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Minority Medicine

Establishment and evaluation of O/W type-based microemulsion on crossbow medicine of the Miao ethnic group

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Author contributions

Yong-Ping Zhang was responsible for the study concept and design. Xue Cheng performed the experiments and manuscript preparation. Xue Cheng and Yong-Ping Zhang drafted the manuscript. Jian Xu and Fang-Fang Yang reviewed and modified the manuscript. Yao Liu helped with data analysis. All authors contributed to the article and approved the submitted version.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

IPM, isopropyl myristate; PDI, polydispersity index; HPLC, high-performance liquid chromatography; TEM, transmission electron microscope; RSD, relative standard deviations.

Citation

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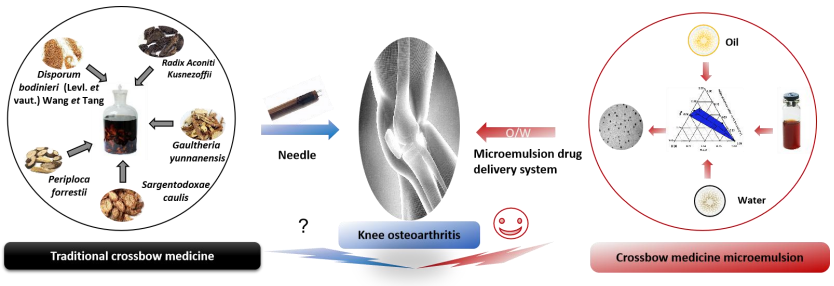
Abstract

Background: Crossbow medicine needle is a traditional external therapy. However, acupuncture pain, inaccurate administration, secondary infection, and poor patient compliance limit the application of specialty therapies. Since the most effective components of crossbow medicine are lipid-soluble, the crossbow medicine needle was prepared as an innovative O/W-type microemulsion to solve these problems. In this study, the crossbow medicine microemulsion preparation method was established and its quality was evaluated. **Methods:** A single factor and pseudoternary phase diagram, combined with a D-optimal mixture design, optimized the formulation of crossbow medicine microemulsion through particle size, polydispersity index, zeta potential, microstructure, and contents of benzoyl neoaconitine, benzoyl hypoaconitine, neoaconitine, periplocin, and methyl salicylate to evaluate the final prescription. **Results:** The best prescription for preparing crossbow medicine microemulsion is 0.1500 g crossbow medicine dry extract, 0.1500 g isopropyl myristate, 0.8625 g polyoxyethylene castor oil-35, 0.2875 g ethanol, 3.700 g water, and 5.1500 g microemulsion. The prepared microemulsion is an oil-in-water type with a clear and transparent appearance. Its average particle size is 19.83 ± 0.78 nm, average polydispersity index is 0.15 ± 0.02, and average zeta potential is −0.51 ± 0.1 mV. The pH value is 5.17 ± 0.02. The microstructure of crossbow medicine microemulsion illustrated a uniform distribution of spherical oil globules. The average contents of benzoyl neoaconitine, benzoyl hypoaconitine, neoaconitine, periplocin, and methyl salicylate were 13.34, 113.05, 43.45, 628.27 and 27.93 μg/g, respectively. **Conclusion:** The preparation process of crossbow medicine microemulsion is reasonable and feasible. The method to determine the content is accurate and straightforward, laying the foundation for further research.

Keywords: crossbow medicine microemulsion; preparation process; content determination; pseudoternary phase diagram; D-optimal mixture design



Tradition

Crossbow medicine, a characteristic of Miao medicine formulation, is the earliest described in *History of Han Continued*, recorded in the Xijin Dynasty of China (266 C.E.–316 C.E.). Crossbow medicine comprises five herbal medicines, *Radix Aconiti Kusnezoffii*, *Disporum bodinieri* (Levl. *et* vaut.) Wang *et* Tang, *Periploca forrestii*, *Gaultheria yunnanensis*, and *Sargentodoxae caulis*, which exhibits multiple pharmacological effects in modern research, including analgesic and anti-inflammatory actions. The crossbow medicine needle, extends from crossbow medicine coupled with a needle, has a significant clinical application, such as the treatment of rheumatic arthralgia diseases. However, complex steps, poor compliance and inaccurate dosage restrict their clinical promotion and application. Microemulsions are thermodynamically stable systems consisting of an oil phase, a water phase, and an emulsifier. They have solubilized various insoluble active ingredients of traditional Chinese medicines, such as oleanolic acid, huperzine A, salvia, and andrographis paniculata extract, suggest the power of microemulsion in traditional Chinese medicine development and application.

Background

Crossbow medicine, a characteristic of Miao medicine formulation, is the earliest described in *History of Han Continued*, recorded in the Xijin Dynasty of China (266 C.E.–316 C.E.). Crossbow medicine comprises five herbal medicines [1–3], *Radix Aconiti Kusnezoffii*, *Disporum bodinieri* (Levl. *et* vaut.) Wang *et* Tang, *Periploca forrestii*, *Gaultheria yunnanensis*, and *Sargentodoxae caulis*, which has the function to anesthetize, invigorate and activate blood circulation, dispel wound, remove cold, and detoxify [4, 5]. Indeed, crossbow medicine exhibits multiple pharmacological effects in modern research, including analgesic and anti-inflammatory actions [6]. These have various therapeutic effects on diseases, such as knee osteoarthritis, lumbar disc protrusion, cervical spondylotic radiculopathy, postherpetic neuralgia, and rheumatoid arthritis [7–10].

The crossbow medicine needle, extends from crossbow medicine coupled with a needle, has a significant clinical application of crossbow medicine [11]. Specifically, crossbow medicine needle therapy involves needles been soaked in the crossbow medicine and applied on the sores and are conducive for transdermal absorption of the drug so that it directly reaches the affected part. The pain and the side effect caused by oral, intravenous, or intramuscular administration to the liver and kidney can be avoided by crossbow medicine needle therapy [12]. However, complex steps, poor compliance, inaccurate dosage, and inconvenient traditional methods restrict their clinical promotion and application. Besides, the cold-soaking method is adopted for the current preparation of crossbow medicine formulation [12]. Nevertheless, the routine cold-soaking method has some limitations, including low extraction efficiency, time-consumption, and high-solvent consumption [13], which does not effectively promote the development of crossbow medicine. Therefore, it is imperative to establish a new dosage form and administration method to improve the efficacy of crossbow medicine.

