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Research paper

Lipid nanoparticles for oral delivery of raloxifene: Optimization, stability, *in vivo* evaluation and uptake mechanism

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ABSTRACT

Raloxifene HCl (RLX) shows low oral bioavailability (<2%) in humans due to poor aqueous solubility and extensive (>90%) metabolism in gut. Lipid nanoparticles (SLN) with glyceryl tribehenate were designed to enhance drug's oral bioavailability. Box–Bhenken design was used to optimize manufacturing conditions. Optimized SLN had particle size of 167 ± 3 nm and high encapsulation efficiency (>92%). Oral bioavailability of RLX from SLN was improved by 3.24 folds compared to free RLX in female Wistar rats. Both clathrin and caveolae mediated endocytosis pathways were involved in the uptake of SLN. Lymphatic transport inhibitor, cycloheximide significantly reduced oral bioavailability of SLN.

1. Introduction

Osteoporosis is a disease characterized by a decrease in bone mass (osteopenia) and a deterioration in bone micro-architecture that leads to an enhanced fragility of the skeleton, and therefore to a greater risk of fracture. Bone strength primarily reflects the integration of bone density and bone quality [1]. There are many causes for osteoporosis, but by far the most common and most important is menopause that is associated with low levels of estrogen in women. Postmenopausal osteoporosis (PMO) affects most women during senescence [2]. It is estimated that 200 million women worldwide are affected by PMO [3]. In Europe, USA and Japan, osteoporosis affects an estimated 75 million women [4].

Selective Estrogen Receptor Modulators (SERMs) are popularly used as first line therapy for prevention and treatment of PMO. They are designed to have tissue-specific effects; acting as either an estrogen agonist or antagonist [5]. Raloxifene HCl (RLX), a SERM is used in prevention and treatment of PMO in women. It shows low solubility and poor oral bioavailability (BA) (less than 2%) with high inter-patient variability in humans. Approximately 60% of oral dose is absorbed, but pre-systemic glucuronide conjugation is extensive, which limits its oral BA [6]. The BA of RLX varies across different species of animals. In rats and dogs, BA of RLX was originally reported as 39% and 17%, respectively [7]. More recently, BA of RLX is reported as 4% and 0% in rats and dogs respectively [8]. Further, BA of RLX in pigs was reported as 7% [9]. Across all these species, low solubility and gut wall glucouronide conjugation seem to be major limiting factors for oral BA of RLX [10].

Several approaches for improvement of solubility and bioavailability of RLX like solid dispersions [11], self-emulsifying drug delivery systems [12] and complexing with beta cyclodextrins [13] have already been explored.

A recent report [14] showed that loading RLX into triglyceride based solid lipid nanoparticles (SLN) improved its oral BA than free drug. However, from studies of Müller et al. [15], we know that triglycerides based nanoparticles of shorter carbon chain length are susceptible to intestinal lipase, co-lipase enzyme system. This can be a deterrent for orally administered SLN. Conversely, higher carbon chain lipids like Compritol 888 ATO (glyceryl behenate) are relatively more resistant to this enzyme system. Besides, nanoparticles stabilized by block co-polymers like poloxamer show distinctly reduced protein adsorption and low phagocytic uptake, achieving longer circulation time in the body [16].

We hypothesized that oral BA of RLX can be improved significantly if higher carbon chain lipid like glyceryl tribehenate was used in conjunction with poloxamer as emulsifier-stabilizer. Therefore, primary objective of this work was to manufacture RLX loaded SLN with glyceryl tribehenate and poloxamer 407 (P407) for oral administration.

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Because manufacture of SLN is a multi-step process with many variables, for reproducibility, it is important to understand the interplay of formulation/process variables and to optimize manufacturing conditions. For this, we used design of experiments (DOE) to optimize the manufacturing process of SLN. However, in a process where many variables are involved, a single design is not always adequate. Therefore, we followed a hybrid-design approach: Plackett Burman design (PBD) for initial screening, followed by Box–Bhenken design (BBD), a sub-type of response surface methodology (RSM) design for process optimization. We selected particle size and entrapment efficiency percentage (EE%) as responses in the design because both of these responses affect *in vitro* and *in vivo* performance of SLN.

The PBD is particularly useful when large number of variables have to be screened with fewer runs. This design uses only a fraction of trials used in full factorial design and is suitable for initial screening of critical variables [17]. However, with PBD alone, we cannot detect interaction effects between variables.

For this, we need more sophisticated RSM design like BBD. We selected BBD because it requires fewer runs and is particularly useful when extreme treatment combinations need to be avoided [18]. Using BBD, we identified multi-factor interactions between manufacturing variables.

We assessed the capability of SLN in enhancing oral BA of RLX by carrying out extensive pharmacokinetic evaluation and tissue distribution studies in female Wistar rats. Finally, to decipher the mechanism of intestinal uptake of SLN, we performed permeation studies in presence and absence of uptake inhibitors. To evaluate the role of lymphatic transport in oral delivery of SLN, we also conducted comparative pharmacokinetic studies in presence and absence of lymph flow inhibitor, cycloheximide.

