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RESEARCH ARTICLE

Preparation and evaluation of novel microemulsion-based hydrogels for dermal delivery of benzocaine

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Abstract

The purpose of the current research was to prepare and evaluate the potential use of microemulsion-based hydrogel (MBH) formulations for dermal delivery of benzocaine (BZN). The pseudoternary-phase diagrams were constructed for various microemulsions composed of isopropyl myristate (IPM) as oil phase, Span 20, Tween 20, Tween 80, cremophor EL and cremophor RH40 as surfactants, ethanol as cosurfactant and distilled water as aqueous phase. Finally, concentration of BZN in microemulsions was 2% (w/w). The physicochemical properties, such as conductivity, viscosity, pH, droplet size, polydispersity index and zeta potential of microemulsions, were measured. Carbopol 940 was used to convert BZN-loaded microemulsions into gel form without affecting their structure. Furthermore, excised rat abdominal skin was used to compare permeation and penetration properties of BZN loaded M3 and M3BHs with BZN solution. According to *ex vivo* study results, BZN-loaded M3BH1 showed highest flux values and high release rate values, and furthermore, this gel formulation had low surfactant content. Finally, in order to learn the localization of formulations within the dermal penetration, formulations and BZN solution were labeled with red oil O and subjected to fluorescence observation. In conclusion, BZN-loaded MBHs could be offered as a promising strategy for dermal drug delivery.

Keywords

Benzocaine, dermal drug delivery, characterization, fluorescence observation, microemulsion, microemulsion-based hydrogel

History

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Introduction

Dermal drug delivery has been an encouraging concept for a long time since skin is easy to access, has a large surface area with vast exposure to the circulatory and lymphatic networks and the route is noninvasive[1,2]. The benefit of dermal systems includes suitability, enhanced patient compliance and elimination of hepatic first-pass effect[3].

Local anesthetics are a class of drugs able to induce pain relief by virtue of their ability to bind to the sodium channel of excitable membranes, thus blocking the influx of sodium ions and the conduction of the nervous impulse[4,5]. Benzocaine (BZN), or para-amino benzoic acid ethyl ester (molecular formula $C_9H_{11}NO_2$, molecular weight 165.189 g/mol), is a local anesthetic used primarily to relieve pain or irritations on the skin and mucosal surfaces[6,7]. It is one of the ester types of long-acting local anesthetics, which has been used for the relief of local pain[8].

Several works describe that micro or nanoencapsulation of local anesthetic greatly prolongs the duration of block and reduces their systemic toxicity[9–12]. Therefore, the development of a new effective topical drug delivery system intended to suitably modulate the BZN release rate, thus prolonging its anesthetic effect, and to enhance its localization in the skin, thus reducing its systemic toxicity, could be particularly useful. Different techniques have been adopted to enhance skin drug absorption. Microemulsions can provide promising technique[13].

Microemulsions are thermodynamically stable, isotropically clear systems that have a droplet size smaller than 0.15 μm. They consist of oil, surfactant, cosurfactant and aqueous phase[14,15]. They have several pharmaceutical advantages, such as ease of preparation, transparency and potentials for solubilizing variety of drugs[16]. In addition to these, they are suitable vehicles for dermal and transdermal delivery of drugs[17,18]. This might be attributed to the interaction of their components with the stratum corneum to reduce its diffusion barrier, their ability to increase concentration and thermodynamic activity of drugs at the site of application, and their hydration effect on the stratum corneum[19]. While microemulsions offer numerous advantages for topical delivery, it is difficult to stabilize them on the skin surface because of low viscosity[20]. In order to overcome this problem, microemulsion-based hydrogel (MBH) formulations could develop by using microemulsions with gelling agents. MBH formulations have produced extensive interest as a potential topical delivery system. These types of formulations combine the advantages of microemulsions and gels. It has been stated that the components of microemulsion may reduce the diffusion barrier of skin and improve drug permeation. Therefore, it is promising for both transdermal and dermal delivery of drugs as an efficient route of drug administration[21].

The aim of this study was to develop novel BZN-loaded oil-in-water microemulsions and MBH formulation and to suggest a better formulation for BZN for dermal delivery. For this aim, the physicochemical characterization, *in vitro* release, stability

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and *ex vivo* permeation/penetration of these formulations were evaluated.

