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Research paper

In vitro/*in vivo* characterization of nanoemulsion formulation of metronidazole with improved skin targeting and anti-rosacea properties

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ABSTRACT

Topical skin treatment was limited due to the lack of suitable delivery system with significant cutaneous localization and systemic safety. The aim of this study was to develop and optimize a nanoemulsion (NE) to enhance targeting localization of metronidazole (MTZ) in skin layers. *In vitro* studies were used to optimize NE formulations, and a series of experiments were carried *in vitro* and *in vivo* to validate the therapeutic efficacy of MTZ-loaded optimal NE. NE type selection and D-optimal design study were applied to optimize NE formulation with maximum skin retention and minimum skin penetration. Three formulation variables: Oil X1 (Labrafil), S_{mix} X2 (a mixture of Cremophor EL/Tetraethylene glycol, 2:1 w/w) and water X3 were included in D-design. The system was assessed for skin retention Y1, cumulative MTZ amount after 24 h Y2 and droplet size Y3. Following optimization, the values of formulation components (X1, X2 and X3) were 4.13%, 16.42% and 79.45%, respectively. The optimized NE was assessed for viscosity, droplet size, morphological study and *in vitro* permeation in pig skin. Distributions of MTZ were validated by confocal laser scanning microscopy (CLSM). Active agent of NE transferred into deeper skin and localized in epidermal/dermal layers after 24 h, which showed significant advantages of the optimal NE over Gel. The skin targeting localization and minimal systemic escape of optimal NE was further proved by *in vivo* study on rat skin. Current *in vitro–in vivo* correlation (IVIVC) enabled the prediction of pharmacokinetic profile of MTZ from *in vitro* permeation results. Further, the *in vivo* anti-rosacea efficacy of optimal formulation was investigated by pharmacodynamics study on mice ear.

1. Introduction

Rosacea was a common skin disease in men and women between the ages of 30 and 50 years [1]. It was characterized by central erythema of the face, with telangiectatic blood vessels, papules and pustules, and could produce skin thickening, even led to rhinophyma. Available evidence supported that rosacea was not caused by an underlying bacterium, and that the pathophysiology of rosacea was better explained as a complex inflammatory disorder involving cross talk among neurovascular dysregulation, augmented immune detection and response, and chronic alteration of superficial cutaneous vasculature [2]. The disease disfigured in a prominent manner, and its treatment was empiric and imperfect [3].

MTZ is a nitroimidazole derivative and was expected to skin enrichment for therapeutics of acne and rosacea. But after traditionally oral administration, it was completely and promptly absorbed. As a result, only a minimal amount of the active agent reached the skin and considerable side effects were also induced [4,5]. Therefore, studies of topical administration of MTZ were promising. The molecular weight of MTZ was 171.15 g/mol which fell in an appropriate range for dermal/transdermal delivery (<500 g/mol) [6]. As its log P value was −0.18, which did not indicate potential retention within lipid domains and the establishment of a reservoir for its low lipophilicity. Thus, when applied

Abbreviations: CLSM, confocal laser scanning microscopy; F, absolute bioavailability; IVIVC, *in vitro–in vivo* correlation; MRT, mean residence time; MTZ, metronidazole; NE, nanoemulsion; Oil, oil phase; O/W, oil-in-water type; PBS, phosphate buffered saline; PDI, Polydispersity Index; PRESS, predicted residual sum of square; Q_{24}, cumulative amount after 24 h; R^2, multiple correlation coefficient; SC, stratum corneum; SD, standard deviation; S_{mix}, mixture of surfactant and co-surfactant; W/O, water-in-oil type.

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to transdermal drug delivery, optimization of vehicles with targeting effect became the crucial step to make MTZ to play maximum dermal therapeutic effects and minimum systemic side effects.

The potentiality of a topical formulation to be used as a delivery system should be evaluated not only in terms of carrier capacity and percutaneous drug absorption, but also in terms of its tolerability and toxicity [7]. Varying approaches had been published to enhance skin retention without increasing systemic side effects, such as making prodrugs [8], adding penetration modifiers [9–11] and using devices, e.g. microneedle [12] and sonophoresis [13]. Recently, using nanoscale vehicles had attracted attention as a method achieved skin targeting effects without using extra devices or complex preparation process [14,15]. Non-toxic and non-irritating ingredients were chosen to prepare nano-vehicles, through a series of optimization procedures, the expected skin targeting effects were obtained.

NE had been proved to have potential in many pharmaceutical applications, not only due to the facile and low cost preparation, but also because of the improved efficacy (i.e. dermal bioavailability when applied to cutaneous diseases) [16]. NE could be formed by numerous oil, surfactant, co-surfactant, and aqueous constituents. As ideal liquid vehicles, the main advantages of NE for drug delivery were its high solubilization capacities for both hydrophilic and hydrophobic molecules, thermodynamic stability, easy formation, low viscosity, high surface area and small droplet size. In some cases [15,17–19], the enhanced accumulation of drug could significantly help optimize the skin targeting formulations without increasing the systemic side effects.