Microemulsions are thermodynamically stable systems consisting of an oil phase, a water phase, and an emulsifier [14, 15]. It promotes the solubility of hydrophilic and lipophilic drugs, increases the transdermal diffusion rate, and maintains an effective blood concentration [16–18]. Recently, microemulsions have solubilized various insoluble active ingredients of traditional Chinese medicines, such as oleanolic acid, huperzine A, salvia, and andrographis paniculata extract [19–23]. These studies suggest the power of microemulsion in traditional Chinese medicine development and application. Therefore, this study initiated the application of microemulsions to establish the skin preparation of crossbow medicine. This strategy can provide novel insight into the development and scientific connotation of ethnic medicine.

Methods

Drug and reagents

Benzoyl hypoaconitine, benzoyl neoaconitine, neoaconitine, periplocin, and methyl salicylate were purchased from Chengdu Desite Biotechnology Co. Ltd. (Lot. DST200620-055, DST191023-056, DST191015-025, DST191106-041, and DST190930-125, purity ≥ 98%, Chengdu, China). *Radix Aconiti Kusnezoffii*, *Disporum bodinieri*, *Periploca forrestii*, *Gaultheria yunnanensis*, and *Sargentodoxae caulis* were purchased from the medicinal market of Taisheng (Huaguoyuan, Guiyang, China) and identified by Sun Qingwen of Guizhou University of Traditional Chinese Medicine. Isopropyl myristate, ethyl oleate, oleic acid polyethylene glycol glyceride, and isopropyl laurate were purchased from Shanghai Yuanye Biotechnology Co. Ltd. (Lot. Y17D9S77792, Z09J9Y63472, D13J8L37819, and T06J9R62846, Shanghai, China). Polyoxyethylene castor oil EL-35, glacial acetic acid, polyoxyethylene hydrogenated castor oil-40, polyethylene glycol-400 were purchased from Shanghai Yien Chemical Technology Co. Ltd. (Lot. R0200056, R009609, R032953, R010818, Shanghai, China). Labrasol was purchased from Dulai Biological Co. Ltd. (Lot. N1810120014, Nanjing, China). Tween-80 and glycerol were purchased from Chongqing Chuanjiang Chemical Reagent Factory (Lot. 20190722 and 20190913, Chongqing, China). 1,2-Propanediol was purchased from Xilong Chemical Co. Ltd. (Lot. 20191120, Shantou, China). Isopropanol was purchased from Shanghai Shenbo Chemical Co. Ltd. (Lot. 20190712, Shanghai, China). Trichloromethane was purchased from Xilong Science Co. Ltd. (Lot. 20190423, Shantou, China). Acetonitrile, glacial acetic acid, and absolute ethanol were purchased from Sinopharm Chemical Reagent Co. Ltd. (Lot. 20200722, 20200822, and 20180712, Beijing, China).

Instruments

Agilent 1260 high-performance liquid chromatography (Agilent Technologies Co. Ltd., Palo Alto, USA), ultraviolet-visible spectrophotometer (UV-9000S, Shanghai Yuanxi Instrument Co. Ltd., Shanghai, China), the chromatographic column is Wondasil C18 (Shimadzu Technology (Shanghai) Trading Co. Ltd., Shanghai, China), DJ-A high-power thermostatic magnetic stirrer (Changzhou Aohua Instrument Co. Ltd., Changzhou, China), DKZ-2 electric heating constant temperature oscillating water tank (Taicang Precision Instrument Equipment Co., Ltd., Suzhou, China), FA1004 electronic balance (Shanghai Sunny Hengping Scientific Instrument Co., Ltd., Shanghai, China), HY-2 thunder magnetic vortex apparatus (Shanghai INESA Science Co., Ltd., Shanghai, China), Malvern Zetasizer Nano ZS90 (British Malvern Instruments Co., Ltd., Shanghai, China), JEM-1400FLASH transmission electron microscope (Japan Electronics, Tokyo, Japan), TGL-16B centrifuge (Shanghai Anting Scientific Instrument Factory, Shanghai, China) and PHS-3B precision pH meter (Shanghai Hongyi Instrument Co., Ltd., Shanghai, China) were used.

Solubility study

The solubility of crossbow medicine extract in different oils (oleic acid polyethylene glycol glyceride, ethyl oleate, isopropyl laurate, and isopropyl myristate), surfactants (Tween 80, polyoxyethylene castor oil-35, polyoxyethylene hydrogenated castor oil-40, and labrosol), and cosurfactants (glycerol, isopropanol, ethanol, and polyethylene glycol-400) was measured by mixing an excess amount of the extract into 3 g of each solvent, swirled for 5 min, ultrasonically treated for 30 min and oscillated at 37 °C for 24 h. Then, the extract suspension was centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a separating funnel, and the concentration of the total alkaloids was analyzed using an ultraviolet spectrophotometer.

Screening of surfactants and cosurfactants based on pseudoternary phase diagram

Pseudoternary phase diagrams screened out the largest microemulsion region. We selected polyoxyethylene castor oil-35 and Tween 80 as a surfactant (outcomes from screening studies described above) to evaluate the effect of surfactant on the microemulsion area. Next, we used the above two cosurfactants (ethanol and glycerol) to evaluate the cosurfactant's effect on the area of the microemulsion region. A fixed surfactant: cosurfactant weight ratio of 2: 1 was used to construct pseudoternary phase diagrams (water titration method) using the isopropyl myristate (IPM) as the selected oil phase and water as the aqueous phase.

Effect of surfactant and cosurfactant weight ratio on the existence of microemulsion region

We selected polyoxyethylene castor oil-35 and ethanol selected based on the outcomes of the screening studies described above to evaluate the effect of the surfactant and cosurfactant mass ratio on the microemulsion region. They were mixed with different weight ratios (1: 3, 1: 2, 1: 1, 2: 1, and 3: 1), and pseudoternary phase diagrams were constructed in the same manner as described previously using the water titration method.

