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Modified pullulan nanoparticles for oral delivery of lopinavir: Formulation and pharmacokinetic evaluation

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ABSTRACT

In this investigation, we report the use of the pullulan acetate, a hydrophobic derivative of pullulan in the formulation of Lopinavir loaded nanoparticles meant for oral delivery. Pullulan was modified to pullulan acetate by acetylation process in the presence of pyridine; acetylation was confirmed by FT-IR and NMR spectra. Lopinavir, an HIV-protease inhibitor was formulated into nanoparticles of pullulan acetate by the well-known emulsion-solvent-evaporation method. The nanoparticles were tested for particle size, entrapment efficiency, in-vitro drug release and stability. Further, extensive pharmacokinetic and tissue distribution studies were performed in Wistar rats. The results showed that, with our method, we could obtain nanoparticles of ~197 nm, high entrapment efficiency (~75%) and monodisperse nature (PDI < 0.2). Stability data showed that the nanoparticles were stable over a period of 3 months. From the pharmacokinetic study data, we found that the relative bioavailability of Lopinavir from nanoparticles was ~2 folds higher than the free drug. Moreover, the tissue distribution study showed a higher distribution of Lopinavir loaded nanoparticles to lymphoid organs (liver, spleen and lymph nodes that are also important viral reservoirs in HIV infection). Thus, we conclude that Lopinavir loaded nanoparticle could be a superior alternative approach to free Lopinavir in treating HIV infection.

1. Introduction

Lopinavir (LPV) is a potent human immunodeficiency virus (HIV) protease inhibitor (PI) and an essential part of Highly Active Anti Retroviral therapy (HAART). It is used as a first line drug in HIV infected patients undergoing anti-retroviral therapy (Farmer et al., 2001). Being substrate of CYP3A4 and P-gp systems, LPV shows poor oral bioavailability in humans (du Plooy, Viljoen, & Rheeders, 2011; Ravi, Vats, Thakur, Srivani, & Aditya, 2012). Therefore, when given alone, like most of the other anti-retroviral drugs, LPV fails to achieve therapeutic concentration in blood and viral reservoirs (Chandwani1 & Shuter, 2008; Haase, 1999). The HIV mainly resides in anatomical (CNS, lymphatic system, liver, lungs and the genitals) and cellular reservoirs (*i.e.* CD+ T lymphocytes and monocytes/macrophages) of the human body (Schrager & D'Souza, 1998). Development of an effective drug delivery approach for the treatment of HIV/AIDS has been a global challenge (Levy, 2007, chap. 10).

Currently, the marketed formulation of LPV (Kaletra®, Abbott Laboratories) contains fixed dose combination of LPV and another PI, ritonavir. Ritonavir acts as a pharmacokinetic booster for LPV's bioavailability by inhibiting CYP3A4 and P-gp system (Zeldin & Petruschke, 2004). Although effective, RTV is known to cause glucose intolerance, gastrointestinal intolerance, lipid elevations, and perioral paresthesia (Shafran, Mashinter, & Roberts, 2005). Thus, there is a need for RTV-free strategy to improve LPV's oral bioavailability and also to achieve optimum LPV concentration in HIV localized sites in the body.

Nanoparticles (NPs) are considered to have wide applications for therapeutic purposes because of their distinctive characteristics (Sundar, Kundu, & Kundu, 2010). Due to their sub-cellular and submicron size, NPs can penetrate deep into the tissues through fine capillaries, cross the physiological barriers and are generally taken up efficiently by the reticulo-endothelial cells (Li & Huang, 2008).

Orally delivered NPs have shown significant improvement in cellular uptake and drug-plasma exposure (Ravi, Aditya, Kathuria, Malekar, & Vats, 2013; Ravi, Vats, Dalal, Gadekar, & Aditya, 2013). They accomplish this by avoiding first pass metabolism, P-gp mediated efflux or by promoting intestinal lymphatic transport (Kingsley et al., 2006). This allows for an efficient delivery of

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therapeutic agents to target sites; the lymphoid organs and other HIV reservoirs of the human body (Trevaskis, Charman, & Porter, 2008).

Among the various NPs, biodegradable polymeric NPs have been widely explored as particulate carriers in pharmaceutical and medical fields (Hunter, Elsom, Wibroe, & Moghimi, 2012). Biodegradable, polymeric NPs provide an attractive alternative for long-term delivery of therapeutic agents meant for chronic administration (Feng, 2004). These drug loaded NPs cut overall cost of medicine, reduce risks of toxicity and are superior to conventional formulation. Control release, improved bioavailability, targeted delivery and improved therapeutic impact (Kumari, Yadav, & Yadav, 2010) are other advantages of NPs.

Pullulan is a non-toxic, non-immunogenic, biodegradable and neutral linear polysaccharide consisting of α-1,6 linked maltotriose residues. It is extensively used in food industry (Leathers, 2003). Pullulan cannot self-associate in aqueous solutions due to its high water solubility. Therefore, hydrophobized pullulan has been investigated as a drug delivery carrier to encapsulate lipophilic compounds (Akiyoshi, Deguchi, Moriguchi, Yamaguchi, & Sunamoto, 1993). Pullulan acetate (PA) is a well explored derivative of pullulan that can form self-aggregating mono-disperse NPs in aqueous media (Hong et al., 2011; Lee et al., 2012; Zhang et al., 2009).