Materials and methods

Materials

BZN was purchased from Acros Organics (Geel, Belgium). IPM, Span 20, carboxymethylcellulose sodium and oil red 0 were purchased from Sigma (Steinheim, Germany). Tween 20 (polyoxyethelene sorbitan monolaurate), Tween 80 (polyoxyethylene sorbitan monooleate), *n*-octanol and ethanol were purchased from Merck, Darmstadt, Germany. Cremophor EL (macrogolglycerol ricinoleate) and cremophor RH40 (PEG-40 hydrogenated castor oil) were kind gifts from BASF, Ludwigshafen Rhine, Germany. Carbopol 940 was purchased from Doga Ilac, Turkey. Dialysis membrane (Spectro/por Dialysis Mebrane, Spectra/por 4, diameter 16 mm, molecular weight of 12–14 kDa) were purchased from Spectrum (Phoenix). Distilled water was used throughout the study. All other chemical reagents and solvents were of analytical grade and used as received.

Preparation of microemulsion formulations

To find out the existence range of microemulsions, pseudoternary-phase diagrams were constructed using titration of series of oil and surfactant/cosurfactant (S/Cos) mixtures with water at ambient temperature ($25 \pm 2\,^{\circ}C$). After being equilibrated, the mixtures were assessed visually and then determined as being microemulsions. The phase diagrams have been constructed by using a software program[15]. All experiments replicated at least four times.

According to the microemulsion areas in the phase diagrams, four different microemulsion formulations were selected at different component ratios. The microemulsion systems were prepared using IPM as oil phase, Span 20, Tween 20, Tween 80, Cremophor EL and Cremophor RH40 as surfactants, ethanol as cosurfactant and distilled water as aqueous phase. After the resulting systems were equilibrated with gently magnetic stirring for 5 min, appropriate amount of BZN was dissolved in the final microemulsion. The final concentration of BZN in microemulsion systems was 2% (w/w).

Characterization of microemulsion formulations

To find out the suitability of microemulsions for dermal applications, the characteristic properties of developed microemulsions, such as pH, viscosity, refractive index, electrical conductivity, droplet size, polydispersity index (PDI) and zeta potential, were evaluated.

The average droplet/particle size and PDI of formulations were measured by dynamic light scattering method (Nano ZS, Malvern Instruments, UK). The particle size and PDI values were obtained by averaging of 10 measurements at an angle of 173° at 25 °C using disposable cells. The measurements were repeated five times at 25 °C.

Zeta potential was measured by using disposable plain folded capillary zeta cells (Malvern Zetasizer Nano ZS). The zeta potential was calculated from the electrophoretic mobility using the Helmholtz–Smoluchowski equation under an electrical field of 40 V/cm. The processing was done by the software installed in the system. The measurements were repeated five times at 25 °C.

The viscosities of formulations were measured by a viscosimeter (AND Vibro Viscometer- SV-10). The pH values of the formulations were determined by a digital pH-meter (Mettler Toledo, Switzerland). The refractive index values of formulations were evaluated using a refractometer (Atago RX-7000 CX, Japan). Electrical conductivity of the formulations was studied using a conductometer (Jenway 4071, UK) to determine the type of microemulsion. Experiments were performed at $25 \pm 2\,^{\circ}C$ five times for each sample, and the results are presented as mean ± SD.

Stability of microemulsions

For physical stability studies, microemulsion formulations were tested by applying centrifugation test. The microemulsions were subjected to centrifugation at $5.175 \times g$ for 30 min and observed for any phase separation. For heating and cooling cycling experiments, 10 mL microemulsion was subjected for six heating/cooling cycles between 4 °C and 45 °C with storage at each temperature for 48 h and assessed for their physical instability like phase separation and precipitation[22].

In addition, microemulsions were stored at $5 \pm 1\,^{\circ}C$ in the refrigerator and $25 \pm 2\,^{\circ}C$ with a relative humidity of $60 \pm 5\%$ and $40 \pm 2\,^{\circ}C$ with a relative humidity of $75 \pm 5\%$ for 3 months in the stability cabinets (Nüve, Turkey). After storage for 3 months, the physical appearance of formulations was analyzed. Microemulsions were also evaluated for the changes in particle size, zeta potential, electrical conductivity, viscosity and pH. The experiments were repeated five times[14,15].

BZN was determined spectrophotometrically at 327 nm. The method was validated for selectivity, linearity, precision, accuracy, recovery and stability. The correlation coefficient (r^2) of determination was 0.998.

Preparation of microemulsion-based hydrogels

MBHs were prepared using 1% or 2% of Carbopol 940 or carboxymethylcellulose sodium by swelling in the microemulsion for 24 h. Carbopol or carboxymethylcellulose sodium was slowly added in to microemulsion under magnetic stirring. Triethanolamine was added dropwise into mixture till a semisolid gel-like consistency was obtained. The pH at gel consistency stage was within 6–8. All developed MBHs were inspected for their homogeneity. Spreadability test, color, syneresis and the presence of lumps by visual inspection after the gels have been set in the container.

