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ORIGINAL ARTICLE

Enhanced skin permeation of glabridin using eutectic mixture-based nanoemulsion

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Abstract This study aimed to investigate the performance of the eutectic mixture of menthol and camphor (1:1, *w*/w) in nanoemulsion formulation for enhanced transdermal penetration of water-insoluble glabridin. Glabridin solubility in different media was determined by a shaking bottle method. The pseudoternary phase diagrams of the oil phase (drug-loaded eutectic mixture or IPM), the surfactant (Tween 80:glycerol = 2:1, *w*/w), and water were constructed using the aqueous titration method. The obtained glabridin nanoemulsions were characterized and compared on their particle sizes, in vitro and in vivo penetration performance on rat skin, and storage stability. The nanoemulsion formulation was optimized as 0.25% glabridin, 5% oil phase, 10% Tween 80, 5% glycerol, and 79.75% water. The obtained nanoemulsions showed a mean droplet size of nearly 100 nm for different oil phases. And the stability of both formulations was similar after storage for 3 months. In vitro skin permeation study showed that the nanoemulsion formulation with eutectic mixture exhibited higher skin permeability (28.26 μg/cm^2) than that with IPM (9.94 μg/cm^2) or the drug solution formulation (3.82 μg/cm^2), which was further confirmed by in vivo skin permeation tests on the rat skin and human skin. The eutectic mixture is a preferable solvent for glabridin, and its nanoemulsion can be used as an excellent nanocarrier for enhanced transdermal delivery of glabridin.

Keywords Glabridin · Eutectic mixture · Nanoemulsions · Skin penetration · Storage stability

Introduction

Transdermal drug delivery system (TDDs), just only next to oral administration, becomes one of the most successful approaches for translation of research to clinical application of therapeutic products [1]. The key obstacle during the development of transdermal products is how to surmount the excellent barrier function of the skin [2]. One long-standing approach to promote drug flux across the skin has been to use penetration enhancers that interact with skin constituents [3]. Terpenes are naturally enhancers as indicated by high percutaneous enhancement ability, reversible effect on the stratum corneum lipids, and low cutaneous irritancy at lower concentrations (1–5%) [4].

Several terpenes are widely used as enhancers, including menthol and camphor. Menthol has been suggested as promising non-toxic, non-irritating transdermal penetration enhancers for successfully delivering a range of different compounds [5]. Studies showed that menthol increased the fluidity of the lipid bilayer to enhance drug permeability [6]. Moreover, menthol is included as an ingredient in a variety of cosmetic products as a cooling and/or flavor-enhancing ingredient [7]. Also, camphor is readily absorbed through

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the skin and produces a feeling of cooling and has been widely used as a fragrance in cosmetics [8]. Previous studies also indicated menthol in combination with camphor enhanced the skin penetration of methyl salicylate [9]. Since menthol and camphor can form eutectic liquids in a wide range of ratios, several reports have adopted their eutectic mixtures in topical formulations as penetration enhancers as well as solvents for drug or excipient [10, 11].

In addition, a variety of nanotechnologies are also commonly used to improve skin penetration of active pharmaceutical ingredients, such as liposomes, nanoparticles, nanocrystals, and nanoemulsions. Among them, nanoemulsions are transparent and have unique tactile and texture properties [12]. During the formulation development of nanoemulsions, oils or triglycerides are commonly used as the oil phase, and those who can dissolve more active ingredients are usually selected as the optimal oil phase to improve drug-loading capacity and prevent drug precipitation with time. Isopropyl myristate (IPM) is frequently applied as the oil phase due to its multi-functions as a drug solvent and as a permeation enhancer in nanoemulsion formulations [13]. However, the performance of IPM and the eutectic mixture in nanoemulsion formulations remains unknown.

