called as ergocalciferol and D3, called as cholecalciferol (Luo et al., 2012). Cholecalciferol, which is generally formed in the skin following sunlight exposure, is more potent that ergocalciferol. Depending on the environmental condition, cholecalciferol may find different chemical versions including calciol, calcidiol, and calcitriol. The active form of cholecalciferol is calcitriol (chemically known as 1, 25-dihydroxyvitamin D3), which is vital for calcium and phosphorus homeostasis, bone metabolism, blood pressure, reabsorption of calcium in the kidney, and secretion of insulin (Gonnet et al., 2010).

The insufficient exposure to sunlight and disease conditions such as hyperparathyroidism, obesity and inflammatory bowel disease can lead to cholecalciferol deficiency (Adams and Hewison, 2010). The sources of cholecalciferol are very limited (e.g., dairy products, beef, liver, egg yolk, and fish), leading to the great demand for enrichment of food and beverages with cholecalciferol (Borel et al., 2015). The enrichment of food products with cholecalciferol or the development of cholecalciferol-based formulations is challenging because it is highly susceptible to degradation under environmental conditions including light, temperature and oxygen that can cause loss of its functionality and physiological benefits. Moreover, it has been reported that the degradation rate of cholecalciferol is high in the low pH range, the rate decreases as pH rises, and the optimum pH for the stability was 6.5 to 8.0 (Makino and Suzuki, 1995). Keeping these aspects into consideration, novel formulation strategies are required for the efficient delivery of cholecalciferol (Gupta et al., 2018). Various delivery options have been explored previously for the delivery of cholecalciferol including casein micelles (Haham et al., 2012), zein nanoparticles coated with

carboxymethyl chitosan (Gonnet et al., 2010), carboxymethyl chitosan-soy protein complex nanoparticles (Teng et al., 2013), solid dispersion (Yuan et al., 2013), and microspheres (Diarrassouba et al., 2015).

In the present study, different approaches were used for improved delivery of cholecalciferol. Solid dispersion is one of the most efficient strategies to tackle poorly soluble molecules. Cholecalciferol is hydrophobic nutraceutical with poor aqueous solubility and stability concern, therefore development of enteric solid dispersion was explored to address these issues. In another approach, cholecalciferol solid dispersion encapsulated in HPMC based delayed release capsules was attempted, which could offer protection of cholecalciferol from the low pH of the stomach because HPMC capsules could delay the release of cholecalciferol until the capsule is in the intestine. In the third, approach, for efficient delivery of cholecalciferol, development of self-emulsifying delivery system was investigated.

Prior to formulation development for cholecalciferol, preformulation studies of cholecalciferol were conducted. The solubility of cholecalciferol in phosphate buffer saline (PBS, pH 7.4) was determined by shake flask method (Konsoula and Jung, 2008). The solubility of cholecalciferol in phosphate buffer saline (PBS, pH 7.4) was found to be 1.8±0.3µg/mL. The partition co-efficient of cholecalciferol was examined in n-octanol: water and n-octanol: PBS (pH 6.8) system and the results indicated the lipophilic nature of the cholecalciferol.

Cholecalciferol is a lipophilic crystalline molecule and it is highly susceptible to degradation under environmental conditions and its degradation rate is high in the low pH range. Therefore, in the first strategy, an enteric solid dispersion-based formulation was developed for the oral delivery of cholecalciferol.

Hydroxypropylmethylcellulose acetate succinate (HPMCAS), is an important enteric polymer and it is more stable than hydroxypropylmethylcellulose phthalate (HPMCP), therefore HPMCAS was chosen in the present study for the preparation of cholecalciferol solid dispersion. HPMCAs was used as solubilizing agent and as an enteric polymer to prevent exposure of cholecalciferol from the acidic environment of the stomach. The effectiveness of HPMCAs as solid dispersion carrier has attracted great attention. HPMCAs is able to initiate and maintain supersaturation of drugs with wide variety of structures and physical properties, and the efficacy advantage of HPMCAs is primarily due to the polymer's superiority as a precipitation inhibitor via formation of colloidal species in aqueous media. HPMCAS based enteric solid dispersion of cholecalciferol was prepared by solvent evaporation method. The percentage yield of HPMCAS-based cholecalciferol solid dispersion (CCF-SD-HPMCAS) and PVP based solid dispersion (CCF-SD-PVP) was found to be optimum. The drug content of the enteric solid dispersions was in the order of 90% with a high degree of content uniformity.