Characterization of MBHs formulations

The viscosity of the MBH formulations was performed using a digital viscometer (Brookfield) equipped with spindle RV2 with 50 rpm. The pH of the MBH formulations was measured with a pH meter (Mettler Toledo, Greifensee, Switzerland). For the determination of BZN in the MBHs, 2 g of each MBH formulation was accurately weighed in 25-mL volumetric flask and dissolved in 20 mL ethanol. This solution was filtered using Millipore filter (0.45 μm). The blend was mixed at 25 °C for 24 h to dissolve the gel completely. Then, the solution was properly diluted and measured by UV-Spectrophotometer at 327 nm. Each experiment was performed in triplicate.

To determine spreadability of MBHs, 1 g of MBHs were transferred to the center of a glass plate (10 cm × 10 cm) and this glass plate was compressed under another glass plate of the same size. Thus, the gel was spread out in between the plates. After one minute, the weight was removed and the diameter of the spread area (cm) was measured. The measurement was performed in triplicate[23].

Stability of MBHs formulations

The MBS formulations were packed in tubes and stored at $5 \pm 1\,^{\circ}C$ in the refrigerator and $25 \pm 2\,^{\circ}C$ at room temperature for 3 months. After storage for 3 months, the physical appearance of formulations was investigated. They were also evaluated for the changes in pH, viscosity, drug content. The experiments were repeated four times. Those formulations, which passed these stability tests, were selected for further study.

Evaluation of *in vitro* benzocaine release

A synthetic membrane (Spectra/por Dialysis Membrane, Spectra/por 4, diameter 16 mm, molecular weight of 12–14 kDa) was mounted on a diffusion cell. The receiver compartment (10 mL) was consisted of ethanol and PBS pH 7.4 (ratio of 20:80) to ensure sink condition. 0.5 mL of the microemulsions and MBHs were applied to the membrane. The donor cell was exposed to ambient temperature and covered with parafilm to prevent evaporation. The temperature of the receptor compartment was maintained at 37 °C, while the buffer solution was stirred continuously with a magnetic bar (600 rpm). Samples (0.5 mL) were withdrawn from the release medium at 0, 1, 2, 3, 4, 5, 6, 7 and 8 h. The samples were analyzed by UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan) at 327 nm.

Ex vivo studies

Preparation of rat abdominal skin

All experiments were performed according to Declaration of Helsinki for the use of animals in research and approved by the Local Animal Ethical Committee (Approval No. 38828770–604.01.01.-E.1823). Male Wistar albino rats weighing 250 ± 20 g were purchased from the Experimental Animal Center of Istanbul Medipol University (Istanbul, Turkey) for the *ex vivo* and microscopic studies. Rats were housed in a room maintained at 22 ± 1 °C with an alternating 12-h light/dark cycle. Animals had free access to pellet diet and water *ad libitum*. The rats were transported to a quiet laboratory at least 1 h before the experiment began. For the *in vitro* studies, all experiments were performed between 09:00 and 12:00 h in normal room light and temperature (22 ± 1 °C). Male rats were sacrificed using anesthetic ether. The hair of test animals was carefully trimmed with electrical clippers and the full thickness skin was removed from the abdominal region. The skin was washed with water, stored at 4 ± 1 °C overnight and then used for *ex vivo* permeability studies.

Permeation and penetration

Franz diffusion cell with an effective diffusion area of 0.53 cm^2 was used for *ex vivo* permeation studies of BZN formulations and BZN solution (2% BZN dissolved in IPM). The skin samples were mounted carefully on diffusion cells. The receiver compartment was consisted of 10 mL ethanol:PBS (ratio of 20:80) to ensure sink condition and its temperature was maintained at 37 ± 0.5 °C with magnetic stirring at 600 rpm throughout the experiment. To prevent evaporation parafilm was used. For each experiment, 1 mL sample of the receiver medium was withdrawn at predetermined time. The samples were analyzed in UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan) at 327 nm. The cumulative amount (%, w/w) of BZN permeated through rat abdominal skin was plotted as a function of time.

After 8 h of contact time, each skin was washed for penetration study. Each skin was cut into minute pieces to determine the amount of BZN deposited in the skin. They were pooled in a tube containing ethanol, vortexed for 20 min and homogenized to obtain a solution. The obtained solution was sonicated for half an hour and again vortexed for 30 min and centrifuged for 30 min. Penetrated BZN (obtained after digestion) were assayed by means of UV spectrophotometer. Each experiment was run in three times.