2. Materials and methods

Raloxifene hydrochloride (RLX) was obtained as a gift sample from Apotex Research Pvt. Ltd. Bangalore, India. High purity glyceryl tribehenate (GB) ($C_{69}H_{134}O_6$, molecular weight 1059.8) was purchased from M/s Himedia Pvt. Ltd. (Hyderabad, India). Kolliphor P407 (poloxamer 407) (P407) and mannitol were procured form Signet Chemicals, Mumbai, India. All other chemicals used were of analytical grade and the solvents were of HPLC grade. Freshly collected Milli-Q water (Millipore, Billerica, MA) was used in preparation of aqueous mobile phase of HPLC analysis.

2.1. Experimental design

We used low resolution PBD to screen critical variables in the manufacturing process (data not shown). Eleven variables were studied at two levels to determine their effect on two responses, viz., EE% and particle size of RLX-SLN formulations. Variables studied were the following: type of surfactant (polysorbate 80 and P407), concentration of surfactant (1% and 5% w/v), temperature of surfactant solution (25 and 75 °C), volume of external phase (10 and 30 ml), speed of homogenization (7500 and 12,500 rpm), time of homogenization (2 and 16 min), amount of lipid (0.5 and 1.5 g), time of ultra-sonication (5 and 15 min), ultra-sonication amplitude (70% and 100%), ultra-sonication pulse (continuous and pulse mode) and temperature during homogenization (60 and 75 °C).

From PBD, we selected three most influential variables (X_1 =surfactant concentration, X_2 = amount of lipid and X_3 = ultra-sonication time) that influence EE (Y_1) and particle size (Y_2) of SLN. Further, these variables were examined for individual and interaction effects using higher resolution BBD, fixing other variables. The BBD comprised 17-runs and 3-factors, studied at 3-levels. Five center point trials were included to assess reproducibility of the method. Using BBD, we also constructed following second order polynomial model for optimization of the manufacturing process:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3$$

where the b_i (for i = 1, 2 and 3) are the linear effects, the b_{ii}'s are the quadratic effects, the b_{ij}'s (for i, j = 1, 2 and 3, $i < j$) are the interactions between the ith and the jth variables; the b_0 is the intercept. Design Expert 8.0.7 software (Full version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN) was used for both designing experiments and for statistical analysis of data. Selection of optimized formulation was done based on 'Goals' mentioned in Table 1.

2.2. Preparation of solid lipid nanoparticles

For preparation of RLX-SLN, previously reported [19] warm oil-in-water micro-emulsion technique was employed, albeit with minor modifications. Briefly, GB (quantity varied as per experimental design) was held in a molten state at 75 °C. In this, accurately weighed quantity of RLX (50 mg) was dispersed thoroughly to form homogenous dispersion. This formed the lipid phase of emulsion. Aqueous phase was prepared by dissolving P407 (quantity varied as per experimental design) into double-distilled high purity water. Aqueous phase was heated until it became isothermal with lipid phase. Both phases were together homogenized (Polytron PT 3100D, Kinematica, Lucerne, Switzerland) at 10,000 rpm for 10 min, while maintaining temperature at 75 ± 0.5 °C. The obtained micro-emulsion was then quickly ultra-sonicated using a probe sonicator (Vibra cell, Sonics, USA) for specific time period at fixed amplitude (300 W output). This resulted in o/w nanoemulsion that was then cooled down in an ice-bath to form SLN. Final volume was adjusted to 200 ml with cold deionized high purity water. SLN dispersions were freeze-dried in lyophilizer (Coolsafe 110-4, Scanvac, Lynge, Denmark) for 12 h with 5% mannitol as a cryoprotectant to obtain free flowing powder. Lyophilized powder was stored in air-tight glass containers at room temperature till further use.

2.3. HPLC method for analysis of raloxifene HCl

2.3.1. Method for analysis of EE, assay and in vitro drug release study samples

Samples from EE, assay and *in vitro* drug release studies were analyzed after suitable dilution and processing by a validated HPLC method. HPLC (Model LC-20AD, Prominence Liquid Chromatograph, Shimadzu Corporation, Kyoto, Japan) with following chromatographic conditions was used: An endcapped C8 reverse-phase analytical column (Zorbax SB-C8, 150 mm long and 4.6 mm internal diameter, particle size 5 μm, Agilent Technologies, Santa Clara, CA); mobile phase comprising of 20 mM ammonium acetate buffer (pH adjusted to 4.5 with glacial acetic acid) and

Table 1
Variables and their levels used in Box–Behnken design.

Factor	Levels used		
	−1	0	+1
Independent variables			
X_1 = Surfactant concentration (% w/v)	2.5	5.0	7.5
X_2 = Lipid amount (mg)	500	1000	1500
X_3 = Ultra sonication time (min)	2.0	4.0	6.0
Dependent factors	Goals		
Y_1 = Particle size (nm)	Minimize		
Y_2 = Entrapment efficiency (%)	Maximize		