In the present study, we intended to develop the nanoemulsion formulation of the eutectic mixture (menthol:camphor = 1:1, w/w) and IPM and compare their physicochemical properties and permeation enhancement performance using glabridin (Glab) as a model drug. Glab (see Fig. 1) is a polyphenolic flavonoid isolated from licorice and can benefit skin with its anti-oxidant, anti-inflammatory, skin-lightening, and ultraviolet-filtering actions. Glab was dissolved into the eutectic mixture or IPM and then introduced into the nanoemulsions using Tween 80 as the surfactant and glycerol as the co-surfactant. The formulation of the nanoemulsion was selected according to the pseudoternary phase diagrams. The physicochemical properties (e.g., morphology, droplet size, zeta potential, and drug content) and short-term stability of the obtained nanoemulsions were investigated. Finally, the enhanced skin permeability of Glab in different formulations was evaluated and compared by in vitro and in vivo methods.

Materials and methods

Materials

Glab (40%, w/w) was procured from Jingzhu Biology Technology Co., Ltd. (Nanjing, China). IPM was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Menthol was purchased from Ryon Biological Technology Co., Ltd. (Shanghai, China). Camphor was a gift from the General Hospital of Ningxia Medical University.
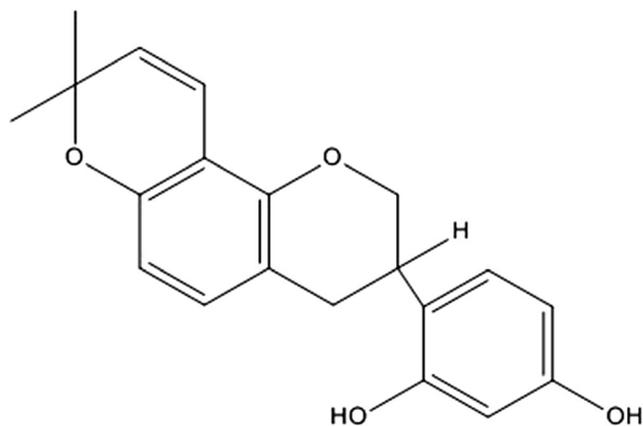

Fig. 1 Chemical structure of Glab

Tween 80 and glycerol were from Damao Chemical Reagent Factory (Tianjin, China). Other reagents used were of analytical grade. All solutions were freshly made using double-distilled water at room temperature before use.

Preparation of eutectic mixture of menthol and camphor

The menthol:camphor eutectic mixture (1:1, w/w) was prepared according to a previous report [14]. The compounds were weighed and mixed. The solid mixture was ground in a mortar until it became a clear liquid.

Determination of Glab solubility

The equilibrium solubility of Glab was assessed by quantifying the amount of Glab dissolved in various media. An excess amount of Glab was dispersed in a 1-mL medium and then shaken for 48 h at 25 °C and 120 rpm using a THZ-100B shaking air bath (Yiheng Scientific Instruments Co., Ltd., Shanghai, China). The samples were centrifuged for 20 min at 4000 rpm by a TDL-40B high-speed centrifuge (Anting Scientific Instrument Factory, Shanghai, China). An aliquot of the supernatant was collected, diluted in ethanol, and then analyzed for drug content.

Analysis of Glab content

The content of Glab was determined by a validated high-performance liquid chromatography (HPLC) method described by Shanker et al. with some modifications [15]. The HPLC system (L2000, Hitachi, Japan) consisted of a pump (L-2130, Hitachi, Japan) with an interface (D-2000 Elite) connected to a UV–VIS detector (L-2455, Hitachi, Japan). The chromatographic separation was performed on a reversed-phase YMC-Pack Pro C18 column (5 μm, 4.6 × 250 mm) at 30 °C. The mobile phase consisted of acetonitrile and 0.44% glacial acetic acid water solution at a volumetric ratio of 62:38

and was delivered at a flow rate of 1.0 mL/min. The detection was performed at 282 nm.