Analysis of the permeation data

The experiments were carried out in triplicate for each sample and the results were presented as an average ± SD. Average cumulative amount of drug permeated per unit surface area of the skin was plotted versus time. The slope of the linear portion of the plot was calculated as flux (*J*ss)[24], and the permeability coefficient was calculated using Equation (2). Permeability coefficient (K_p, cm/h) was calculated by dividing the flux with initial concentration of drug in the donor compartment. Lag time (h) was calculated from the x intercept of the linear portion of the plots of cumulative amount of drug permeated versus time in steady state conditions. Diffusion coefficient (*D*) values were calculated from the release rate values.

$$K_p = \frac{Jss}{C_v}$$

where K_p was the permeability coefficient, and C_v was the total amount of the drug.

The enhancement of drug penetration due to microemulsion-based formulations was noted as enhancement ratio (ER)[25], which was calculated using the following Equation (3).

$$\mathrm{ER} = \frac{\text{Flux from formulations}}{\text{Flux from BZN SOL}}$$

Fluorescence observation

To study the formulations localization within the dermal penetration, formulations were labeled with oil red 0 (0.01% w/w) and subjected to fluorescence observation. Full-thickness abdominal rat skins without subcutaneous tissue were applied to catch a whole view of penetration. 0.5 mL oil red O-labeled formulations was added to rat abdominal skins. After 1-h exposure, the skin was removed, cleaned remaining formulation and frozen at −80 °C. Cryotome sections of 20 μm thickness using a cryostat (LEICA CM 1950, Germany) and they were subjected to normal and fluorescence light microscopy (Axiozoom V16, equipped with AxioCam HRM, Germany).

Histological examination of skin

Abdominal rat skin without subcutaneous tissue was exposed to formulations for 1 h. After 1-h exposure, the skin was removed, cleaned remaining formulation and frozen at −80 °C. Cryotome sections of 20 μm thickness and these samples were then observed under microscope (AxioCam HRM camera, Axiozoom, Germany).

Results and discussion

For topical administration, local anesthetic drugs are used to alleviate unpleasant sensations by preventing or diminishing the conduction of sensory nerve impulses near to the site of their application[26]. In order to produce an effective nerve block in reasonable time, anesthetic drugs are not only localized on the skin surface but also are prompted to pass into the skin. Nowadays, EMLA® is the most successful commercially available formulation to obtain topical anesthetic effect. In addition, it is a water-in-oil emulsion consisting of lidocaine and prilocaine, thickened with Carbopol[27]. However, this commercial product has some disadvantages, for example, long action time like 1 h or more due to high pH value of the formulation. Another disadvantage, at least for dermal usage, is that it requires occlusive dressing for deep dermal penetration. Therefore, there is need for new drug delivery systems in order to have local anesthetic effect. To overcome these disadvantages for topical anesthetic drug delivery, there are basically two different approaches. The first one is development of novel topical drug delivery systems, such as microemulsions, nanoparticles, liposomes, etc.[28]. Microemulsions have emerged as favorite colloidal

carriers of formulators around the world due to their simple and economical preparation, long-term stability, biocompatibility and high solubility degree of poorly soluble drugs[29]. It has been shown that dermal drug delivery may benefit from the characteristics of nanotechnology-based drug delivery systems especially microemulsions[14]. Recent studies indicated that microemulsions could increase the penetration of bioactive agents into dermal tissues and improve the dermal bioavailability with a good topical tolerance[2,13,16,29]. The second strategy has been to increase the drug bioavailability by prolonging the contact time of the formulation with stratum corneum. For this purpose, hydrogels have been used for prolonging drug residence time as well as reducing the application frequency and amount of drug administered and also for increasing patient compliance and acceptance[2]. These two strategies were combined in our present study; BZN-loaded microemulsions and MBHs were developed.

Preparation of BZN-loaded microemulsion formulations

IPM was selected as oil phase for the preparation of microemulsion systems. IPM as a permeation enhancer has a strong permeation enhancing effect and can increase the diffusion coefficient in skin[30]. In addition, for the production of microemulsions, usually high concentrations of surfactants and cosurfactants are required to develop these diagrams and thus attain the microemulsion region. Therefore, it is important to assess the dermal tolerance of these systems due to the possibility of irritation. In the present study five different surfactants namely Span 20, Tween 20, Tween 80, Cremophor EL and Cremophor RH40 were investigated for their suitability to form microemulsions possessing requisite formulation characteristics. Ethanol, which is commonly used in dermal microemulsions[13], was selected as a cosurfactant to prepare BNZ-loaded microemulsions. Moreover, Tween 20 and 80 are a nonionic surfactant, which are nontoxic when compared to ionic surfactants and has appropriate blend of low and high hydrophilic lipophilic balance (HLB), (HLB = 15–16) which can result in stable microemulsion[31]. In addition, the HLB value of all formulations was determined as 11.5.