The developed enteric solid dispersion was characterized by Fourier transform–infra red spectroscopy (FTIR), differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and X-ray diffraction analysis. Furthermore, as the developed solid dispersion comprising surfactant was intended for oral administration, therefore, the effect of formulation on the viability of the mimics of intestinal cells (Caco-2 cells) was investigated. Subsequently, the dissolution behavior of the developed enteric formulation was evaluated in the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), and the stability of the formulation at various storage conditions was also determined. The FTIR analysis indicated that the characteristics functional group of the solid dispersion formulation had almost the same features as

that of native drug and polymer physical mixture. The study indicated that molecular interactions did not occur, ruling out the possibility of alteration in the chemical structure of the active molecule. The SEM study revealed the crystalline form of cholecalciferol with irregular orthorhombic crystals. However, the solid dispersion formulation of cholecalciferol appeared to be small aggregates of amorphous particles. The solid dispersion formulations, CCF-SD-HPMCAS and CCF-SD-PVP did not reveal any crystal morphology of cholecalciferol and there was a remarkable change in the appearance of polymer. The morphological changes in the particles thus indicated the formation of a new solid phase, correlated with the changes in the crystalline state, leading to a single amorphous phase. The thermal behavior of cholecalciferol can be witnessed by a sharp endothermic peak corresponding to the temperature 85.14˚C characteristics of its crystalline structure as seen under the SEM. On the other hand, the solid dispersion formulation did not show any endothermic peak event corresponding to the melting of cholecalciferol indicating the formation of solid dispersion. X-ray diffractograms of cholecalciferol exhibited peaks attributed to its crystalline characteristics. On the other hand, in the solid dispersion formulation, the characteristics peaks of cholecalciferol were not appeared. This could be attributed to the conversion of cholecalciferol in amorphous or molecular dispersion form in the solid dispersion formulations. The amorphous form, owing to higher free energy, is commonly associated with improved solubility compared to the crystalline form.

Surfactants could improve drug's solubility via micellar solubilization and play a vital role in the modulation of membrane permeability. They also possess the potential to inhibit p-glycoprotein, thereby improving the drug's bioavailability and intracellular drug concentration, which is having an implication in tackling drug resistance. However, surfactants are associated with the limitation of local irritation, membrane

disruption, and cellular death. Hence, surfactant-based formulations should be investigated for their cytotoxic potential. The viability assay indicated that the surfactant used in the developed solid dispersion of cholecalciferol had no cytotoxic effect on Caco-2 cells. Dissolution study of enteric solid dispersion in two-stage dissolution under biomimetic medium indicated pH-dependent release of cholecalciferol from the HPMCAS-based solid dispersion. The improved drug dissolution from HPMCAS based solid dispersion can be due to the reduction in particle size of the drug and possible amorphization of solid dispersion, enhanced wetting of the drug and the possible solubilization effect of the polymer/surfactant. Another mechanism of dissolution enhancement could be complexation of cholecalciferol with hydrophilic PVP macromolecules in water, which is prominent for its intrinsic solubility. PVP also avoids crystallization of dissolved molecules. In addition, the enteric polymer can offer pH-dependent controlled release and improved bioavailability as it restricts recrystallization of cholecalciferol in gastric media and drug degradation in acidic conditions. Also, HPMCAS-based solid dispersion of cholecalciferol exhibited higher relative bioavailability as compared to non-enteric solid dispersion and native molecule. The improved relative bioavailability of solid dispersion-based cholecalciferol formulation could be attributed to the better solubility of the cholecalciferol. Further, enteric solid dispersion could offer protection to the cholecalciferol which in turn lead to marked improvement in the relative bioavailability of the cholecalciferol. Moreover, the water-soluble nature of the developed formulation was compatible with the aqueous characteristics of the lining of the gastro-digestive tract, which can also contribute to the improved relative bioavailability of cholecalciferol (Li *et al.*, 2011; Sun *et al.*, 2012). The stability of the CCF-SD-HPMCAS and CCF-SD-PVP was investigated by storing the formulation at different

conditions of temperature and humidity The stability study showed no significant changes in the cholecalciferol content in the developed formulation under storage at experimental conditions. Solid dispersion offers immobilization of the cholecalciferol in polymer carriers. Alternatively, the other explanation for the improved stability is the physical barrier for the penetration of oxidizing agents, whose exposure to cholecalciferol is obstructed in the polymeric matrix-based solid dispersion formulation.