Construction of phase diagram

The pseudoternary phase diagrams were constructed using the aqueous titration method as previously reported [16]. Surfactant (Tween 80) and co-surfactant (glycerol) were mixed in a 2:1 (*w*/w) ratio and used as S_{mix}. For each diagram, the oil (eutectic mixture or IPM) and S_{mix} were mixed thoroughly in a series of weight ratios in vials (0.5:9.5, 1:9,…9:1, 9.5:0.5). For each combination of oil and S_{mix}, water was slowly dropped into the mixture, then visual observation was made and the physical state of the system was recorded. The percentage of each phases was calculated at different physical states and plotted on a pseudo three-component phase diagram using the Origin software (version 8.0) according to the method reported by Chen et al. [16].

Preparation of Glab nanoemulsions

An optimum formulation of Glab-loaded nanoemulsion was prepared by spontaneous emulsification method. Glab powder was accurately weighed and dissolved in 1.0 g oil phase at a concentration of 50 mg/g. Tween 80 (2.0 g) and glycerol (1.0 g) were then added to the oil phase and vortexed for 3 min. Finally, 14.95 mL water was slowly dropped into the mixture under vortexing to obtain a clear and transparent nanoemulsion.

Characterization of Glab nanoemulsions

The drug-loaded nanoemulsions were characterized in terms of the morphology, mean droplet size, polydispersity index (PI), and zeta potential. The morphology of nanoemulsions was observed under a transmission electron microscopy (TEM, H-7650, Hitachi, Japan) by placing one drop of the samples on a carbon-coated copper grid and drying at room temperature. The droplet size distribution and zeta potential of nanoemulsions were analyzed by a NICOMP™ 380 ZLS submicron particle analyzer (PSS Nicomp, Santa Barbara, CA, USA).

In vitro skin permeation study

The skin permeation study was performed to investigate the effect of different nanoemulsion formulations on the enhancement of the skin permeation of Glab. For in vitro skin permeation study, the dorsal skin was obtained from male Sprague Dawley rats (7–9 weeks old, 200 ± 20 g) and subcutaneous fat and tissue were removed. The skin was fixed between the donor and receptor chambers with the stratum corneum side upwards, and 0.2-mL Glab nanoemulsion was applied to the skin surface with the effective diffusion area of 2.89 cm^2. A mixture of acetonitrile and pH 7.4 phosphate buffer saline (38:62, *v*/v) was used as the receptor medium to maintain a sink condition according to a previous report with some modifications [17], which was continuously stirred with a magnetic stirrer at 500 rpm and maintained at 32 ± 1 °C throughout the experiment. At predetermined time intervals (1, 2, 3, 4, 5, and 6 h), the receptor phase was taken out and immediately replaced with an equal volume of fresh medium. The amount of Glab in the samples was analyzed by the HPLC method.

The value of permeation flux (J_{ss}, μg/cm^2/h) was calculated by the following equation:

$$J_{ss} = \frac{Q_t}{(t \times A)} = K_p C_0$$

Where Q_t is the total amount of drug penetrated through the unit diffusion surface, and C_0 is the initial donor drug concentration.

In vivo skin permeation study

In vivo skin permeation study on rat skin was also performed according to the methods described by Tiossi with some modifications [18]. The dorsal hair of rats was shaved and applied with a piece of filter paper (1 cm^2) preabsorbed with 30 μL of Glab-loaded formulations. At fixed intervals of time, the paper was removed from the skin, and three strips of adhesive tape were consecutively applied and removed. The paper and tapes were cut into small pieces and extracted with 4 mL ethanol twice, and the collected solution was centrifuged at 4000 rpm for 2 min. The supernatant was quantified by HPLC for Glab content.