The construction of phase diagrams makes it easy to find out the optimum concentration range of components for the existence range of microemulsions. The construction of pseudoternary-phase diagrams was used to obtain appropriate concentration ranges of components in the areas of forming microemulsions. The drug-free microemulsion formulation was selected from the gravity center of the phase diagram. All formulations were clear and transparent. The pseudoternary-phase diagrams for drug-free microemulsions were given in Figure 1. Compositions of microemulsion formulation according to the pseudoternary-phase diagrams and area values were presented in Table 1. The area of M1, M2, M3 and M4 microemulsions was determined as 670, 494, 732 and 711, respectively. M3 microemulsion has higher area than the other microemulsions. After BZN (2% (w/w)) was entirely dissolved in the final microemulsion, the clear microemulsion-based formulation was obtained with no phase change.

Characterization of microemulsions

In order to determine the physicochemical properties of each microemulsion, pH, viscosity, droplet size, PDI, zeta potential, refractive index and conductivity were measured. The physicochemical parameters and characterization of microemulsions in the presence and absence of BZN were listed in Table 2. The drug content in BZN-loaded microemulsions was within the range between 95.81 ± 0.21 and $100.67 \pm 0.31\%$.

Differences in droplet size of microemulsion formulations were noted. The droplet size of the formulations was found between 61.62 ± 1.423 nm and 167.9 ± 7.807 nm. Different surfactants were optimized in order to obtain the minimal droplet size distribution randomness with narrow PDI range. There are several mechanisms affecting the particle size of microemulsions. When the surfactant concentration increases, the particle size of microemulsions decreases. In addition, oil concentration and cosurfactant concentration affect the particle size as well. In this study, it is easily can be seen Span 20 that was used as surfactant is affecting the particle size. For example, microemulsion M3 that possesses the lowest Span 20 concentration (12%) has the lowest particle size. Microemulsion M2 and M4, which have the similar particle size, show that both have the same Span 20 concentration (16%). Microemulsion M1 has the highest Span 20 concentration (25%) and it has increased particle size than other developed microemulsions (M2, M3 and M4).

Small PDI of the developed microemulsions showed uniformity in the size distribution of droplets. PDI below 0.3 could be used as an indication of uniformity of droplets. This parameter could be used as an indication of stability of microemulsions. Zeta potential values of microemulsions were found neutral due to microemulsion components like nonionic surfactants.

The conductivity of four microemulsion formulations was found between 36.7 ± 0.043 mS/cm^{-1} and 53.8 ± 0.027 mS/cm^{-1}. The results of electrical conductivity meter analysis showed that microemulsions were in the form of oil-in-water phase system that would be regarded as suitable for dermal applications of BZN. The refractive indexes of all microemulsions were ranged between 1.408 ± 0.003 and 1.456 ± 0.001 and thus signify that prepared microemulsions were clear and transparent.

All formulations were found clear on visual inspection. The pH of a dermal preparation is an important factor for patient compliance. The pH of the formulations was found in between 4.962 ± 0.043 and 6.460 ± 0.116. Ideally, dermal formulations should possess pH in the range of 5–6, for minimizing discomfort of patient or irritation on the skin due to acidic pH and microbial growth on the skin because of basic pH[22]. The results of characterization study indicate development of successful BZN loaded microemulsion formulations with optimum characteristics.

Stability studies of microemulsions

Stability test is performed to ensure that developed microemulsions retain their suitability for use until the end of their expiration date. Centrifuged microemulsion formulations showed no phase separation or drug precipitation, indicating that prepared microemulsion was physically stable. Moreover, they showed no signs of breaking or cracking even when subjected to heating/cooling cycles. In contrast to regular emulsions, microemulsion vehicles are formed spontaneously when admixing the appropriate quantities of the components, without requiring additional mechanical energy, and they are 'infinitely' physically stable due to their thermodynamic nature[32].