In the second approach, PVP-K30 based solid dispersion formulation was developed to improve the solubility of cholecalciferol and developed solid dispersion was subsequently encapsulated in the hydroxypropylmethyl cellulose (HPMC) based delayed release capsules which offer protection of cholecalciferol from the low pH of the stomach. The HPMC capsules formulation (DRHCap-SD) delay the release of cholecalciferol until the capsule is in the intestine (i.e. pH>5.5). The solid dispersion-based formulation of cholecalciferol was characterized by FTIR, DSC, SEM, and X-ray diffraction analysis. Furthermore, as the developed solid dispersion comprising surfactant is intended for oral administration, therefore, the influence of formulation on the activity of Caco-2 cells was investigated. Subsequently, the dissolution profile of DRHCap-SD was evaluated in the biorelevant gastric and intestinal fluid, and thereafter stability profile of the formulation at various storage conditions was assessed. The results demonstrated improved solubility of cholecalciferol in solid dispersion-based formulation. The enhancement of solubility of cholecalciferol from developed solid dispersion formulation may be due to various reasons including surfactant aided solubilization and improved wetting property, conversion into amorphous form and size reduction. The drug content of solid dispersions was in the order of 91±2.3%.

The analysis of FTIR spectra of solid dispersion formulation showed that the characteristics functional group cholecalciferol is retained in the solid dispersion formulation. Hence the chemical structure of cholecalciferol was likely to be unaffected during solid dispersion formulation development. The developed solid dispersions formulation exhibited no crystalline feature of cholecalciferol and formulation appeared as small aggregates of amorphous particles. The changes in the morphology of formulation suggested transitions from crystalline to amorphous phase in the process of obtaining solid dispersions. Solid dispersion obtained by solvent evaporation technique in our study did not show any endothermic peak event corresponding to melting of cholecalciferol, indicating the formation of solid dispersion where the drug converted from crystalline to amorphous form. In x-ray diffraction analysis, cholecalciferol exhibited crystalline peaks at 2θ due to its crystalline characteristics. On contrary, the characteristics peaks of cholecalciferol were not appeared in the solid dispersion formulation.

The cell viability assay in Caco-2 cells demonstrated that the surfactant used in the solid dispersion formulation of cholecalciferol had no adverse effect on intestinal cells. Further, dissolution profile of HPMC capsule encapsulated solid dispersion showed improved dissolution of cholecalciferol. The improvement in the dissolution profile of cholecalciferol from DRHCap-SD can be attributed to the protection of the cholecalciferol in acidic SGF condition and this can also be associated with the reduction in particle size of the cholecalciferol and its possible amorphization of solid dispersion, enhanced wetting of the drug and the possible solubilization effect of the polymer or surfactant. The bulk properties of the developed solid dispersion and their physical mixture were determined and compared with the USP specifications. The results demonstrated that the flow properties were improved by formulation of solid

dispersion. Moreover, the stability study indicated no significant changes in the cholecalciferol content in the developed formulation under storage at experimental conditions. The developed solid dispersion-based formulations could lead to immobilization of the cholecalciferol in polymer carriers leading to decreases in cholecalciferol mobility. In addition, improved stability may be attributed to the physical barrier for the penetration of oxidizing agents, whose exposure to cholecalciferol is hindered in the polymeric matrix-based solid dispersion formulation. Also, encapsulation in delayed release HPMC capsule further contribute to the improved stability of cholecalciferol in DRHCap-SD.

