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Zero order controlled release delivery of cholecalciferol from injectable biodegradable microsphere: *In-vitro* characterization and *in-vivo* pharmacokinetic studies

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ABSTRACT

Poly(lactic-*co*-glycolic acid) microspheres loaded with cholecalciferol (CL), more bioactive form of vitamin D was developed as an injectable controlled drug release system and was evaluated for its feasibility of once a month delivery. The CL loaded microspheres (CL-MS) were prepared by simple oil in water (O/W) emulsion–solvent evaporation technique incorporated with a stabilizer, Tocopherol Succinate (TS). Different formulation as well as process parameters were investigated namely concentration of emulsifier, concentration of stabilizer and drug: polymer mass ratios. The prepared CL-MS were evaluated for particle size, drug loading, *in-vitro* drug release and *in-vivo* pharmacokinetics in rats. The optimized formulation was found to have a mean particle size of 28.62 ± 0.26 µm, Encapsulation Efficiency (EE) of 94.4 ± 5.4% and drug loading of 5.19 ± 0.29% with CL:TS ratio of 2:1. It was found that the EE drastically decreased (26 ± 5.9%) in the absence of stabilizer (TS) indicating its role in stabilization of CL during formulation. DSC and XRD studies indicated that CL existed in an amorphous structure in the polymer matrix. SEM of the CL-MS revealed the spherical morphology and confirmed the particle size. *In-vitro* release showed that the CL release from CL-MS followed near zero–order drug release kinetics over nearly 1 month. *In-vivo* pharmacokinetic study of CL-MS showed higher $t_{1/2}$ (239 ± 27.5 h) compared to oily CL depot (32.7 ± 4.8 h) with sustained release of CL plasma concentration for 1 month. The labile CL could thus be effectively encapsulated and protected against degradation during microspheres formulation, storage and release in presence of stabilizer. This novel CL loaded PLGA MS is stable and may have great potential for clinical use.

1. Introduction

Deficiency of Vitamin D (VD) affects almost 50% of the population worldwide (Holick, 2007). This insufficiency in VD among people can be attributed to the changed lifestyle and environmental factors that reduce exposure to sunlight and in turn reduces the Ultra Violet B (UBV) induced VD production in the skin (Krishnan and Feldman, 2011). VD deficiency and its relationship with most of diseases is becoming the main topic of discussion among scientists and doctors off late. VD is found in different chemical forms such as Cholecalciferol (CL), ergocalciferol, calcidiol and calcitriol (Ramalho et al., 2015). CL is an inert form and must be converted to its calcitriol in the liver (Trump et al., 2010). Bioactive VD is a steroid hormone that has long been known for its important role in regulating body levels of calcium and phosphorus, and in mineralization of bone (Glade, 2013). Clinically, there are promising studies which demonstrate the protective role of VD against cancer, heart diseases, obesity, autoimmune diseases, influenza and type-2 diabetes (Luo et al., 2012; Wang et al., 2013; Nair and Maseeh, 2012). VD also plays an important role in skeletal homeostasis by modulating bone metabolism (Ignjatović et al., 2013).The major challenge with the use of CL in formulations is the labile nature of the molecule. It is extremely sensitive to external and environmental factors such as temperature changes and light which in turn may affect the stability and efficacy of the molecule (Luo et al., 2012). CL rapidly gets oxidized and inactivated on exposure to atmospheric air and aqueous conditions (Boardman et al., 2005). Other challenges in the delivery of CL include its short half-life in the blood stream (Yin et al., 2010) and its high first pass metabolism (Plum and DeLuca, 2010).

Copolymers of lactic and glycolic acids, poly(lactic-*co*-glycolic acid) (PLGA) are one of the extensively researched polymers (Sahoo et al.,

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2005) for the development of controlled release formulations for many macromolecules such as proteins and peptides, DNA and RNA, as scaffolds for tissue engineering and medical devices (Liechty et al., 2010; Mundargi et al., 2008; Blanco and MaJ, 1998). This polymer, approved by the United States Food and Drug Administration (USFDA) (Makadia and Siegel, 2011) can be modulated for degradation rate by varying the copolymer composition (ratio of lactic acid to glycolic acid). PLGAs are biocompatible and biodegradable polymers which undergo hydrolysis in aqueous environments such as body fluids to yield lactic acid and glycolic acid monomers which are biocompatible and rapidly cleared from the body (Davis et al., 1996). Lupron Depot®, drug releasing PLGA particles were first of its kind to be approved by USFDA in 1989 (Ramalho et al., 2015). PLGA microspheres have been effectively developed for the delivery of anticancer drug, cisplatin with an aim to increase the efficacy and reduce the adverse effects (Moreno et al., 2008). PLGA nanoparticles have been successfully developed for the delivery of Vitamin D for cancer therapy to enhance its short half-life and its low bioavailability (Ramalho et al., 2015).

Despite the spectra of health benefits demonstrated by CL, it continues to remain a challenge to formulate CL into a successful and stable formulation owing to its labile nature. Encapsulating such a labile molecule within PLGA particles may help protection of the drug against variety of formulation parameters and on storage. The use of a stabilizer may be able to increase the stability of CL (Sawicka, 1991). Exenatide, a peptide molecule has been effectively loaded in PLGA microparticles with high drug loading and stability for long term delivery to over-come the limitation of commercially available exenatide (Byetta®) to be injected twice daily (Qi et al., 2013).