Screening of the concentration range of components of microemulsion based on pseudoternary phase diagrams

For the concentration range of components of the microemulsion, we prepared the oil and the surfactant mixture or Km (surfactant: cosurfactant weight ratio = 2: 1) using volume ratios of 1: 9, 2: 8, 3: 7, 4: 6, 5: 5, 6: 4, 7: 3, 8: 2, and 9: 1, respectively.

Formulation optimization of microemulsion by D-optimal mixture design

We selected the optimal proportion of Smix (the best excipients selected through the pseudoternary phase diagrams, including IPM oil, mixed emulsifiers and water) for further study according to the pseudoternary phase diagrams. Next, a D-optimal mixture design was used to develop the different proportions of oil (A), Km (B), and water (C) (Table 2). We homogenized IPM oil and Km using a magnetic stirrer (DJ-A, Aohua Instrument, Changzhou, China) at 25 °C and 300 r/min. Then we added water to prepare the microemulsion. The particle's diameter (nm, Y1), polydispersity index (PDI) (Y2), and solubility of the extract in the microemulsion (μg/mL, Y3) and were selected as the dependent variables. The particle diameter (Y1) and PDI (Y2) was measured using a Malvern Zetasizer Nano ZS90. The solubility of the extract in the microemulsion (Y3) was measured using the ultraviolet-visible spectrophotometer method described above. The polynomial equation, contour plots, and curved surface were performed in Design-Expert software (V.8.0.6; Stat-Ease Inc., MN, USA).

Preparation of crossbow medicine microemulsions

The five crude drug materials were dispersed in 12 times 70% aqueous ethanol (v/v) and heated with refluxing in a water bath at 80 °C for 1 h (extracting for three times). The extracting solution was collected and filtered, and then the fluids were concentrated to extract powder.

We selected the oil phase, surfactants, cosurfactants, and Km ratio with maximum microemulsion region based on the pseudoternary phase diagrams. The microemulsion's optimal proportion mixture depended on the D-optimal mixture design (Table 2). The optimal oil, surfactant, and cosurfactant were mixed in the 15 mL vial under magnetic stirring at 25 °C. The crossbow-extract powder was added and stirred into the mixture until the powder completely dissolved.

The drug loading amount of crossbow medicine extract in microemulsion

The various proportions of extract (1%, 1.25%, 1.5%, 1.75%, 2%, 2.25%, 2.5%, 2.75%, 3%, and 3.5%) was added to the blank microemulsion formulation obtained from the section "formulation optimization of microemulsion by D-optimal mixture design" and then stirred with a magnetic stirrer for 0.5 h. The solubility, appearance, particle size, and PDI of crossbow medicine microemulsion was determined using the above method.

Detection of five components in crossbow medicine microemulsion by high-performance liquid chromatography (HPLC)-DAD

Wondasil C18 column (4.6 × 250 mm, 5 μm, Shimadzu Technology Trading, Shanghai, China) was used, and the temperature was maintained at 30 °C. An optimum mobile phase was composed of 0.1 mol/L ammonium acetate solution (A, containing 0.1% glacial acetic acid) and acetonitrile-tetrahydrofuran (B, 25: 15). The flow rate was maintained at 1 mL/min with gradient elution conditions set as follows: 0–1 min, 5%–15% (B); 10–50 min, 15%–15.5% (B); 50–85 min, 15.5%–17.5% (B); 85–105 min, 17.5%–18% (B); 105–125 min, 18%–22% (B); 125–145 min, 22.00%–22.00% (B); 145–150 min, 22%–5% (B). The sample injection volume was kept at 10.00 μL. The wave was maintained at 235.00 nm. Method validation was performed for specificity, linearity, precision, stability, repeatability, and recovery.

Preparation of the test solutions of crossbow medicine microemulsion samples

An aliquot of 5.15 g crossbow medicine microemulsion was ultrasonicated in 5 mL ammonia and 40 mL isopropanol: ethyl acetate (1: 1) for 30 min. The fluids evaporated in a water bath at 40 °C. The residues were dissolved in 10 mL of methanol (containing 0.05% hydrochloric acid) as the test solutions.

Physicochemical characterization of crossbow medicine microemulsion

According to *Chinese Pharmacopoeia* [24], the physicochemical characterization of crossbow medicine microemulsion (appearance, pH value, particle size, PDI, zeta potential, stability, drug loading amount, and microstructure) was evaluated. The appearance of the microemulsion was visually inspected and recorded. The pH value of the microemulsion was determined using a digital pH meter. The Malvern Zetasizer Nano ZS90 instrument evaluated the particle size, PDI, and zeta potential of the microemulsion. HPLC-DAD analyzed the five component contents of the microemulsion. The crossbow medicine microemulsion was investigated using transmission electron microscope (TEM) (JEM-1400FLASH, Tokyo, Japan) [25, 26]. The microemulsion was stained using 2% phosphotungstic acid and positioned on a carbon-coated copper grid. The grid was examined under TEM to obtain point-to-point high-resolution microemulsion images (80 kV) using different combinations of bright-field imaging at increasing magnification (100,000 × and 200,000 ×). To understand the thermodynamic stability, we centrifugated the microemulsion at 15,000 rpm for 10 min and exposed/stored at −4 °C, followed by 4 °C and 25 °C for 1 month to observe the phase separation situation, respectively.

Results

Solubility study

The solubility of crossbow medicine extract in different types of oils, surfactants, and cosurfactants is shown in Table 1. Among the selected oils (oleic acid polyethylene glycol glyceride, ethyl oleate, isopropyl laurate, and isopropyl myristate), the extract had the greatest solubility in IPM oil (497.73 μg/mL). Among the selected surfactants (Tween 80, polyoxyethylene castor oil-35, polyoxyethylene hydrogenated castor oil-40, and labrosol), the extract had greater solubility in polyoxyethylene castor oil-35 (271.73 μg/mL) and Tween 80 (107.6 μg/mL). Among the selected cosurfactants (glycerol, isopropanol, absolute ethanol, and polyethylene glycol-400), the extract had greater solubility in ethanol (841.44 μg/mL) and glycerol (654.25 μg/mL). Therefore, IPM oil was the oil phase for microemulsion preparation; surfactant and cosurfactant still need to be optimally selected.

