Journal of Drug Delivery Science and Technology 43 (2018) 107–112

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Short communication

Development of liposomal formulations to potentiate natural lovastatin inhibitory activity towards 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase

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ARTICLE INFO

Keywords:
Liposomes
Lovastatin
Red Yeast Rice
HMG-CoA

ABSTRACT

Liposomal formulations were obtained mixing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) with synthetic lovastatin or lovastatin extracted from Red Yeast Rice (RYR) to prepare a vehicle able to overcome both the disadvantage of lovastatin, i.e its poor oral bioavailability and side effects. Liposomal formulation obtained combining DOPC, DOPE and hydro-alcoholic extract of RYR showed optimal physico-chemical, mechanical and thermal characteristics and the strongest inhibition activity versus 3-hydroxy-3-Methyl glutaryl coenzyme A (HMG-CoA) reductase.

1. Introduction

3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase is a key enzyme in cholesterol synthesis. Statins are inhibitors of cholesterol and are considered first-choice therapy for controlling dyslipidaemia reducing levels of low-density lipoproteins (LDL) [1]. Lovastatin can be considered as one of the most well tolerated statins, however, it shows some disadvantages, i.e. poor oral bioavailability (lower than 5%) because of its rapid metabolism in the gut and liver [2], and some side effects, as myalgia. Several polymeric formulations have been tested in order to increase its oral absorption [3,4]. However, the best strategy to circumvent the solubility limit consists in loading insoluble drugs into water soluble carriers able also to offer chemical and biological protection. Liposomes are biocompatible carriers which represent the first choice vehicle to enhance hydrophobic drug activity. Moreover, liposomes enable slow release at the target site over prolonged periods of time [5]. We developed a liposomal formulation for lovastatin and evaluated the effect of lipidic vesicles on lovastatin inhibitory activity. Moreover, to overcome statins side-effect, lovastatin from Red Yeast Rice (RYR) was also tested. RYR is a natural food, produced by fermenting the *Monascus Purpureus* fungus on steamed rice and it is well known that lovastatin by RYR is more effective in lowering cholesterol than the synthetic one (i.e. 5–6 mg/day of natural lovastatin has a comparable efficacy of 20–40 mg/day of synthetic lovastatin [1]) with a better side-effect profile as regards myalgia, and lack of drug-drug interactions [6]. The aim of this paper is the evaluation of effectiveness of liposomes on synthetic and RYR lovastatin inhibitory activity against HMG-CoA-reductase.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC -purity 99%) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE - purity 99%) were purchased from Avanti Polar Lipids, Inc., Alabaster (US) and used without further purification. All chemicals, solvents and HMG-CoA reductase assay kit were purchased from Sigma Aldrich. RYR was kindly provided by MediBase s.r.l. (Prato, Italy).

2.2. Methods

2.2.1. Lovastatin extraction and quantification

Lovastatin was extracted from RYR using two different solvents. 1 g

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http://dx.doi.org/10.1016/j.jddst.2017.09.019
Received 26 July 2017; Received in revised form 25 September 2017; Accepted 26 September 2017
Available online 28 September 2017
1773-2247/

of RYR was suspended in 10 mL of ethanol (sample A) and 10 mL of ethanol:H_2O 1:1 (sample B) by a vortex for 10 min. Then, it was subjected to agitation by an oscillation plate (15 h) and magnetic stirrer (6 h). Samples were centrifuged (2650 g for 3 min). The liquid phases were used to obtain liposomal formulations after quantifying lovastatin amount.

Lovastatin was quantified via HPLC-UV method already reported [3] using a R.P-C18 column and a mixture of acetonitrile, water and methanol (5:3:1 ratio v/v) as the mobile phase (flow rate: 1.0 mL/min; injection volume: 20 μL), detector 230 nm. All the measurements were carried out in triplicate.

2.2.2. Liposomes preparation

Appropriate amounts of DOPC and DOPE stock solution (4×10^{-2} M in $CHCl_3$) and alcoholic and hydro-alcoholic RYR extracts were mixed, dried and kept under vacuum overnight at 30 °C, to remove traces of the organic solvent. The dried films were dissolved in H_2O, vortexed and multilamellar vesicles obtained. Subsequent treatment with nine freeze/thaw cycles and 27 extrusion passages through polycarbonate filters with 200 nm pore diameter allowed obtaining unilamellar vesicles of restricted size distribution [5]. To determine the exact total content of lovastatin, 100 μL of liposomal suspension were mixed with 50 μL of methanol to break down the liposomal structure. Then, 300 μL of acetonitrile were added dropwise to precipitate the lipids. The solution was centrifuged (1490 g) for 15 min. The liquid phase was analyzed by HPLC-UV following the procedure previously reported [3].

2.2.3. DLS and ζ-potential measurements

DLS and ζ-potential measurements were performed on a Zetasizer Instrument Nano ZS 90, light source He-Ne laser 633 nm, Max 4 mW, (Malvern Instrument Ltd, UK). The liposomes were allowed to equilibrate for 2 min at 25 °C, and three measurements were performed for each sample. The data were analyzed with the instrument software to generate the intensity-based size distribution and polydispersity index (PDI), and averaged based on the three measurements. ζ-potential was calculated from the electrophoretic mobility by means of the Helmholtz –Smoluchowski relation.