In addition, the permeation performance of these formulations was also tested and compared on human skin using the same method. Three female volunteers were applied with drug-loaded preparations on the medial side of their left
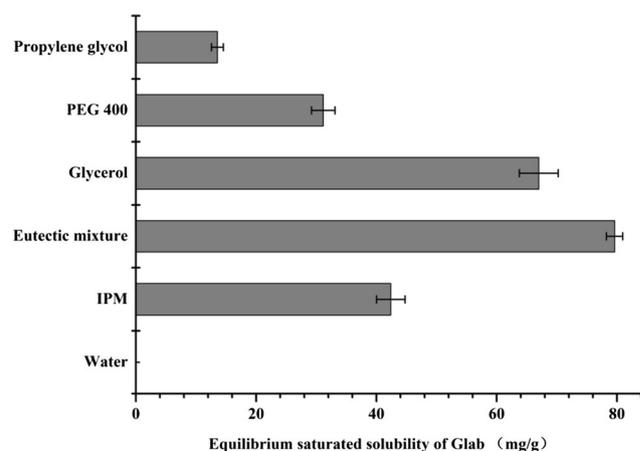

Fig. 2 Equilibrium saturated solubility of Glab in various media (*n* = 3)
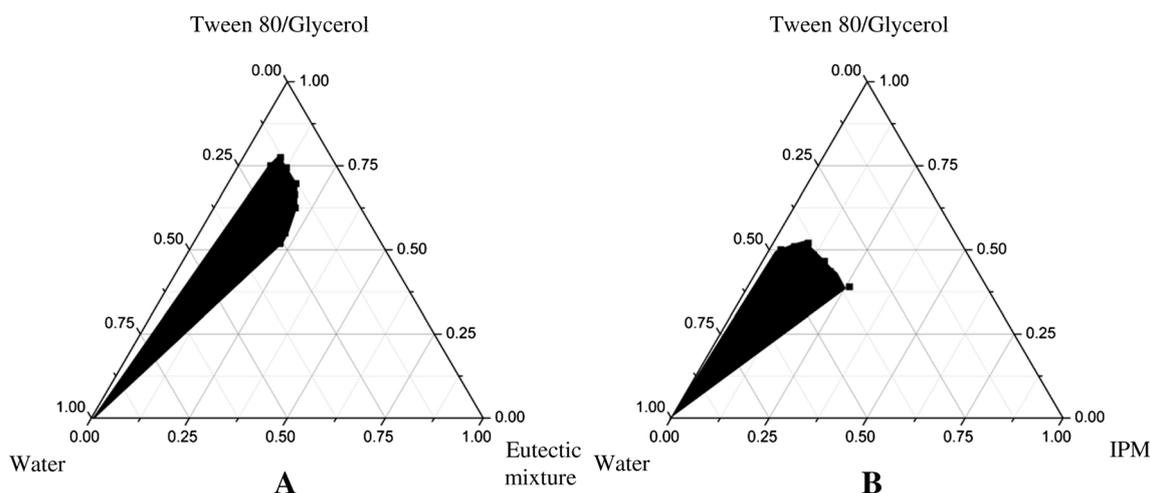
Fig. 3 Pseudoternary phase diagrams of eutectic mixture (**a**) and IPM (**b**) formulations. *Black area* shows the oil/water nanoemulsion region

forearm that were changed with another formulation every other day. The amount of drug remaining on the paper and tapes at different time points was analyzed and the amount of drug which penetrated into the skin was calculated by subtracting the remaining part from the initial drug dose.

All procedures were approved by the Research Ethics Committee, General Hospital of Ningxia Medical University.

Short-term stability test

Glab nanoemulsions with different oil phases were stored at room temperature for 3 months. And the analyses on their droplet size, PI, zeta potential, and drug content were carried out on the day of preparation and after 30, 60, and 90 days of storage.

Statistics analysis

All the results are expressed as the means ± standard deviation. SPSS version 17.0 was used to analyze the data. ANOVA and Student's *t* test were used to investigate statistical differences. The $p < 0.05$ was considered as statistically significant.