Construction of pseudoternary phase diagrams of surfactants and cosurfactants

As shown in Figure 1A–B, polyoxyethylene castor oil-35, as a surfactant, provided more microemulsion regions (shaded in blue) than Tween 80. Therefore, polyoxyethylene castor oil-35 was selected as the surfactant for microemulsion preparation.

Figure 1C–D shows the microemulsion systems of IPM oil as the oil phase, polyoxyethylene castor oil-35 as the surfactant, and water as the aqueous phase with ethanol and glycerol. As a cosurfactant, ethanol provided more microemulsion regions (shaded in blue) than glycerol. Therefore, ethanol was the cosurfactant for microemulsion preparation.

Effect of surfactant and cosurfactant weight ratio on microemulsion region

From the five-phase diagram (Figure 2), the largest microemulsion region was observed in surfactant mixture 3: 1. The microemulsion region (shaded in blue) expanded with the decreased proportion of ethanol in the phase diagram of 3: 1, while in the phase diagram of 1: 3, the microemulsion region shrunk.

Table 1 Solubility of crossbow medicine extract in different oils, surfactants, and cosurfactants.

Classification	Oils/surfactants/cosurfactants	Solubility (μg/mL)
Oil phase	Oleic acid polyethylene glycol glyceride	36.20
	Ethyl oleate	261.09
	Isopropyl laurate	270.73
	Isopropyl myristate	497.73
Surfactants	Tween 80	107.60
	Polyoxyethylene castor oil-35	271.73
	Polyoxyethylene Hydrogenated castor oil-40	71.82
	Labrosol	45.22
Cosurfactants	Glycerol	654.25
	Isopropanol	549.52
	Ethanol	841.44
	Polyethylene glycol-400	36.20

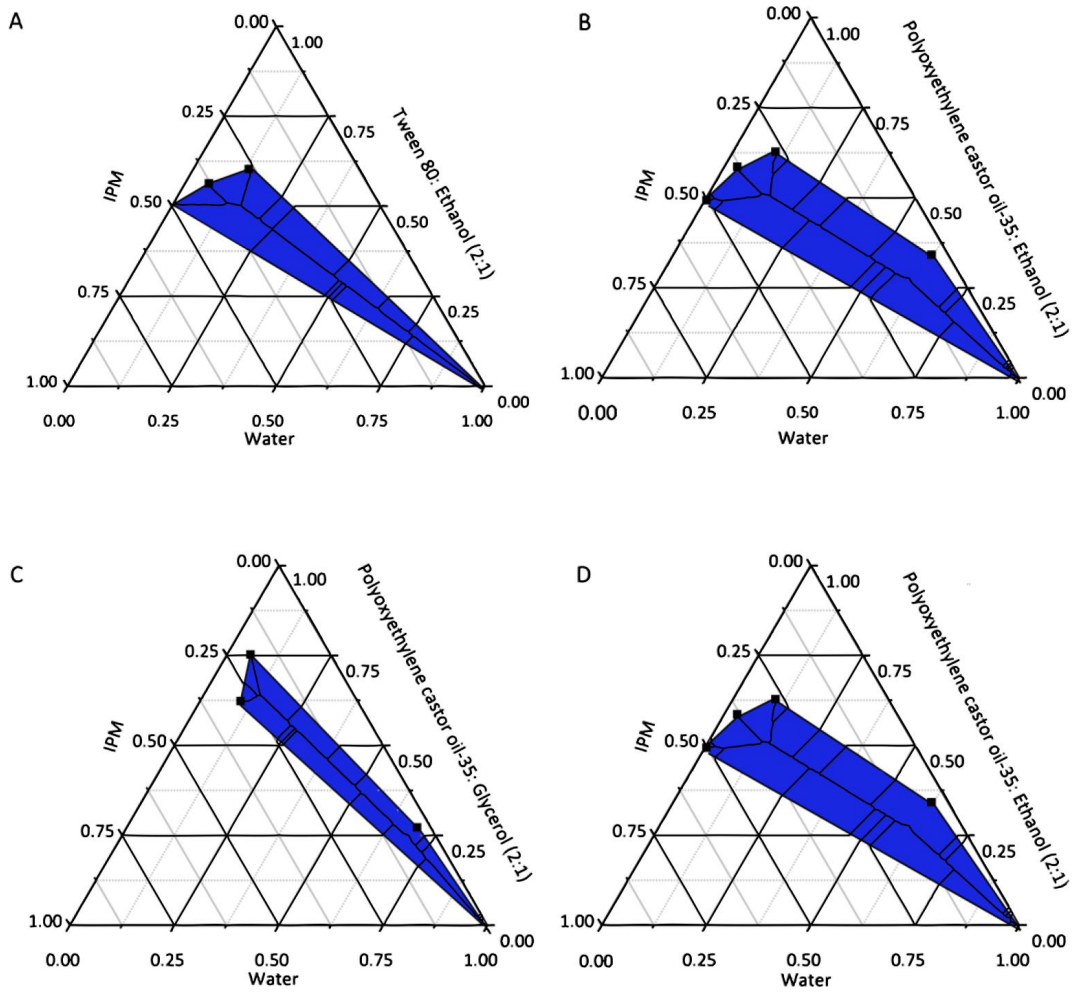


Figure 1 Screening of surfactants and cosurfactants on the basis of pseudoternary phase diagrams. A, pseudoternary phase diagram of IPM + Tween 80: ethanol (2: 1) + water; B, pseudoternary phase diagram of IPM + polyoxyethylene castor oil-35: ethanol (2: 1) + water; C, pseudoternary phase diagram of IPM + polyoxyethylene castor oil-35: glycerol (2: 1) + water; D, pseudoternary phase diagram of IPM + polyoxyethylene castor oil-35: ethanol (2: 1) + water. IPM, mixture of oil phase.