In this work, MTZ was used as a model drug. Formulation was optimized based on *in vitro* studies. Therapeutic efficacy of MTZ-loaded optimal NE was validated by a series *in vitro* and *in vivo* experiment. Therefore, the current study was conducted with multiple aims: (1) to explore the optimal NE formulation which performed significant skin targeting effect; (2) to investigate the therapeutic effect of MTZ-loaded NE depended on *in vitro* and *in vivo* studies; and (3) to indicate the potential of the optimal NE as an ideal vehicle for hydrophilic drug in cutaneous therapeutics.

2. Materials and methods

2.1. Materials and skin membranes

Metronidazole (MTZ) was purchased from ALFA AESAR (ZhongAn pharmaceutical, Tianjin, China). Labrafil M1944CS was obtained from GATTEFOSSé (Saint-Priest Cedex, France). Tetraethylene glycol was purchased from HEOWNS (Tianjin, China). Cremophor EL was purchased from BASF SE (Ludwigshafen, Germany). Klucel® MF was obtained from Hercules, Inc. (Wilmington, DE, USA). All other reagents were of analytical grade.

2.2. Skin membranes and animals

Skin samples were taken from the pig of about three months old. After removing the hair and the subcutaneous fatty tissue, the skin was cleaned in normal saline, then divided into smaller pieces and stored at −20 °C prior to use. Male Sprague-Dawley rats weighing 200 ± 20 g were used for pharmacokinetic studies. Male Kun-Ming mice weighing 20 ± 2 g were used for pharmacodynamics studies. All the animals were purchased from Chinese Academy of Medical Sciences (Tianjin, China).

Animal protocols were performed under the guidelines for humane and responsible use of animals in research set by Tianjin University School of Pharmaceutical Science and Technology. Rats and mice sacrifice by cervical dislocation were allowed by the protocols.

2.3. Construction of pseudo-ternary phase diagram and preparation of formulations

Labrafil was used as oil phase (Oil), Cremophor EL was used as surfactant and tetraethylene glycol was used as co-surfactant. First, the mixture of surfactant and co-surfactant at the w/w ratio of 2:1 was prepared as mixed surfactant (S_{mix}). Next, the mixture of Oil and S_{mix} was prepared at varying w/w ratios, e.g. 1:9, 2:8, 3:7. Then, 1 g of Oil/S_{mix} mixture of the certain ratio was titrated with double distilled water drop by drop. The water/Oil/S_{mix} mixture was stirred by a magnetic mixer. The sample was checked by visual observation. If the sample was an isotropic and clear solution, it was defined as a NE; if the sample was cloudy or showed the phase separation, it was not a NE. The boundary point between NE and non-NE was determined and corresponding component ratio was recorded during the titration. The NE system pseudo-ternary phase diagram was constructed by labeling the recorded boundary points in a ternary plot depended on SigmaPlot 10.0 (Systat, USA) software.

Hydrophilic drug MTZ (1%) was dissolved in aqueous phase and was added into Oil/S_{mix} mixture by water titration method as described above.

2.4. Formulation optimization of MTZ-loaded NE

2.4.1. Optimization along water dilution line

NE microstructures could significantly affect active agent percutaneous behavior, and water content had been reported as a key factor that strongly influenced active agent transdermal permeation potential from NE formulations [20]. NE formulations contained 20%, 60% and 80% w/w aqueous phase along water dilution line (Oil-to-S_{mix} ratio of 1:3, which could be infinitely diluted by water) were used in this study, represented for NE with low (W/O), medium (O/W) and high (O/W) water contents, respectively. The components of these three formulations are demonstrated in Fig. 1 and Table 1.

2.4.2. Optimization by D-optimal design

The further optimization study was designed based on a three component system: the oil phase X1 (Labrafil), the mixed

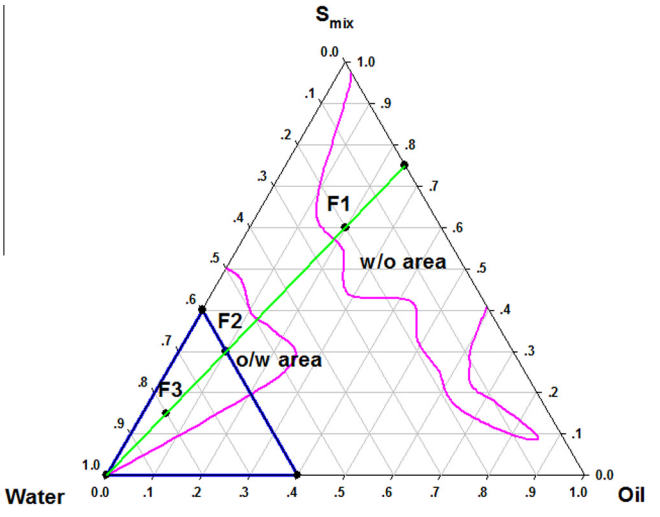

Fig. 1. Pseudo-ternary phase diagram of a NE system (area surrounded by pink line) made of Labrafil (Oil), mixture of Cremophor EL and tetraethylene glycol (S_{mix} with surfactant/co-surfactant ratio was 2:1 w/w) and water. Green line represented water dilution line at a constant S_{mix}-to-Oil ratio of 1:3 (w/w). Area surrounded by blue line was used for D-optimal design. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)