The stability studies of BZN-loaded microemulsion formulations ($M1_{BZN}$-$M4_{BZN}$) were performed at $5 \pm 1\,^{\circ}C$, $25 \pm 2\,^{\circ}C$ and $40 \pm 2\,^{\circ}C$ for 3 months. After storage for 3 months, the physical appearance of formulations was found stable except $M4_{BZN}$ formulation. Phase separation was observed at $M4_{BZN}$ after 3 months storage. Microemulsions were also evaluated for the changes in particle size, zeta potential, electrical conductivity, viscosity and pH. Figure 2 shows changes in particle size and PDI of BZN loaded microemulsions at the end of 3 months. No significant changes of drug content, zeta potential, viscosity, pH and clarity of microemulsions ($M1_{BZN}$ and $M3_{BZN}$) were observed ($p > 0.05$). In addition to this, particle size and PDI value of $M2_{BZN}$ microemulsion was increased after 3 months at $5 \pm 1\,^{\circ}C$, $25 \pm 2\,^{\circ}C$ and $40 \pm 2\,^{\circ}C$ ($p < 0.05$).

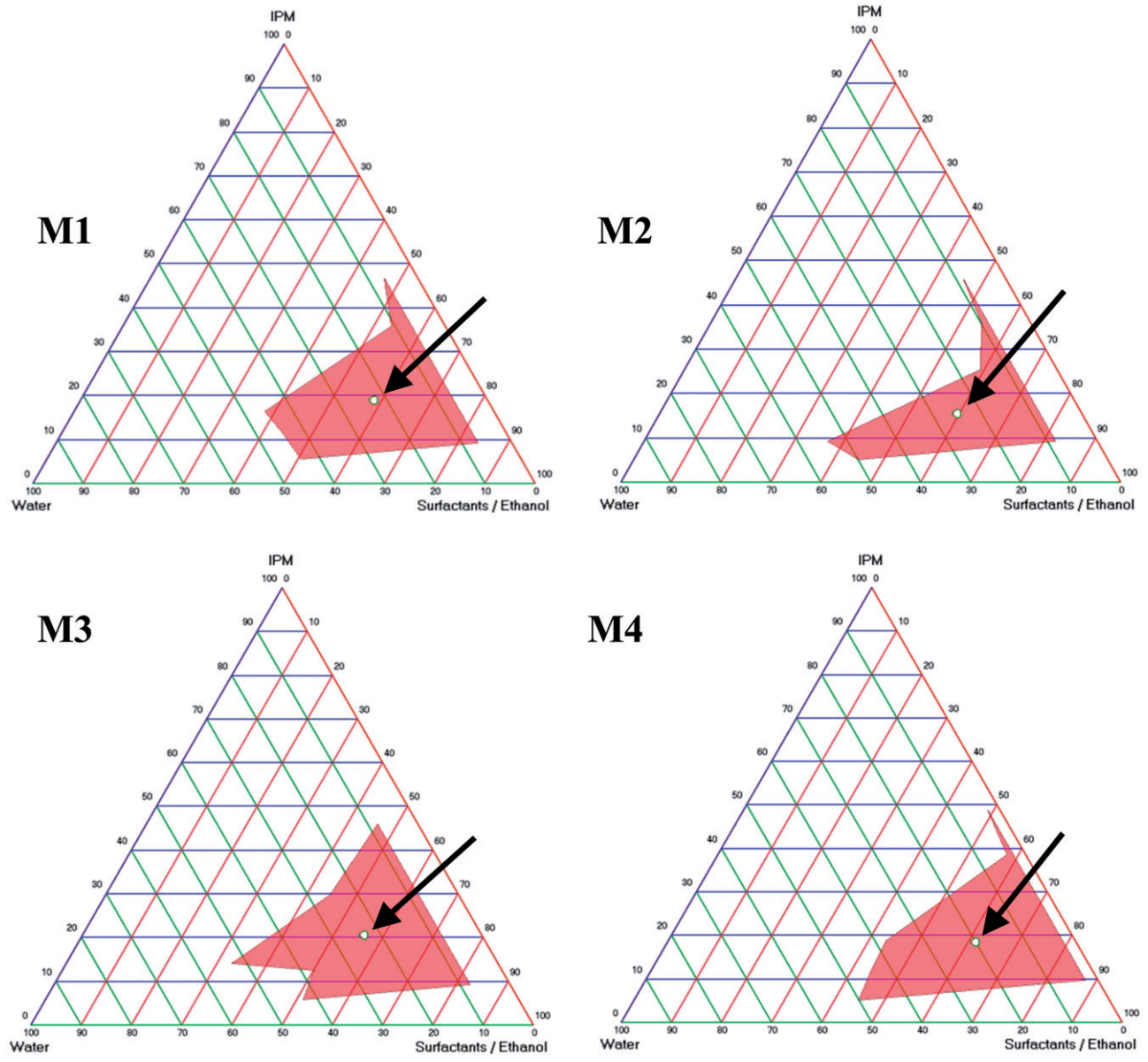

Figure 1. The pseudoternary-phase diagrams of microemulsion formulations (M1, M2, M3 and M4) composed of IPM, Span 20, ethanol and different surfactant (M1 Tween 20, M2 Tween 80, M3 Cremophor EL, M4 Cremophor RH40).