Formulation optimization of microemulsion by D-optimal mixture design

As shown in Table 2, the composition of 16 experimental runs was performed. The influence of the oil phase, mixed emulsifier, and water on relative particle size and PDI are shown in (Figure 3). All variables remained at the central level when the other three variables changed within the optimization range. The relative influence of the oil phase and water relative to PDI was small. PDI decreased with an increase in the proportion of mixed emulsifiers. Water has a relatively small effect on average particle size and oil phase. Also, mixed emulsifiers have a greater effect on average particle size. When the proportion of the oil phase decreases and the proportion of mixed emulsifiers increases, the average particle size decreases. When the oil-phase ratio increases, the average particle size continuously increases with an increase in the

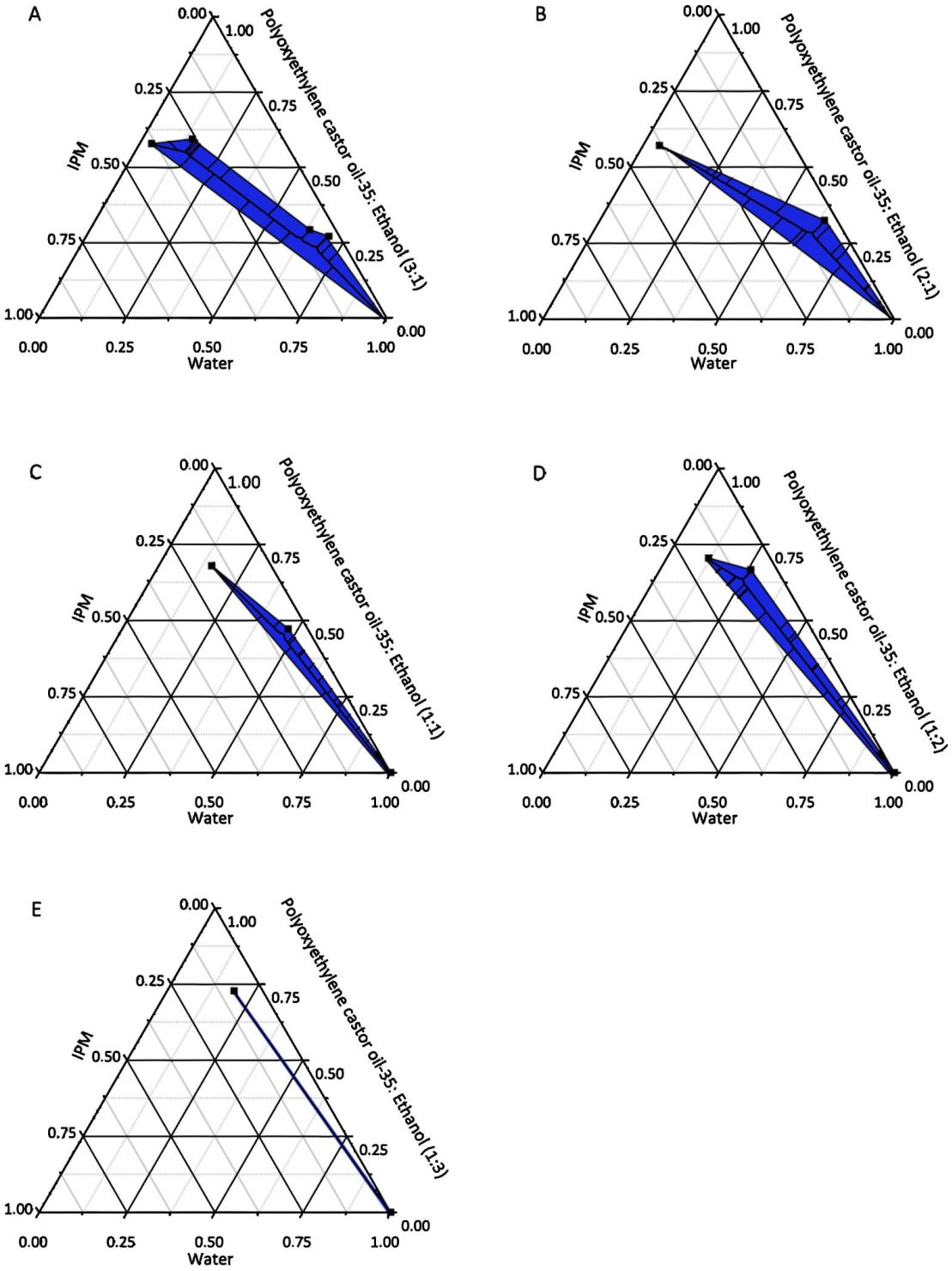


Figure 2 Pseudoternary phase diagrams of microemulsions composed of oil, water, and surfactant mixture (polyoxyethylene castor oil-35: ethanol) at various surfactant: cosurfactant ratios. A, pseudoternary phase diagram of IPM + polyoxyethylene castor oil-35: ethanol (3: 1) + water; B, pseudoternary phase diagram of IPM + polyoxyethylene castor oil-35: ethanol (2: 1) + water; C, pseudoternary phase diagram of IPM + polyoxyethylene castor oil-35: ethanol (1: 1) + water; D, pseudoternary phase diagram of IPM + polyoxyethylene castor oil-35: ethanol (1: 2) + water; E, pseudoternary phase diagram of IPM + polyoxyethylene castor oil-35: ethanol (1: 3) + water. IPM, mixture of oil phase.

Table 2 Experimental runs of D-optimal design and their responses.

Experimental runs	IPM (%)	Km (%)	Water (%)	Globule size (nm)	PDI	Solubility (μg/mL)
1	0.05	0.21	0.74	265.50	0.57	356.32
2	0.03	0.35	0.62	15.84	0.11	282.44
3	0.05	0.28	0.68	24.41	0.28	327.06
4	0.03	0.23	0.74	18.02	0.40	310.74
5	0.07	0.31	0.62	299.10	0.10	334.18
6	0.03	0.26	0.71	17.19	0.60	333.68
7	0.03	0.35	0.62	15.71	0.19	330.98
8	0.05	0.28	0.68	36.23	0.20	327.26
9	0.05	0.21	0.74	226.60	0.76	312.71
10	0.07	0.21	0.72	122.80	0.42	332.37
11	0.03	0.32	0.65	16.56	0.40	306.72
12	0.05	0.28	0.68	35.45	0.21	296.01
13	0.06	0.24	0.70	271.00	0.23	298.19
14	0.07	0.31	0.62	462.60	0.08	273.99
15	0.07	0.26	0.67	195.60	0.23	259.90
16	0.07	0.28	0.65	204.50	0.17	374.27

Data are expressed as a mean of 3 ± standard deviation. IPM, mixture of oil phase; PDI, polydispersity index.

