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Propolis wax nanostructured lipid carrier for delivery of β sitosterol: Effect of formulation variables on physicochemical properties

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β sitosterol (PubChem CID: 222284)
Glyceryl behenate (PubChem CID: 5362585)
L-alpha-phosphatidyl choline (PubChem CID: 122218)
N,O-Bis (trimethylsilyl) trifluoroacetamide (Pubchem CID: 9601896)
Trimethyl-chlorosilane (Pubchem CID: 6397)
Potassium hydroxide (Pubchem CID: 14797)
Potassium bromide (Pubchem CID: 253877)

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ABSTRACT

Preparation and characterization of novel functional nanostructured lipid carriers containing β sitosterol has been studied. The nanostructured lipid carrires (NLCs) were formulated with propolis wax (PW) alone or in mixture (1:1 w/w) with glyceryl behenate (GB), and pomegranate seed oil (PSO) and produced by a hot melt emulsification method. Response surface methodology was used to optimize formulations with respect to β sitosterol concentration, liquid lipid content and solid lipid composition. The NLCs formulated with less oil and higher drug content showed higher size and lower encapsulation efficiency. Solid state analysis exhibited lower crystallinity of optimal formulations compared to raw lipids and a drug amorphization into the NLC matrix. The compatibility between drug and encapsulating materials was confirmed by Fourier transform infrared spectroscopy. Transmission electron microscopy showed spherical particles ranged around 100 nm confirming the applicability of such formulations for the production of functional foods.

1. Introduction

As consumers desire healthful food products, the demand for functional and new enriched products is growing very fast. β sitosterol is one of several phytosterols with chemical structure similar to that of cholesterol and has beneficial effects on reducing total and low density lipoprotein cholesterol in human. Beside its hypocholesterolemic properties, β-sitosterol exhibits a wide range of *in vivo* biological functions such as anti-tumor, antioxidant, antidiabetic, anti-inflammatory, and gallstone reducing activities and can improve symptoms of prostatitis (Engel & Schubert, 2005; Hamedi, Ghanbari, Saeidi, Razavipour, & Azari, 2014). People consume plant sterols daily through the ingestion of fruits and vegetables, vegetable oils, and cereals; however, the amount they receive (150–400 mg/day in typical diets) is often not great enough to provide significant blood-cholesterol lowering effects. The results of over 200 clinical trials assert that phytosterol intake of 2 to 3 g/day provide patients with clinically relevant reductions in circulating cholesterol levels (Abumweis, Marinangeli, Frohlich, & Jones, 2014). Food fortification provides the possibility to enhance nutraceutical intake. However, incorporation of β sitosterol in food and beverages is rather challenging. β sitosterol has very low bioavailability in the crystalline form and is almost insoluble in water and only slightly soluble in edible oils. Although esterification improves the solubility of phytosterols in oil phase, the applications of esterified forms are limited to high-fat based food products such as spreads and margarines (Leong et al., 2011). Thus, they need to be either suspended or emulsified for application into wider range of food products and aqueous-based formulations.

Nano emulsification offers a promising means for improving solubility and bioavailability of lipid-soluble bioactive substances. Increasing interest in developing colloidal nano lipid dispersions like nano emulsions, solid lipid nano particles (SLNs) and nanostructured lipid carriers (NLCs) emanates from their large interfacial area which facilities the absorption of encapsulated compound and good stability against creaming or sedimentation (Lacatusu, Badea, Stan, & Meghea, 2012). Furthermore, these delivery systems are particularly attractive as they allow the introduction of lipophilic substances in liquid products with a negligible effect on product appearance and stability.

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However, few investigations (Lacatusu et al., 2012; Ribeiro et al., 2016) have been conducted concerning their application in phytosterols encapsulation. Ribeiro et al. (2016) investigated the ability of long and medium chain triacylglycerols (TAGs) in combination with a non-ionic surfactant in formation of lipid-based colloidal dispersions containing phytosterols. Results indicated the formation of super-cooled emulsions using liquid TAG (myritol), whereas amorphous particles formed in the case of solid TAG (trilaurin). In both cases, the complete suppression of the crystallization of both pytosterols and lipids were observed due to the nanoscale confinement. The work carried out by Lacatusu et al. (2012) involved blend of natural oils (grape seed oil, fish oil and squalene) and solid lipids. This led to the formation of β sitosterol NLC with enhanced antioxidant activity. In our previous work (Soleimanian, Goli, Varshosaz, & Sahafi, 2018), the formation of stable NLC with long term physical and oxidative storage stability has been attested using propolis wax (PW) and pomegranate seed oil (PSO) as soild lipid and liquid lipid matrix. We found that propolis wax as a novel ingredient could be used in formulation of lipid nano particles. There are also few applications of PSO as a functional ingredient in foods and no investigation has been conducted on preparation of food bioactive-containing NLCs using PSO. The high β sitosterol content of PSO makes it proper for developing a NLC system for the modulation of cholesterol absorption.

Therefore, the aim of this project was to investigate the ability of PW and PSO to obtain a water-dispersible β sitosterol formulation for its incorporation into different kinds of foods and beverages. Response surface methodology (RSM) was applied to examine the effect of solid lipid composition, β sitosterol and PSO content on droplet characteristics and entrapment efficiency of NLCs. The optimized formulations were further characterized by differential scanning calorimetry (DSC), X-ray diffraction (XRD), Fourier transform infrared (FTIR), and Transmission electron microscopy (TEM).