Results and discussion

Solubility of Glab

The equilibrium saturated solubility of Glab in different solvents is shown in Fig. 2. Glab is practically insoluble in water (0.91 μg/g), but soluble or sparingly soluble in other solvents, indicating its hydrophobic nature. Among the various solvents tested, the eutectic mixture of menthol and camphor at 1:1 (*w/w*) provided the highest solubility of Glab (79.60 mg/g), and it could dissolve 1.9 times of Glab than IPM (42.38 mg/g). The

solubility of Glab in three types of polyhydric alcohols was as follows: glycerol (66.99 mg/g) > PEG 400 (31.15 mg/g) > propylene glycol (13.55 mg/g).

Ternary phase diagrams

Two ternary phase diagrams using the eutectic mixture or IPM as the oil phase were constructed to identify the nanoemulsion regions. Tween 80 as an emulsifier additive has been widely used in oral and dermal pharmaceutical products. Thus, Tween 80 was selected as the surfactant in the nanoemulsion system because of its low toxicity/cost and excellent emulsification capability. Glycerol was introduced into the nanoemulsion formulation as a co-surfactant due to its good solving power for Glab, as well as its permeability enhancement and moisture retention capabilities.

Figure 3 shows the pseudoternary phase diagrams of the blank oil phase (without drugs), mixed surfactants, and water. A larger area of the nanoemulsion region was depicted for the system containing the eutectic mixture (15.9%) than IPM (12.8%). From the diagrams, one point was taken and further applied to prepare the drug-loaded nanoemulsions. Table 1 summarizes the composition of the developed nanoemulsion formulations. The prepared nanoemulsions were subsequently

Table 1 Composition of the Glab-loaded formulations

Code	Oil phase	Drug (%)	Oil (%)	Tween 80 (%)	Glycerol (%)
NE-1	Eutectic mixture	0.25	5	10	5
NE-2	IPM	0.25	5	10	5
SS[a]	/	0.25	0	10	5

"/" means there is no oil phase in this formulation

[a] Indicates the water solution of Glab
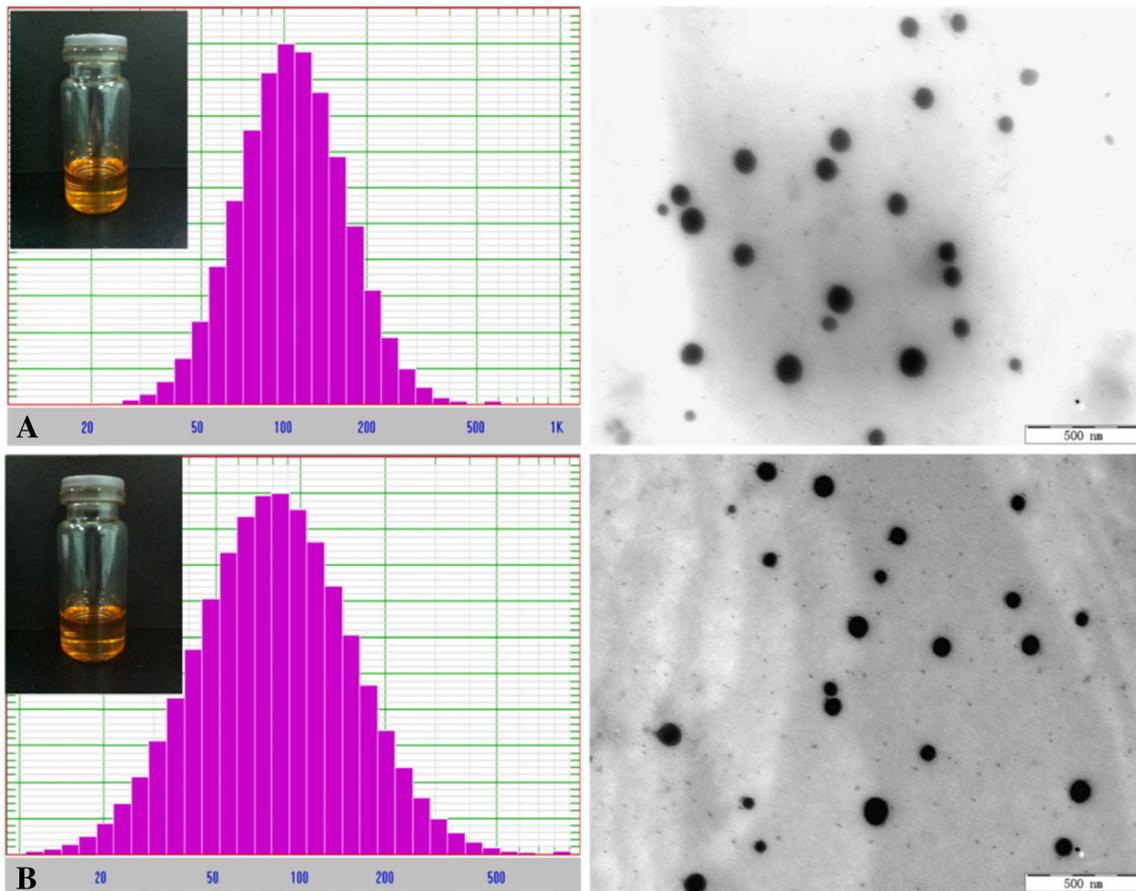
Fig. 4 Appearance, size distribution, and TEM images (*size bar* = 500 nm) of Glab NE-1 (**a**) and NE-2 (**b**)