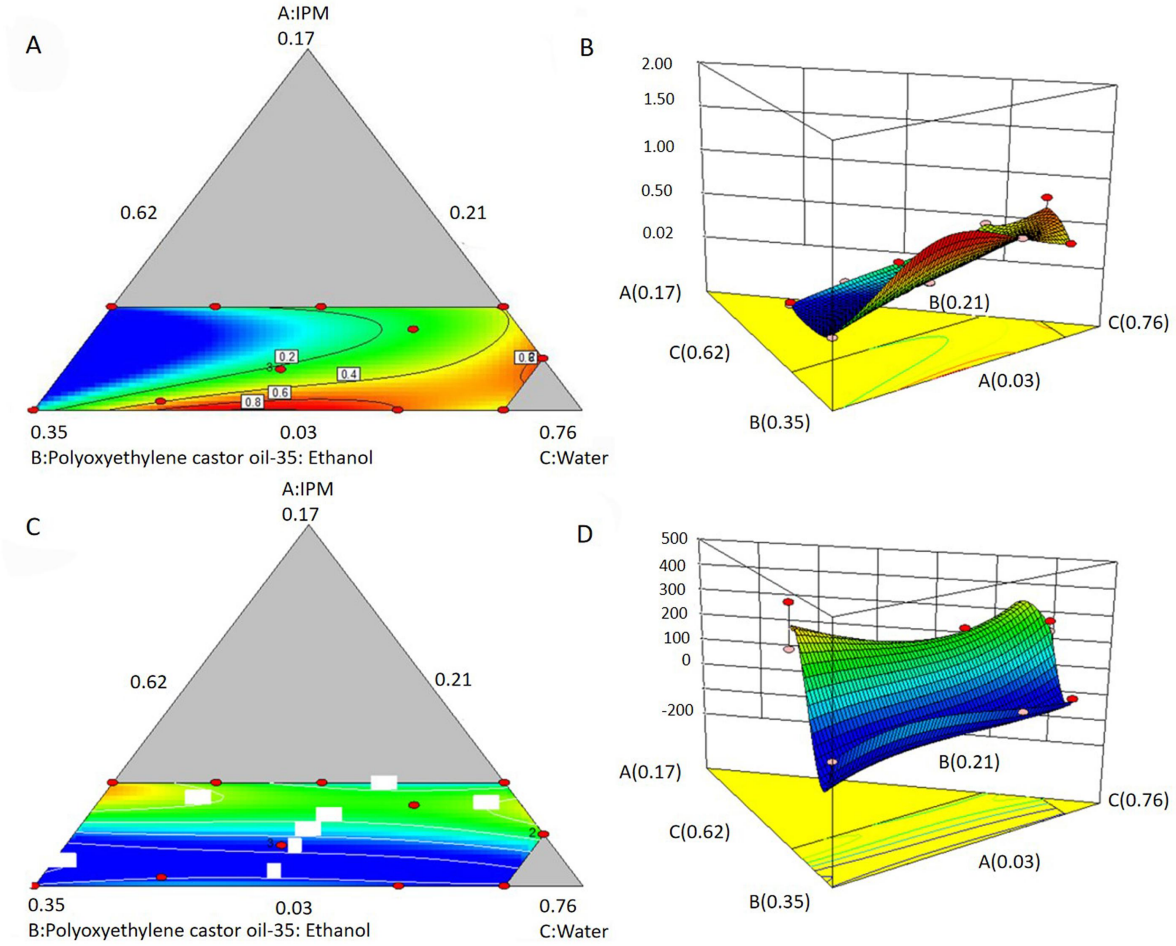


Figure 3 Effect of variables on microemulsion. A&C, contour plots for the effect of variables on globule size (Y1) and PDI (Y2); B&D, three-dimensional response surface plots for the effect of variables on globule size (Y1) and PDI (Y2). PDI, polydispersity index.

A good model is considered to accurately predict the experimental results if the *P*-value is less than 0.05. The detuning degree value is proportion of mixed emulsifiers.

Then, we performed polynomial equation analysis in the Design-Expert software and determined the composition that yields a microemulsion formulation with ideal attributes.

greater than 0.05, and the adjusted R^2 and predicted R^2 values of the selected models were comparatively higher than other models for both the responses. As shown in Table 3, the fourth power model showed a superior fit for Y1, while the cubic model showed a superior fit for Y2. The special equation of Y1 is as follows: Y1 = 63446.13*A + 20.36*B + 19.16*C + 1.18E + 5*A*B + 1.09E + 5 *A*C + 162.3*B*C − 1.07E + 5*A*B*C + 56275*A*B* (A − B) + 47312.25*A*C (A − C) + 331.85*B*C* (B − C). The *P* and detuning degree values of the model were 0.0026 and 0.1858, respectively. Similarly, the equation of Y2 is as follows: Log_{10}(PDI) = 27034.61*A − 482.78*B − 0.57*C − 35117.5*A*B − 44642.29*A*C + 933.86*B*C + 24451.32*A*B*C − 8247.06*A*B (A − B) − 18477.78*A*C* (A − C) + 610.82*B*C* (B − C). The *P* and detuning degree values of the model were 0.0055 and 0.8767, respectively. These results indicate that the regression equation has a well-fit ability.

Then, an overall optimum region plotted by contour plots and three-dimensional response surface plots showed that factors A (3%), B (17.25% and 5.75%), and C (74%) provided the optimum response of Y1: 18.02 nm and Y2: 0.40. Table 4 shows the above prescription proportion's verified results. The average experimental values of Y1 and Y2 were 17.88 and 0.12, while predicted values 18.38 and 0.40, respectively. These results indicate that the model can predict the experimental results well.

