International Journal of Pharmaceutics 511 (2016) 455–461

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Development of a novel nanoparticle formulation of thymoquinone with a cold wet-milling system and its pharmacokinetic analysis

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ARTICLE INFO

Article history:
Received 20 April 2016
Received in revised form 8 July 2016
Accepted 17 July 2016
Available online 19 July 2016

Keywords:
Bioavailability
Cold wet-milling system
Dissolution
Nanoparticle
Thymoquinone

ABSTRACT

The present study aimed to develop a nanoparticle (NP) formulation of thymoquinone (TQ), a potent anti-oxidant chemical, with use of a cold wet-milling (CWM) system to improve its dissolution behavior and pharmacokinetic properties. The NP formulation of TQ (TQ/CWM) was prepared by CWM system, and its physicochemical properties were characterized in terms of particle size distribution, morphology, crystallinity, and dissolution. The photochemical properties of TQ were also examined upon UV/VIS absorption, reactive oxygen species (ROS) generation, and photostability. Pharmacokinetic studies were carried out in rats. Application of the CWM system to TQ led to successful development of nano-sized TQ. The mean diameter of TQ in TQ/CWM was calculated to be 143 nm, and TQ particles in TQ/CWM were found to be amorphous. There was a marked improvement in dissolution rate compared with TQ. TQ showed significant generation of singlet oxygen and superoxide upon exposure to simulated sunlight, suggesting its high photoreactivity, and solid samples such as TQ and TQ/CWM exhibited higher photostability than TQ solution. In comparison with TQ, enhanced TQ exposure was observed with a ca. 6-fold increase of oral bioavailability, and the T_{max} was shown to be a quarter. From these findings, the NP approach employing the CWM system might be a promising dosage option for improving the nutraceutical values of TQ.

1. Introduction

Thymoquinone (TQ) is a bioactive constituent derived from *Nigella sativa* (Ranunculaceae) (Abukhader, 2013). TQ has attractive nutraceutical properties such as anti-inflammatory, anti-oxidant, and anti-cancer (Abukhader, 2013; Amin and Hosseinzadeh, 2015; Nagi et al., 1999). On the basis of these biological functions, TQ has been considered as a promising drug candidate or nutraceutical agent for treatment of these diseases. However, some issues remain to be resolved for clinical application in terms of the oral absorbability and photostability of TQ. Generally, orally-taken compounds with poor dissolution rate exhibit limited absorbability in gastrointestinal media, suggesting poor oral bioavailability (Dizaj et al., 2015). Poor dissolution rate of TQ leads to low oral bioavailability, possibly limiting clinical outcome (Schneider-Stock et al., 2014). Improvement on poor dissolution behavior of TQ could serve enhanced oral bioavailability and therapeutic efficiency of TQ. A very limited number of studies has been undertaken to improve the therapeutic potential of TQ, including micelle nanoparticles, chitosan nanoparticles, and liposomes, suspension and solution formulations (Alam et al., 2012; Ganea et al., 2010; Odeh et al., 2012). Although these oral liquid formulations enhanced the solubility and bioavailability of TQ, the TQ samples in solution state were more prone to photodegradation than solid state (Salmani et al., 2014).

Abbreviations: ANOVA, analysis of variance; AUC_{0-24}, area under the curve of blood concentration vs. time from $t=0$ to $t=24$ after oral administration; BA, oral bioavailability; C_{max}, maximum concentration; CWM, cold wet-milling; CoQ_{10}, coenzyme Q_{10}; DSC, differential scanning calorimetry; HPC, hydroxypropyl cellulose; HPLC, high-performance liquid chromatography; LDS, laser diffraction scattering; NaPB, sodium phosphate buffer; NBT, nitroblue tetrazolium; NC, negative control; NP, nanoparticle; RNO, *p*-nitrosodimethylaniline; ROS, reactive oxygen species; PC, positive control; PLM, polarized light microscopy; PXRD, powder X-ray diffraction; SEM, scanning electron microscopy; T_{max}, time to maximum concentration; TQ, thymoquinone; UV, ultraviolet; VIS, visible light.

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http://dx.doi.org/10.1016/j.ijpharm.2016.07.038
0378-5173/

These observations prompted us to develop a solid formulation of TQ with high photostability, while improving the dissolution behavior and oral bioavailability. Recently, the nanoparticle (NP) approach has been considered to be one of the most promising strategies for enhancing the dissolution behavior and oral bioavailability of poorly soluble drugs in water (Pathak and Raghuvanshi, 2015). Oral bioavailability of poorly soluble chemicals has been improved by NP approach with wet-milling technologies (Kawabata et al., 2011). However, nano-pulverization with high energy might cause partial melting of TQ particles and the formulation of large aggregates because of its low melting point (46 °C) (Yuminoki et al., 2012). Previously, to suppress melting of a chemical with a low melting point during nano-pulverization, a cold wet-milling (CWM) system was developed under controlled system temperature (Onoue et al., 2014). Although the biopharmaceutical properties of TQ might be improved by enhancing its dissolution behavior with use of the CWM system, the applicability of CWM technology for TQ has never been elucidated.

The present study was undertaken to develop a NP formulation of TQ (TQ/CWM) with the CWM system. The physicochemical properties of TQ/CWM were evaluated, focusing on particle size distribution, morphology, crystallinity, and dissolution behavior. Photochemical characterization of TQ was conducted by UV/VIS absorption, ROS assay, and photostability test. The pharmacokinetic behavior of TQ in rats was evaluated after oral administration of TQ and TQ/CWM.