subjected to characterization tests to compare the formulations with different oil phases.

Characterization of Glab nanoemulsions

Figure 4 shows that the resulted nanoemulsions were optically transparent with a monophasic appearance. The nanoemulsion prepared with the eutectic mixture exhibited an average size of 109.0 nm with its PI value of 0.146. The formulation using IPM as the oil phase showed a mean droplet size of 105.6 and a PI value of 0.391. These results were further confirmed by TEM observation (Fig. 4). The developed emulsion drops were spherical and discrete under TEM. The zeta potential of the nanoemulsions was neutral, which could be mainly attributed to the non-ionic surfactant in the formulation.

In vitro skin permeation study

The amount of Glab permeated through the skin in a constant area for 6 h is shown in Fig. 5, and the related parameters are depicted in Table 2. NE-1 showed a higher amount of skin permeation with no lag time than that of other formulations.

As shown in Fig. 5, the amount of Glab permeated through the skin in the initial 1 h was found to be 7.1, 1.9, and 0.3 $\mu g/cm^2$ for NE-1, NE-2, and SS, respectively. NE-1 enabled drug
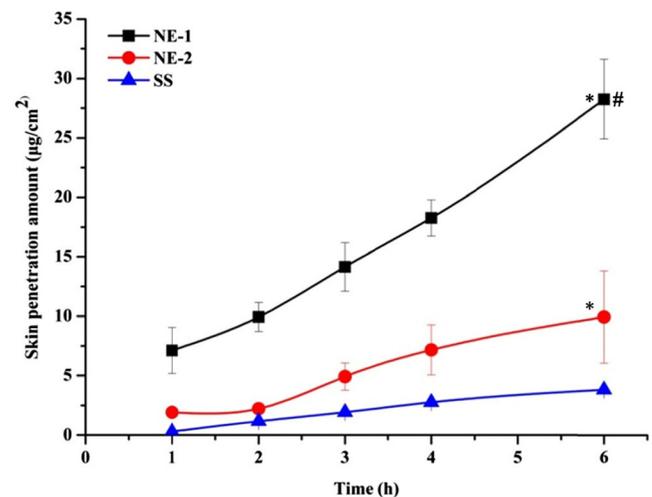

Fig. 5 In vitro percutaneous permeation of Glab through fresh rat skin after application of Glab-loaded nanoemulsions containing the oil phase of eutectic mixture (NE-1) and IPM (NE-2) compared with Glab-saturated solution (SS) at 32 ± 1 °C ($n = 6$). *$p < 0.01$ when compared with SS formulation. #$p < 0.01$ when compared with NE-2 formulation