HPLC method validation

As shown in Figure 4, the method has good specificity. The regression equations for benzoyl neoaconitine, benzoyl hypoaconitine, neoaconitine, periplocin, and methyl salicylate were y = 15.173x − 26.74, y = 12.063x − 58.3, y = 13.215x − 40.18, y = 5.129x − 55.92, y = 21.353x + 25.6, the concentration ranges were, and the correlation coefficients (r) were 0.9992, 0.9995, 0.9997, 0.9993, and 0.9995, respectively. It indicated that benzoyl neoaconitine, benzoyl hypoaconitine, neoaconitine, periplocin, and methyl salicylate showed a good linear relationship with peak area of 3–9, 39.1–117.3, 11.7–35.1, 129.3–387.9, and 5.8–17.3 μg/mL, respectively. The average peak area of benzoyl neoaconitine, benzoyl hypoaconitine, neoaconitine, periplocin, and methyl salicylate was 50.72, 839.78, 277.12, 1,001.92, and 287.27, and the relative standard deviations (RSDs) were 1.39%, 1.77%, 1.83%, 1.54%, and 1.13%, respectively. The average peak area of the stability of benzoyl neoaconitine, benzoyl hypoaconitine, neoaconitine, periplocin, and methyl salicylate for 12 h were 83.53, 648.03, 255.6, 1,553.73, and 342.95, and the RSDs were 1.6%, 1.28%, 1.09%, 1.39%, and 1.47%, respectively. The average content of the repeatability of benzoyl neoaconitine, benzoyl hypoaconitine, neoaconitine, periplocin, and methyl salicylate were 479.33, 3,912.8, 1,517.11, 21,606.78, and 990.6 μg/g, and the RSDs were 2.91%, 2.73%, 2.63%, 2.58%, and 1.59%, respectively. The average recoveries of benzoyl neoaconitine, benzoyl hypoaconitine, neoaconitine, periplocin, and methyl salicylate were 101.34%, 100.16%, 98.46%, 100.59%, and 101.27%, and the RSDs were 1.82%, 2.62%, 2.96%, 2.97%, and 2.81%, respectively. The RSDs were all less than 3%, which indicates that the established content determination method met the analysis requirements.

Detection of five components in crossbow medicine microemulsion by HPLC-DAD

The benzoyl neoaconitine, benzoyl hypoaconitine, neoaconitine, periplocin, and methyl salicylate content in crossbow medicine microemulsion were determined to be 13.34 ± 0.63, 113.05 ± 2.43, 43.45 ± 0.86, 628.27 ± 13.86, and 27.93 ± 0.8 μg/g, respectively.

Physico-chemical characterization of crossbow medicine microemulsion

The three batches of crossbow medicine microemulsion had a transparent liquid with tawny, low viscosity, well distributed, and strong Tyndall effect. The pH value of the microemulsion was 5.17 ± 0.02, which is suitable for application to the skin. The mean value of particle size, PDI, zeta potential was 19.83 ± 0.78 nm, 0.15 ± 0.02, and −0.51 ± 0.1 mV, respectively. The TEM image of the microstructure of microemulsion is shown in Figure 5. The microemulsion showed a uniform distribution of spherical oil globules without any agglomeration. It indicated that the prepared microemulsion formulations had a uniform distribution. The three batches had no phase separation in the centrifugation and freeze-thaw tests, indicating that the microemulsion has good stability.

Table 3 Comparison of drug loading, particle size, and PDI fitting equations.

Response	Model	*P*-value	Detuning degree	Adjusted R^2	Predicted R^2
Globule size/nm	Linear	0.0026	0.0580	0.5369	0.3692
	Fourth power	0.0255	0.1858	0.7529	0.5070
	Special cubic	0.9349	0.1349	0.7257	−0.0674
	Cubic	0.1236	0.2358	0.8321	−3.4952
PDI	Linear	< 0.0001	0.2414	0.7668	0.6761
	Fourth power	0.7852	0.1493	0.7261	0.4822
	Special cubic	0.3029	0.1413	0.7314	0.2497
	Cubic	0.0055	0.8767	0.9440	0.8109

PDI, polydispersity index.

Table 4 Predicted and actual values of optimized prescription indexes.

Parameter	Predicted value	Experimental value	Average value
Globule size/nm	18.38	17.90	17.88
		17.98	
		17.76	
PDI	0.40	0.11	0.12
		0.14	
		0.12	

PDI, polydispersity index.

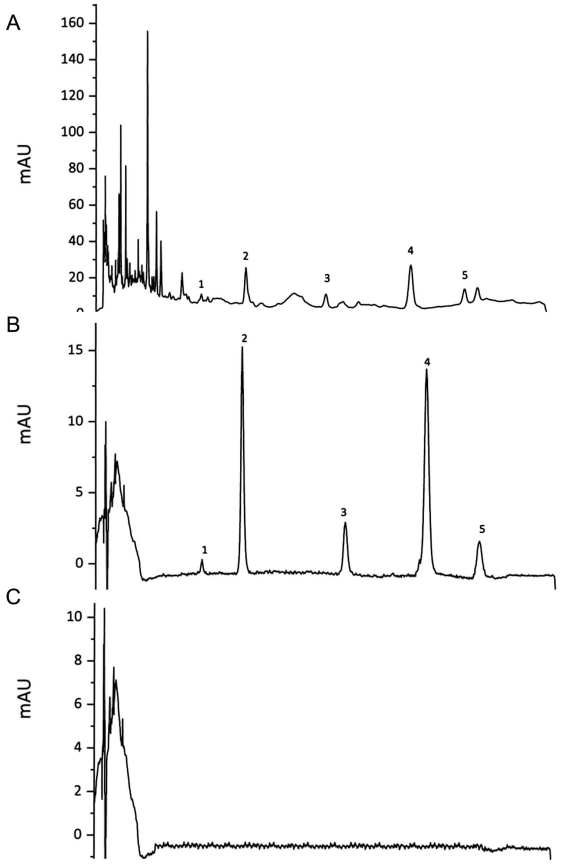


Figure 4 HPLC chromatograms of a mixed reference substance (A), sample (B), and blank solvent (C). Peak 1 for benzoyl neoaconitine, peak 2 for benzoyl hypoaconitine, peak 3 for neoaconitine, peak 4 for periplocin, and peak 5 for methyl salicylate. HPLC, high-performance liquid chromatography.