2. Material and methods

2.1. Chemicals

TQ (purity: 99%) and sulisobenzone were bought from Sigma-Aldrich (Tokyo, Japan). Quinine HCl, nitroblue tetrazolium (NBT), *p*-nitrosodimethylaniline (RNO), imidazole, hydroxypropyl cellulose-SSL (HPC-SSL), isobutyl *p*-hydroxybenzoate, and ammonium acetate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Methanol (liquid chromatography grade) was purchased from Kanto Chemical (Tokyo, Japan).

2.2. Preparation of TQ/CWM

The NP formulation of TQ was prepared with a CWM system based on a previous report with some modification (Yuminoki et al., 2012). Briefly, 50 mg of TQ was weighted into the vessel of a rotation/revolution mixer (custom-made NP-100 equipped with cooling unit, Thinky Co., Ltd., Tokyo, Japan). Zirconia (zirconium oxide) balls (5.0 g) with a diameter of 1.0 mm (Nikkato Co., Ltd., Osaka, Japan) were put into the vessel, and the indicated volume of HPC-SSL solution (10 mg/mL) dissolved in distilled water was also added. The TQ suspension was nanosized by 2-step wet-milling with pulverizing conditions as follows: the first step (pulverization process), 1000 rpm for 5 min with solution of HPC-SSL (0.5 mL); and the second step (dispersion process), 400 rpm for 1 min after the addition of 4.5 mL of HPC-SSL aqueous solution. After nanosizing for TQ with wet-milling, the TQ suspension containing 50 mg of milled TQ and 50 mg of HPC-SSL in a 20 mL vial was frozen with liquid nitrogen and freeze-dried using an FD-81 freeze dryer (Tokyo, Rikakikai, Tokyo, Japan). Primary drying was conducted for 12 h at −20 °C under a chamber vacuum pressure of 150 mTorr, and secondary drying was undertaken for 12 h at room temperature under a chamber vacuum pressure of 150 mTorr. To prepare TQ/CWM, pulverization was carried out under temperature control (−10 °C) in the presence of cold water solution of HPC-SSL (−20 °C, 0.5 mL).

2.3. Laser diffraction scattering (LDS)

Before freeze-drying, each TQ sample (ca. 2.5 mg) was dispersed in 18 mL of distilled water, and the particle size distribution of dispersed TQ particles was evaluated by an LDS method using a Mastersizer 2000 (Malvern Instruments, Malvern, UK) equipped with small-volume dispersing unit (Hydro 2000 μP, Malvern Instruments). The particle size distribution is expressed as the volume median diameter at 3 times.

2.4. Microscopic experiments

2.4.1. Scanning electron microscopy (SEM)

TQ samples were coated with platinum on HITACHI Ion Sputter E-1010 (Hitachi, Tokyo, Japan) before taking an image. Representative scanning electron microscopic images of TQ samples were taken with a scanning electron microscope, VE-7800 (Keyence Corporation, Osaka, Japan). Each sample was fixed on an aluminum sample holder using double-sided carbon tape for SEM observations.

2.4.2. Polarized light microscopy (PLM)

Representative PLM images of TQ samples were taken with a CX41 microscope (Olympus Co. Ltd., Tokyo, Japan). TQ samples were examined under conditions including differential interference contrast, slightly uncrossed polars, and using a red wave compensator.

2.5. Powder X-ray diffraction (PXRD)

The PXRD pattern was collected using a Mini Flex II (Rigaku Corporation, Tokyo, Japan) with Cu Kα radiation generated at 15 mA and 30 kV. Data were obtained from 3° to 33° (2θ) at a step size of 0.1° and a scanning speed of 4°/min.

2.6. Thermal analysis

Differential scanning calorimetry (DSC) was performed using a DSC Q1000 (TA Instruments, New Castle, DE). DSC thermograms were collected in an aluminum close-pan system using a sample weight of ca. 3 mg and a heating rate of 5 °C/min with nitrogen purge at 70 mL/min. The temperature axis was calibrated with indium (ca. 5 mg, 99.999% pure, onset at 156.6 °C).

2.7. Characterization of photochemical properties

2.7.1. UV spectral analysis

TQ was dissolved in 20 mM sodium phosphate buffer (NaPB, pH 7.4) at a final concentration of 100 μM. UV–vis absorption spectra were recorded with a HITACHI U-2010 spectrophotometer (HITACHI, Tokyo, Japan) interfaced to a PC for data processing (software: Spectra Manager). A spectrofluorimeter quartz cell with 10 mm pathlength was employed.

2.7.2. Irradiation conditions

Samples were stored in an Atlas Suntest CPS + solar simulator (Atlas Material Technology LLC, Chicago, USA) equipped with a xenon aarc lamp (1500 W). A UV special filter and a window glass filter were installed to adapt the spectrum of the artificial light source to natural daylight. The irradiation test was carried out at 25 °C with irradiance of 250 W/m^2 (300–800 nm).

2.7.3. Reactive oxygen species (ROS) assay

Both singlet oxygen and superoxide anion generated from irradiated TQ (25, 50, 100, and 200 μM), quinine (200 μM) and sulisobenzone (200 μM) were measured in accordance with