The aim of the present investigation was to formulate a stable, zero order controlled release delivery of cholecalciferol from injectable biodegradable microspheres of PLGA for once a month delivery. The cholecalciferol loaded microspheres (CL-MS) were successfully prepared by simple O/W emulsion – solvent evaporation technique. The different formulation and process parameters were optimized during the course of the study. Another major goal of this study was to stabilize the labile molecule against degradation during formulation, release study as well as storage by optimizing the concentration of stabilizer used.

2. Materials and methods

2.1. Materials

Cholecalciferol and D-α-Tocopherol Succinate (TS) were purchased from Sigma Aldrich, USA. PLGA with molecular ratio of D,L-lactide/glycolide 75/25 (Resomer 752H Mw 13 kDa) was kindly gifted by Evonik (Mumbai, India). Poly(vinyl alcohol) (PVA) (Mowiol® 4–88 Mol wt 36,000 Da) was purchased from Sigma Aldrich, USA. Acetonitrile and methanol used were of HPLC grade and were purchased from Merck Ltd., India. All other chemicals used were of chemical grade without further purification.

2.2. Preparation of PLGA microspheres

PLGA microspheres loaded with CL were prepared by an oil-in-water (O/W) emulsion - solvent evaporation technique as described in literature (Fig. 1) (Freitas et al., 2005; Zidan et al., 2006). Briefly, specified amount of PLGA and CL were dissolved in 1 mL of dichloromethane (DCM) with or without stabilizer (TS). This organic phase was slowly added to 100 mL of 1% w/v PVA or different concentrations of TPGS aqueous solution. The formed emulsion was homogenized at 7000 rpm using an Ultra-Turrax® (T-25 digital Ultra-turrax, IKA India Private Ltd., Bangalore, India) for 1 min followed by stirring at 250 rpm, 25 °C for 4 h for removal of organic solvent. The solid microspheres were recovered by filtration through a paper filter (0.45 μm, millipore) and washed 3 times with distilled water to remove residual PVA and non-encapsulated CL. These filtered microspheres were frozen at − 70 °C for 12 h followed by freeze-drying (Labconco, USA) under vacuum (1 mbar, − 30 °C) for 24 h.

2.3. Effect of different formulation variables on CL-MS

Effects of different formulation variables were studied on the particle size, drug loading, release study and morphology of CL-MS. Different drug: stabilizer ratios by weight (1:0, 1:1, 2:1 and 6:1) were tried out to prevent oxidation of CL during formulation, release and storage. Effect of 6, 8. 10, 15 and 20% of CL theoretical loading were tried to check its effect on % Encapsulation Efficiency of CL in microspheres. D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS) was tried out at different concentrations (0.1% w/v, 0.3% w/v, 0.5% w/v) instead of PVA as a steric stabilizer to obtain stable emulsion and was evaluated for particle size and Encapsulation Efficiency of CL. TPGS has dual role of PEG as well as Vitamin E, hence can act as a surfactant as well as an antioxidant simultaneously (Zhang et al., 2012).

2.4. Particle size distribution

The particle distribution of CL-MS was determined in triplicate using a Master-sizer 2000 laser particle analyzer (Malvern Instruments Ltd., Malvern, UK). The dried microspheres were dispersed in a vial by bath sonication for 10 s in deionized water to prevent aggregation before examination. The particle size distribution was characterized by volume mean diameter d 10%, d 50% and d 90% which are diameters below which 10%, 50% and 90% of the particles are present. The results are expressed as mean ± standard deviation. The width of particle size distribution is measured by parameter Span value which is calculated by following equation,

$$\text{span value} = \frac{d(0.9) - d(0.1)}{d(0.5)} \quad (1)$$

The span value is a dimensionless number which illustrates whether the spread of the distribution is narrow or wide (Ito et al., 2008).

2.5. Scanning electron microscope (SEM)

The surface morphology and shape of microspheres were examined using a Scanning Electron Microscope (SEM, JSM-5600LV, JEOL, Tokyo, Japan). Double sided carbon tape was affixed on aluminium stub. The sample of CL-MS was sprinkled onto the tape. The aluminium stubs were coated with platinum plasma beam using JFC-1600. Auto fine coater was used to make layer of 2 nm thickness above the sprinkled powder. These stubs were placed in the vacuum chamber of SEM and the microspheres were observed using a gaseous secondary electron detector (working pressure: 0.8 Torr, acceleration voltage: 10–20 kV) under different magnifications.

2.6. High performance liquid chromatography

The amount of CL for routine analysis was determined using High Pressure Liquid Chromatography (HPLC) system (Waters, USA) unit consisting of a pump and a UV–Vis detector. Separation was achieved using a Inertsil C-18 column (100 × 4.6 mm, 5 μ) at a flow rate of 1.0 mL/min and a mobile phase consisting of acetonitrile: methanol: water at a ratio of 90: 8: 2 (v/v). The detection wavelength was set at 265 nm and injection volume was kept at 50 μl. The peak areas of CL were recorded and the concentrations were calculated from a standard curve. The retention time of CL was found to be 8 mins.

2.7. Encapsulation Efficiency

To determine loading percentage of CL in the microspheres, 5 mg of freeze dried CL-MS was dissolved in 0.2 mL of acetonitrile. After