2. Material and methods

2.1. Materials

β sitosterol was supplied by Sigma-Aldrich. Propolis sample obtained from Espadana Mokamel Co. (Isfahan, Iran). PW (melting point of 62–64 °C) was extracted using soxhlet apparatus and petroleum ether as solvent. PSO (with fatty acid composition of 78.12 ± 0.16% punicic acid, 7.6 ± 0.04% linoleic acid, 7.12 ± 0.04% oleic acid, 3 ± 0.04% palmitic acid and 2.52 ± 0.02% stearic acid) was provided by local supplier. Compritol® 888 ATO US/NF (glyceryl behenate: GB), a mixture of ~15% monoglycerides, 50% diglycerides and 35% triglycerides of behenic acid, melting point of 71–74 °C) was kindly donated by Gattefossè (Saint-Priest, France). Lecithin (L-α-phosphatidylcholine) was purchased from Daejung Co. (Korea). All other chemicals were analytical grade and supplied by Merck Co. (Darmstadt, Germany).

2.2. Methods

2.2.1. Lipid screening

Prior to the NLC production, solubility and miscibility of the β sitosterol in lipid phase was checked. This was done by heating the lipid matrix 10 °C above its melting point and dissolving increasing amounts of the drug therein. The obtained hot solutions were observed for transparency and phase separation. The presence of crystals was then investigated visually and under normal light after cooling down of lipid mixture (Nikolić, Keck, Anselmi, & Müller, 2011).

2.2.2. Preparation of β sitosterol-loaded NLC

The nanostructured lipid carriers were prepared by emulsification using high-speed homogenization followed by ultrasonication method according to the previous literature (Soleimanian et al., 2018). The disperse phase was formulated using either PW or its binary mixture (1:1w/w) with GB, lecithin and varying amounts of PSO and β sitosterol and heated to 85 °C. Then, an aqueous phase, a solution of Tween 80 in phosphate buffer solution (10 mM; pH = 7) with the same temperature was gradually added to the oil phase under intense stirring. The resulted emulsions were subjected to a hot high shear homogenization (Ultra-Turrax T25 basic, IKA Staufen, Germany), by applying 14000 rpm for 10 min. The samples were further processed by probe-type sonicator (Adeeco, Iran, power: 250), for 8 min (on for 2 s at intervals of 2 s, 250 W) while maintaining the temperature around the melting point of the lipids. The attained emulsion was cooled down in an ice bath for 30 min to recrystallize lipid and form NLC. Total concentration of lipid phase (solid lipids, PSO, and β sitosterol) and surfactant mixture (Tween 80: lecithin with the ratio of 1:0.25 w/w) was 10% and 6% of total formulation weight, respectively. The drug-free NLC suspensions were prepared with exactly the same procedures except the drug. The freshly prepared NLC formulations were diluted (1:1) with ultrapure water, frozen at −80 °C for 24 h, and then lyophilized (ALPHA 2–4, Martin Christ Inc., Osterode, Germany) at −70 °C and 0.001 bars for 24 h for evaluation of thermal behavior, crystalline structure and drug-lipid interaction.

2.2.3. Gas chromatography (GC) analysis of β sitosterol content

2.2.3.1. Sample preparation. A total of 300 μl of PSO or NLC dispersion was placed in a 100 ml flat-bottom flask. After adding 100 μl of 5a-cholestane (1 g/ml) as internal standard and 5 ml of ethanoic KOH (2N), the sample was saponified under reflux and continues agitation at 90 °C for 1 h. Then, 10 ml of hexane were added to extract the unsaponifiable fraction. The flask was shaken and the contents carefully transferred to a 100 ml separatory funnel for liquid–liquid extraction. The organic phase was then washed three times with 5 ml of distilled water to remove the soap, water separated, and sodium sulphate was added to hexane to absorb moisture. The solvent containing phytosterols was transferred into 15 ml tubes and dried under a nitrogen stream. The dry residue was derivatised by dissolving the sample in 50 μl of pyridine and addition of 50 μl N,O- bis (trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane solution in a water bath at 60 °C for 60 min. A total of 2 μl of the mixture was analyzed by gas chromatography (Leong et al., 2011).

Stock solution of β sitosterol was prepared in methanol. β sitosterol standard solutions were analyzed by placing 100 μl standard and 100 μl internal standard solution into 15 ml tubes and drying under a nitrogen stream. The dry residue was derivatised as already described.

β sitosterol was determined with the Agilent technologies network GC system, series 6890N (Wilmington, DE, USA), with flame ionization detection (FID) via injection in split less mode. Separation was conducted on a HP-5MS column of 30 m length × 0.32 mm internal diameter × 0.25 mm film thickness. Injection was made at 250 °C. Helium was used as the mobile phase at flow rate of 1 ml/min. The column initial temperature, 200 °C, was kept for 1 min after which it was increased 20 °C/min until achieving 300 °C, which was maintained for 10 min. The detection temperature was set at 280 °C (Santos et al., 2007).

β sitosterol content was calculated based on multiple point internal standard method by plotting the ratio of peak areas for standards to IS on the y-axis vs the ratio of the β sitosterol to the IS amount on the x-axis. IS concentration was kept constant.

2.2.4. Dynamic light scattering

The analysis of particle size (PS) as Z-average and polydispersity index (PDI) of NLC formulations was performed by photon correlation spectroscopy (PCS) using a Zetasizer (NanoSizer 3000, Malvern Instruments, Malvern, UK) at an angle of 90° in 0.01 m width cells at 25.0 ± 0.1 °C. The surface charge (zeta potential) of lipid nanoparticles was determined in a capillary cell using the same instrument which utilized the Helmholtz–Smoluchowski equation to convert the

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