Discussion

In this study, pseudoternary phase diagram, D-optimal mixture design, and new dosage form were used to improve the dosage form of crossbow medicine of the Miao ethnic group and an O/W microemulsion skin preparation was prepared. Simultaneously, the physical and chemical properties of crossbow medicine microemulsion were evaluated, which provided an experimental basis for its popularization and application.

The pseudoternary phase diagram is widely used in the prescription screening of the self-microemulsion drug delivery system, often used in screening oil, surfactant, and cosurfactant [27–29]. Isopropyl myristate was used as an oil phase, which reportedly has a high affinity with the skin and increases the solubility of water-insoluble components [30, 31]. The hydrophilic lypophilic balance value of polyoxyethylene castor oil-35 as emulsifier was 13.3, and the best hydrophilic lypophilic balance value was between 11 and 15 [32]. Polyoxyethylene castor oil-35 was often used to prepare a microemulsion of insoluble components [33]. Additionally, in the microemulsion preparation process, cosurfactants can help surfactants reduce interfacial tension and make surfactants have more adsorption at the interface between oil and water [34]. Related studies showed that [35, 36], low-chain alcohols could be freely distributed between the oil and water phases, reducing interfacial tension and contributing to microemulsion formation. Ethanol was often selected as a cosurfactant in microemulsions.

Optimal mixture design is an experimental design method integrating mathematics and statistics. It accurately obtains the relationship between factors and effects with the least number of experiments and is often used to optimize the formulation process of preparations [37, 38]. D-optimal mixture design was used to optimize the best proportion of auxiliary materials and evaluated by particle size, solubility, and PDI. In the preparation process, the change of the proportion in microemulsion excipients had little effect on the solubility. The contour and response surface diagrams revealed that the best ratio of water: oil: surfactant: cosurfactant was 74: 3: 17.25: 5.75. The particle size was 19.83 ± 0.777, and PDI was 0.147 ± 0.02, which indicated that the particle size distribution of the crossbow drug microemulsion was uniform, and the whole system was relatively stable. Therefore, the ratio of water: oil: surfactant: cosurfactant was 74: 3: 17.25: 5.75.

Additionally, traditional therapy of crossbow medicine liquid is made by soaking in white wine for 14 days. Still, this method takes too long and is not conducive to modern research and production. Some studies showed that the best extraction process of the prescription could be screened through the orthogonal design combined with a pharmacodynamic test [39]. Therefore, the research group used $L_9(3^4)$ combined with anti-inflammatory and analgesic tests to screen out the alcohol extraction process of crossbow medicine in the early stage. Since most of the effective components of crossbow medicine are water-insoluble components, microemulsion can be used as the carrier of insoluble components [14]. Thus, crossbow medicine is prepared into O/W microemulsion to increase the solubility of fat-soluble components. Simultaneously, we evaluated the appearance, particle size, PDI, and zeta potential of the crossbow microemulsion, which all met the requirements of the *Chinese Pharmacopoeia* [22]. A method for

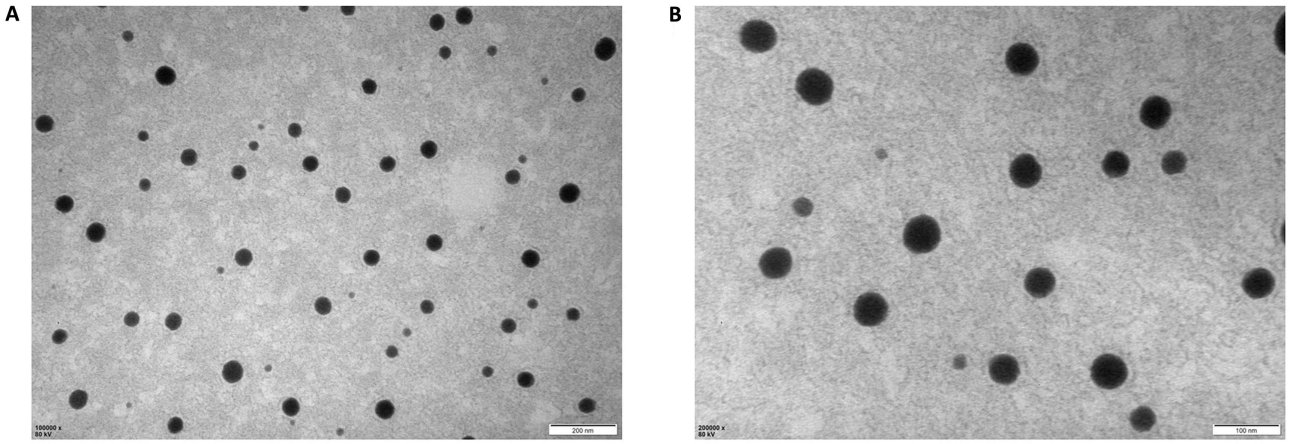


Figure 5 TEM of the crossbow medicine microemulsion. A, scale: 200 nm; B, scale: 100 nm. TEM, transmission electron microscope.

determining five components in crossbow medicine microemulsion was established, which had strong specificity, high sensitivity, and good accuracy. These studies showed that the preparation process of crossbow medicine microemulsion was reasonable and feasible.

This study has some limitations. The drug release mechanism of crossbow drug microemulsion in vivo and in vitro has not been studied. In the future, the drug release mechanism of crossbow drug microemulsion, in vivo and in vitro, and its pharmacokinetic process in rats will be investigated.

Conclusion

Crossbow medicine, an external treatment preparation with Miao medicine characteristics, has obvious advantages in treating chronic inflammatory diseases, such as knee osteoarthritis. However, traditional extraction and preparation methods are difficult to meet the clinical needs and the modern development of the Miao ethnic group. Alcohol extraction was used as the extraction method. The pseudoternary phase diagram and D-optimal mixture design were used to screen the types and proportions of microemulsion components. Thus, this study provides an experimental basis for the modernization of crossbow medicine and provides feasibility and robustness for microemulsion in ethnic medicine.

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