Table 1. composition of optimum microemulsion formulations (M1, M2, M3 and M4).

Components (%)	M1[a]	M2[b]	M3[a]	M4[b]
IPM	18.63	15.41	20.64	18.54
Span 20	25.07	16.30	12.75	16.81
Tween 20	13.98	-	-	-
Tween 80	-	13.50	-	-
Cremophor EL	-	-	24.65	-
Cremophor RH40	-	-	-	13.93
Ethanol	19.52	29.81	18.70	30.75
Distilled water	22.60	24.99	23.25	19.97

[a]Surfactant/cosurfactant ratio = 2:1.
[b]Surfactant/cosurfactant ratio = 1:1.

When characterization and stability studies were taken into consideration, $M1_{BZN}$ and $M3_{BZN}$ formulations were selected for further studies because of high viscosity values and good stability. M1 and M3 microemulsions have higher viscosity than M2 and M4, but this is not enough for extending the residence time of formulations on the skin surface. To overcome this problem, MBH formulations were prepared with gelling agent Carbopol 940 and carboxymethylcellulose sodium.

Preparation and characterization of MBHs

Carboxymethylcellulose sodium salt and Carbopol 940 were evaluated for their potential use in the microemulsions as different gelling agents. Selection of the suitable gelling agent was made on the basis of compatibility with the components of microemulsions. It was detected that carboxymethylcellulose sodium salt was not able to convert the BZN-loaded microemulsions into gel. This inefficiency could be attributed to their susceptibility to clot in the presence of high concentrations of surfactants. Similar with our results, Olariu et al. prepared propranolol hydrochloride loaded MBH with methylcellulose, carboxymethylcellulose sodium salt and hydroxypropylmethylcellulose. They found that cellulose derivatives were not able to gel[33]. For the preparation of the MBH, gelling agent Carbopol 940 was selected and dispersed in microemulsion at 1% (MBH1) or 2% (MBH2) concentration (w/w). Carbopol is mainly used gelling agent in the preparation of MBH formulations[20,33].

Table 2. Characterization of the developed blank and BNZ-loaded microemulsion formulations (mean ± SD, $n = 5$).

Formulation/ parameters	pH	Droplet size (nm)	PDI	Zeta potential (mV)	Refractive index	Conductivity (mS/cm)	Viscosity (cP)
M1	5.051 ± 0.045	141.8 ± 3.909	0.127 ± 0,091	0.325 ± 0.047	1.415 ± 0.001	47.7 ± 0.027	38.5 ± 0.12
$M1_{BZN}$	4.962 ± 0.043	167.9 ± 7.807	0.107 ± 0.072	0.319 ± 0.079	1.438 ± 0.002	40.4 ± 0.050	38.63 ± 0.15
M2	5.875 ± 0.020	91.40 ± 2.304	0.184 ± 0.026	0.0694 ± 0.018	1.408 ± 0.003	53.8 ± 0.027	29.6 ± 0.2
$M2_{BZN}$	6.010 ± 0.039	98.50 ± 2.889	0.148 ± 0.012	0.0442 ± 0.017	1.417 ± 0.002	45.0 ± 0.016	29.8 ± 0.1
M3	5.404 ± 0.055	65.45 ± 1.497	0.337 ± 0.018	0.425 ± 0.018	1.442 ± 0.001	44.0 ± 0.004	60.9 ± 0.1
$M3_{BZN}$	5.516 ± 0.140	61.62 ± 1.423	0.291 ± 0.021	−0,774 ± 0.013	1.456 ± 0.001	36.7 ± 0.043	61.13 ± 0.15
M4	6.383 ± 0.095	97.03 ± 1.974	0.117 ± 0.036	0.0022 ± 0.013	1.435 ± 0.001	44.9 ± 0.022	32.8 ± 0.12
$M4_{BZN}$	6.460 ± 0.116	108.1 ± 2.374	0.098 ± 0.055	−0,477 ± 0.028	1.462 ± 0.002	37.7 ± 0.03	33.13 ± 0.15