Table 2 Permeation parameters of the nanoemulsion from in vitro skin permeation studies (mean ± S.D., $n = 3$)

Preparation	J_{ss} (μg/cm²)/h	$P \times 10^3$ (cm/h)	L (h)
NE-1	4.71*·**	1.91*·**	–
NE-2	1.68*	0.67*	0.56
SS	0.64	0.26	0.40

*$p < 0.01$ when compared with SS formulation, **$p < 0.01$ when compared with NE-2 formulation

permeation at an amount of 28.3 μg/cm² after 6 h. However, NE-2 and SS permeated only 9.9 and 3.8 μg/cm² for the same duration. Compared with those of SS and the NE-2, the J_{ss} of NE-1 was significantly increased by approximately 7.4- and 2.8-fold, respectively. And, the J_{ss} of NE-2 was 2.6 times higher than that of SS. Nanoemulsions are extensively utilized for a variety of pharmaceutical purposes including transdermal and dermal drug delivery [19]. They are versatile systems, possess high solubilizing capacity of diverse compounds, and, in particular, is capable of poorly solubilizing water-soluble drugs. The increase in solute concentration and the tendency of the vehicle to favor partitioning into the stratum corneum make microemulsion a promising vehicle for enhanced transdermal drug permeability [20]. On the other hand, both menthol and camphor were used for improving skin penetration of poor soluble drugs [14, 21]. In the present study, the combination of menthol-camphor eutectic mixture and nanoemulsion technology significantly improved Glab permeation compared with that of the water solution, which corresponds well with that of previous reports.

In vivo skin permeation study

For in vivo skin permeation study (Fig. 6), the total amount of Glab permeated into rat skin at 6 h was 47.5, 41.5, and 28.0 μg/cm² for NE-1, NE-2, and SS, respectively. When these values were compared with initially applied amount of Glab, the percentage of total drug permeation for NE-1, NE-2, and SS was 65.13, 57.18, and 37.92%, respectively. Furthermore, drug permeation on human skin was similar to that on rat skin; the drug permeation rate followed the same order of NE-1 > NE-2 > SS on both skin models, which further suggested that rat skin could be useful for evaluating different formulations and for predicting human skin permeability [22].

Taking the in vitro and in vivo results together, NE-1 is the optimal formulation enabling better penetration of Glab through the skin in comparison with other formulations, NE-2 and SS (control). Both nanoemulsion-based formulations exhibited good permeation properties in comparison to drug solution formulation, which is also observed in a previous

report [20]. In addition, the eutectic mixture showed as a better penetration enhancer than IPM when applied as the oil phase of the nanoemulsion formulation at the same level.

Stability of Glab nanoemulsions

Table 3 shows the stability data of Glab nanoemulsions. During the test period, the value of zeta potential fluctuated but still remained nearly neutral. The drug content showed a loss of 2.83 and 8.37% for NE-1 and NE-2, respectively. Compared with the drug loss of 12.7% after storage for only 10 days at 25 °C and 75% humidity in another report [23], Glab exhibited a better chemical stability performance even in the aqueous surroundings of this study. The enhanced stability may be attributed to the protection of the oil phase in nanoemulsion formulation, which was also reported previously [24]. The droplet size and PI value were slightly increased,
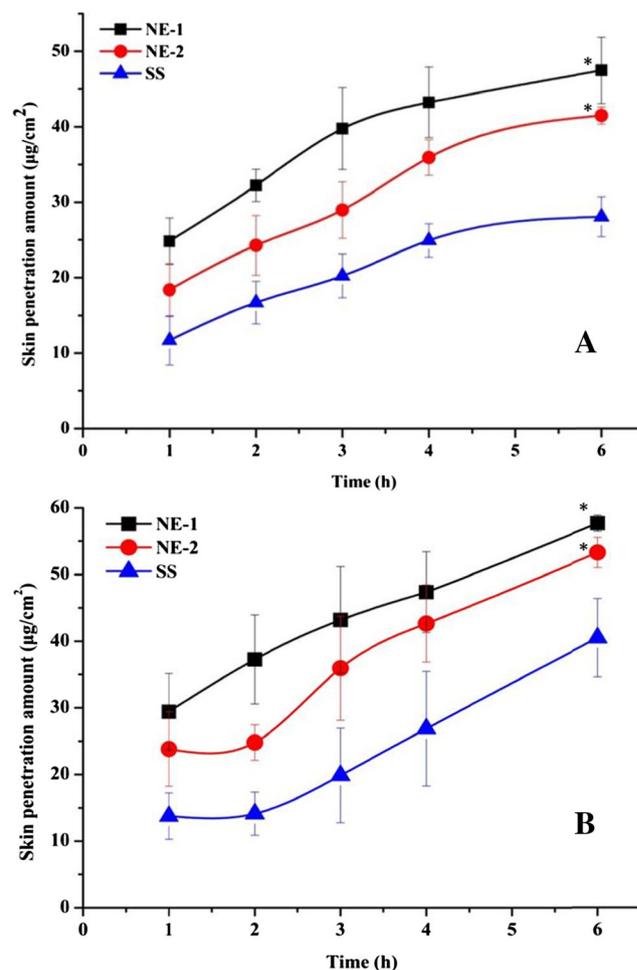