This investigation deals with the synthesis and characterization of pullulan acetate (PA) polymer, its use in preparing LPV loaded pullulan acetate nanoparticles (PANPs). Further, characterization of PANPs such as particle size, morphology, drug encapsulation efficiency and *in vitro* drug release are reported. Comparative oral pharmacokinetic studies and tissue distribution studies of free LPV and LPV loaded PANPs were conducted in male Wistar rats to evaluate *in vivo* performance of prepared PANPs.

2. Experimental

2.1. Materials

LPV (purity > 99%) was obtained as a gift sample from Matrix Laboratories, Hyderabad, India. Pullulan (M_w = 200 kDa) was purchased from Hayashibara (Tokyo, Japan). Rat intestinal microsomes (RIM), rat liver microsomes (RLM) and NADPH were procured from BD Gentest, Woburn, USA. HPLC grade acetonitrile, ammonium acetate, heparin, methanol, methylene chloride (DCM), potassium dihydrogen phosphate and sodium citrate were purchased from Merck Laboratories, Mumbai, India. Methyl cellulose (molecular weight 14 kDa, viscosity 15 cps) and Tween 80 were purchased from S.D. Fine Chemicals Ltd, Mumbai, India. A Milli-Q water purification system (Millipore, MA, USA) was used for obtaining high quality HPLC grade water.

2.2. Methods

2.2.1. Synthesis of pullulan acetate

Pullulan acetate (PA), as hydrophobized pullulan, was synthesized by previously reported Motozato's method (Motozato, Ihara, Tomoda, & Hirayama, 1986). Briefly, 2 g of pullulan was dissolved by vigorous stirring in 20 ml of formamide maintained at 54 °C. For acetylation of pullulan, 6 ml pyridine and 15 ml of acetic anhydride was added to the above solution while maintaining the temperature at the 54 °C for 48 h. A dark-brown precipitate was obtained that was further purified by triturating with 1000 ml distilled water and 500 ml methanol. The solid material was vacuum-dried for 24 h, to finally obtain the product.

2.2.2. FT-IR spectroscopy

Fourier transform infrared (FTIR) spectra of pullulan and PA were recorded on a FT-IR 4200 (JASCO, USA) spectrometer in the range of 4000–400 cm^{-1} by KBr pellet method (1% sample in KBr). The samples were vacuum dried before FT-IR scan. Total of 40 scans were taken for each sample.

2.2.3. ^1H NMR spectroscopy

The proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a Shielded Varian Inova spectrometer at 500 MHz (International Equipment Trading Ltd., Vernon Hills, USA) using tetramethylsilane (TMS) as an internal standard. Samples were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) before analysis. All NMR spectra were acquired at ambient temperature.

The degree of substitution (DS) in PA for acetyl groups was calculated from the integration value of acetyl protons (A) observed at 1.8–2.2 ppm and the OH protons and H-1 to H-6 protons (B) of pullulan moiety observed at more than 3.5 ppm. The DS values were calculated by the NMR method using following equation: DS = 10A/(3B + A), derived from the equation: $A/3x = B/\{7 + (3 - x)\}$ (corresponding to the area for one hydrogen in a glucose unit), where x equals DS [17].

2.2.4. Preparation of PANPs

LPV loaded PANPs were prepared according to the previously reported oil-in-water emulsion-solvent evaporation technique (Byun et al., 2011) with minor modifications. Briefly, accurately weighed quantities of LPV (10 mg) and PA (100 mg) were dissolved in 5 ml of DCM, this constituted organic phase. To form the primary emulsion, the organic phase was dropped into 50 ml aqueous PVA (0.5%, w/w) solution by means of a syringe fitted with needle (Internal diameter – 0.75 mm), positioned a few centimeters above the surface of the medium under gentle magnetic stirring (800 rpm). The primary emulsion was further subjected to high speed homogenization (at 5000 rpm, Polytron PT 3100D, Kinematica, Switzerland) for 15 min. The resulting colloidal preparation was centrifuged at 20,000 × g for 45 min to obtain LPV loaded PANPs. To remove the adherent free drug and excess PVA, the pellet was re-suspended in deionized water and centrifuged three times at 20,000 × g for 15 min each. Washed NPs were re-suspended in deionized water and subjected to pre-freezing at −80 °C for 6 h. Further, freeze-drying was carried for 24 h at −110 °C in a lyophilizer (Coolsafe 110-4, Scanvac, Denmark). Mannitol (5%, w/v) was used as a cryoprotectant. This lyophilized powder was stored in sealed glass containers at room temperature till further use.

2.2.5. Particle size analysis

Particle size, size distribution and zeta potential of the prepared PANP dispersions were measured by Zetasizer (Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). All the samples were suitably diluted with double distilled water before measurement. Backscattering was measured by a detector at an angle of 173°. Instrument temperature was set at the 37 °C during the measurement. The result was described as mean ± S.D. (n = 6).

2.2.6. Scanning electron microscopy (SEM) analysis

PANPs were examined for morphology under scanning electron microscope (JSM-6360LV Scanning Microscope; Jeol, Tokyo, Japan). Before analysis, 100 μl of PANPs dispersion was dried overnight on an aluminum stub under vacuum. This was then sputter-coated with gold-palladium layer under an argon atmosphere using a gold sputter module in a high-vacuum evaporator (JFC-1100 fine coat ion sputter; Jeol, Tokyo, Japan). Coated samples were then scanned and photomicrographs were taken at an acceleration voltage of 15 kV.