In the third approach, development and characterization of self-emulsifying drug delivery systems was explored for oral delivery of cholecalciferol. The solubility of cholecalciferol in various oils, surfactants, and co-surfactants was determined and 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 to obtain pseudo-ternary phase diagram to identify good emulsification region. On the basis of phase diagram, different compositions were selected. The formulations were prepared by incorporating cholecalciferol in the specified mixture of oleic acid, Cremophor EL and polyethylene glycol 400 with continued stirring till the homogenous mixture formed. The cholecalciferol loaded SEEDS formulations were characterized on the basis of different parameters including emulsification tendency, precipitation of drug, phase separation, density of globules and globules uniformity. The morphology of the SEDDS formulation was investigated by transmission electron microscopy. The size, zeta potential and the polydispersity of the formulation was determined using the zeta sizer. The SEDD formulation exhibited size in nanometre range with narrow particle size

distribution. The SEDD formulation was subjected to in-vitro stability assessment in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The formulation was observed for coalescence of droplets, precipitation or phase separation after 2h incubation. There were no coalescence, precipitation or phase separations in the formulation. Subsequently, dissolution profile of cholecalciferol SEDDS formulation was studied in SGF (pH 1.2), wherein, SEDD exhibited significantly improved dissolution profile of cholecalciferol compared to the native molecule. Evaluation of biocompatibility of SEDDS with caco-2 cells was determined by using MTT assay and the results indicated that the formulation had no toxic effects on the caco-2 cells. The bio-accessibility of cholecalciferol using SEDDS in GIT or more specifically, the amount of cholecalciferol available for absorption after the lipid digestion from the formulation was also determined using a two-stage GIT model consisting of a gastric and small intestinal phase and the formulation exhibited good bio-accessibility. The observed good bioaccessibility of the choleclciferol could be attributed to its micellar solubilization. One of the constituents of developed formulation is long chain fatty acid, which is promptly digested in the intestine. Thereafter lipolysis took place and the resultant digestion moieties are subsequently solubilized by bile salt mixed micelles, which is colloidal in nature with great potential of facilitating absorption (Pouton and Porter 2008). Further, it has been reported that the reduction of the crystallinity is associated with the improved bioaccessibility and bioavailability of molecule (Lindfors et al., 2007). Further, the stability study indicated no significant changes in the cholecalciferol content in the developed formulation under storage at experimental conditions. The lipid based SEDDS formulations cause encapsulation of cholecalciferol in lipid carriers leading to decreases in cholecalciferol mobility. Additionally, improved stability may be attributed to the physical barrier for the penetration of oxidizing agents,

whose exposure to cholecalciferol was hindered in the SEDDS formulation encapsulated in capsule.

7.2 Conclusion

In the present research work, in the first approach, enteric solid dispersion of cholecalciferol was successfully developed using hydroxypropylmethylcellulose acetate succinate. The enteric solid dispersion of cholecalciferol was characterized by FTIR, DSC, SEM, and X-ray diffraction analysis, and these techniques indicated the successful formation of the enteric solid dispersion-based products. Solid dispersion formulation in this study comprises cholecalciferol, polymer and surfactant. Hence, the safety profile of the formulation in terms of cytotoxic potential in Caco-2 cells was investigated. The results indicated that the polymer/surfactant used in the enteric solid dispersion formulation of cholecalciferol had no cytotoxic effect on Caco-2 cells. Subsequently, the dissolution behavior of cholecalciferol was studied using two-stage dissolution in a biomimetic medium comprising SGF and SIF. The study indicated pH-dependent release profile and improved dissolution of cholecalciferol from enteric solid dispersion-based products. The developed enteric formulation of cholecalciferol also showed higher relative bioavailability as compared to native molecule. Finally, the stability study indicated no significant difference in the cholecalciferol content in the developed formulations under the experimental conditions. The developed enteric solid dispersion for cholecalciferol exhibited potential for further translational studies. In the second approach, PVP-K30 based solid dispersion of cholecalciferol was successfully developed which were encapsulated in delayed release HPMC capsule (DRHCap-SD). The solid dispersion of cholecalciferol was characterized by FTIR, SEM, DSC and X-ray diffraction analysis, and these techniques indicated the successful formation of the solid dispersion of cholecalciferol. Also, solid dispersion formulation exhibited no

cytotoxic effect on Caco-2 cells. The dissolution study of DRHCap-SD and SD formulation of cholecalciferol in SGF and SIF medium demonstrated improved dissolution of cholecalciferol from DRHCap-SD formulation. The storage stability study demonstrated no significant difference in the cholecalciferol content in the DRHCap-SD formulations. In the third approach, SEDDS based formulation for cholecalciferol delivery was prepared, characterized and they exhibited improved dissolution and bio-accessibility. In summary, the formulation strategies explored in this research work exhibited potential for improved oral delivery of cholecalciferol. The findings in the present research work are seminal, which could have implications in the further development in this potential area of research.