2.2.4. Rheological measurements

Flow curves and frequency-sweep experiments were performed using a AR2000 Rheometer (TA-Instruments, US) with a cone-plate measuring device of 40 mm diameter, employing a gap size of 0.1 mm. The temperature was maintained at 37 °C. The dynamic viscosity η under a controlled shear rate ranging from 1 to 100 s^{-1} and back from 100 to 1 s^{-1} was measured. Once the linear viscoelastic region was identified by performing a strain sweep test, the elastic modulus G′, the viscous modulus G″ were determined by a dynamic time sweep test (ν = 10 Hz; strain = 10%). All the measurements were carried out in triplicate [7,8].

2.2.5. Thermal analysis

Thermogravimetric analysis (TGA) was performed using a Q600 thermogravimetric analyzer (TA Instruments). Liposomal formulations (10 mg) were inserted in a platinum crucible and heated from room temperature to 600 °C, with a rate of 10 °C/min, under nitrogen purge gas.

2.2.6. HMG-CoA reductase inhibition test

The in vitro inhibitory effect of liposomal formulations against HMG-CoA was determined. Samples were prepared as previously reported [3] 100 μL of each sample were added to the assay kit. The rate of NADPH oxidation by HMG-CoA was monitored every 15 s at 340 nm for a period of 5min using a Ultraspec 200 UV (Biotech, USA) following the provided protocol.

2.2.7. Statistical analysis

Multiple comparisons were performed by one-way ANOVA and individual differences tested by Fisher's test after the demonstration of significant intergroup differences by ANOVA. Differences with $p < 0.05$ were considered significant [9].

3. Results and discussion

3.1. Liposomes preparation

Several approaches have been tested to improve bioavailability of drugs with a low aqueous solubility and intestinal permeation, such as lovastatin. The most promising route to enhance the bioavailability of such a drug is the use of liposomes, or lipid nanovesicles since lovastatin is a lipophilic compound (log P = 3.91) [10]. Similarly to synthetic lovastatin also lovastatin from RYR must be uploaded into a suitable carrier. In fact, RYR cannot be administered as it is or as a tablet, if a prolonged or delayed release must be achieved. Previous studies showed that lovastatin is quickly released from bare RYR, i.e. up to 42% of the total lovastatin amount was released within the very first hour and it increased to 54% (3 h), 67% (5 h) and 83% after 7 h, reaching 100% after 24 h [3].

Phospholipids like 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) are known for their propensity to form lamellar phases, as in biological membranes. Lipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), on the other hand, form non lamellar phases which are not common in biology. However, mixed phospholipid systems constitute biomembrane models that have stable lamellar structure, and moreover, phase changes between lamellar and non lamellar phases can influence certain major membrane functions, such as modulation of the action of membrane proteins [11].

The phospholipids DOPC/DOPE were judged suitable precisely for their capacity to construct very fluid liposomes, with some loss of stability as well as capacity to retain the drug. In fact, membrane fluidity is a major factor affecting encapsulation efficiency of the drug [12] and unsaturated phospholipids increase membrane fluidity, which is expected to facilitate drug leakage [13]. This physicochemical property was exploited to favour insertion of both synthetic and natural lovastatin. The melting temperature (Tm) of the liquid crystalline phase transition can be used to kwon the thermodynamics of the lipid bilayer system of interest as a function of their composition. In particular, the Tm values for DOPC and DOPE (−17 °C and −16 °C, respectively) permit to prepare easily the fluid liposome. In fact, for processing (hydration and formulation) of phospholipids, the temperature of the medium should be above the gel-liquid crystal transition temperature (Tm). Starting from DOPC and DOPE four liposomal formulations were obtained (Table 1). A significant difference was found according to the extraction solvent. Starting from 1 g of RYR, which corresponded to 50 mg of lovastatin, absolute ethanol extracted up to 35.4 mg of

Table 1
Physico-chemical characterization of liposomal formulations (A: liposomes obtained mixing DOPC, DOPE and RYR alcoholic extract; B: liposomes obtained mixing DOPC, DOPE and RYR hydro-alcoholic extract; C: liposomes obtained mixing DOPC DOPE and standard lovastatin in EtOH; D: empty liposomes) by particle size, zeta potential and lovastatin encapsulation efficiency (EC: encapsulation efficiency; D: mean diameter ± standard deviation; PI: polydispersity index; ζ: potential).

Composition	mg[a]	mg[b]	E.C	D (nm)	PI	ζ (mV)
A:DOPC-DOPE + R EtOH	20.6	3.2 ± 0.1	9%	155 ± 17	0.40	−37 ± 7
B:DOPC-DOPE + R (EtOH:H_2O)	35.4	6.4 ± 0.2	31%	450 ± 23	0.35	−32 ± 7
C:DOPC-DOPE + Lov	28	3.9 ± 0.1	14%	263 ± 15	0.47	−29 ± 5
D: DOPC-DOPE	–	–	–	85 ± 11	0.30	−18 ± 5

[a] mg of extracted lovastatin.
[b] mg of encapsulated lovastatin.