Fig. 6 In vivo percutaneous permeation of Glab through rat skin (**a**, $n = 5$) or human skin (**b**, $n = 3$) after application of Glab-loaded nanoemulsions containing the oil phase of eutectic mixture (NE-1) and IPM (NE-2) compared with Glab-saturated solution at room temperature. *$p < 0.01$ when compared with SS formulation

Table 3 Stability study of two formulations at room temperature

Time (d)	Formulation	Mean particle size (nm)	PI	Zeta potential (mV)	Drug loading (%)
0	NE-1	109.0 ± 2.0	0.146 ± 0.043	−2.61 ± 0.08	0.247 ± 0.045
	NE-2	105.6 ± 7.0	0.391 ± 0.017	−3.42 ± 0.05	0.251 ± 0.042
30	NE-1	115.6 ± 3.1	0.117 ± 0.008	−1.96 ± 0.06	0.249 ± 0.036
	NE-2	115.0 ± 4.1	0.414 ± 0.018	−3.03 ± 0.10	0.247 ± 0.047
60	NE-1	119.2 ± 2.1	0.204 ± 0.012	−2.01 ± 0.08	0.240 ± 0.053
	NE-2	118.4 ± 9.9	0.437 ± 0.045	−1.09 ± 0.07	0.236 ± 0.033
90	NE-1	127.4 ± 10.6	0.174 ± 0.048	−2.21 ± 0.07	0.240 ± 0.012
	NE-2	130.7 ± 2.0	0.443 ± 0.037	−1.13 ± 0.05	0.230 ± 0.011

indicating occurrence of crystal growth or agglomeration. The addition of some hydrophilic polymers, such as carbomer and hydroxypropyl methylcellulose (HPMC), may improve the physical stability of these nanoemulsions. Thus, further studies are needed.

Conclusion

In this study, nanoemulsions were prepared with different oil phases. By comparing the physicochemical characterizations such as apparent solubility, droplet size, zeta potential, and in vitro/in vivo skin permeation and stability, as well as the optimized oil phase for nanoemulsion formulation, the eutectic mixture of menthol and camphor was confirmed as a preferable solvent for Glab. The drug permeation through the skin was enhanced by formation of nanoscale emulsion droplets, which was further improved when the eutectic mixture was adopted into the formulation. These results suggest that the nanoemulsions based on the eutectic mixture of menthol and camphor could be used as a useful carrier for transdermal delivery of Glab.

Compliance with ethical standards The experiments comply with the current laws of the country in which they were performed. All procedures were approved by the Research Ethics Committee, General Hospital of Ningxia Medical University.

Conflict of interest The authors declare that they have no conflict of interest.

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