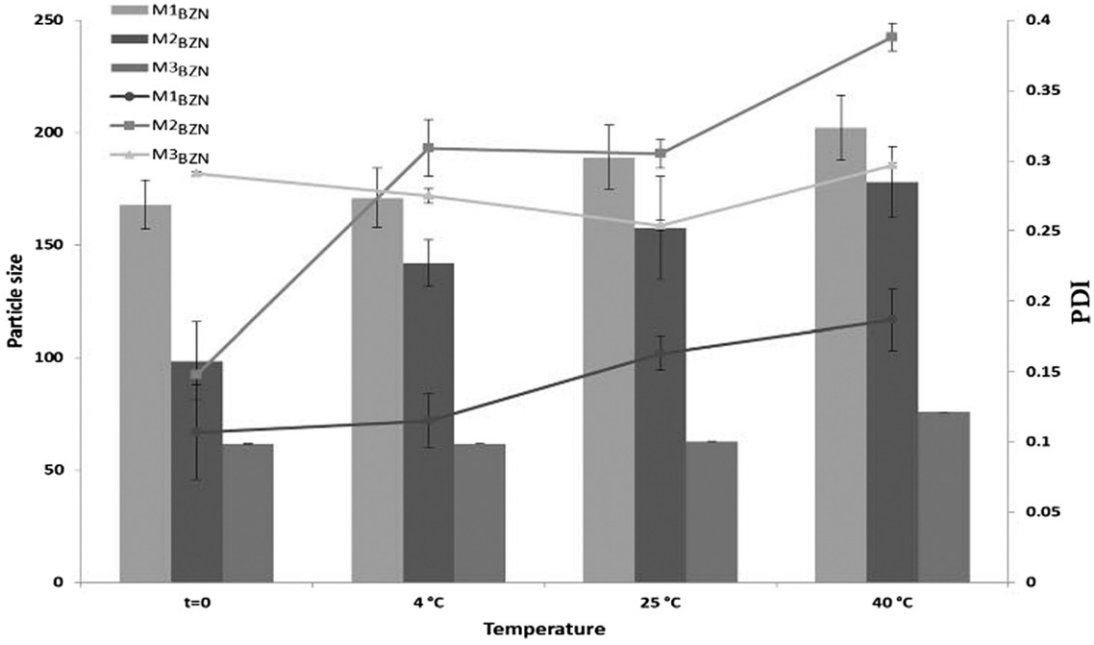

Figure 2. The changes in particle size and PDI of $M1_{BZN}$, $M2_{BZN}$ and $M3_{BZN}$ at $t = 0$ (initial time), 4 °C, 25 °C and 40 °C (mean values ± SD; $n = 3$) at the end of 3 months.

The prepared BZN-loaded MBHs were evaluated for drug content, viscosity, spreadability and pH. The drug content in BZN-loaded MBHs was within the range between 95.91 ± 0.26% and 99.21 ± 0.19%. The developed MBHs had pH values varying from 6.72 ± 0.013 to 7.75 ± 0.032, suitable for topical application. The viscosities of the $M1BH1_{BZN}$ (BZN-loaded M1 microemulsion-based hydrogel containing 1% Carbopol), $M1BH2_{BZN}$, $M3BH1_{BZN}$ and $M3BH2_{BZN}$ were detected 241 ± 0.001, 387 ± 0.003, 273 ± 0.002, 395 ± 0.001 cP, respectively (Table 3). $M1BH1_{BZN}$ formulation was found 10 times viscous than $M1_{BZN}$. $M3BH1_{BZN}$ formulation was 4.5 times viscous than $M3_{BZN}$. Thus, it is clear that MBHs can stay longer than MEs on the skin surface. Spreading diameter of the developed MBHs was found similar results. In addition to this, MBH1s (containing 1% Carbopol) is higher than MBH2s (containing 2% Carbopol). Similarly, Chaudhary et al were found that with increase in the concentration of the polymer, viscosity of the solution was increased; spreadability of the formulation was reduced[34].

To evaluate the stability of MBH formulations, they were packed in tubes and stored at 5 ± 1 °C in the refrigerator and 25 ± 2 °C at room temperature for 3 months. After storage for 3 months, no change was observed for physical appearance of MBHs. Moreover, no changes of pH, viscosity and drug content of formulations were determined.

In vitro BZN release studies

In order to assess the performance of the formulations, the BZN-loaded microemulsions and MBHs were studied for *in vitro* release through synthetic membrane. The results are listed in Table 4. As shown in Table 4, the addition of 1% Carbopol was not significantly affect the flux value ($p > 0.05$) because of slightly increase viscosity of microemulsions. However, the flux values of 2% Carbopol MBHs were significantly lower ($p < 0.05$) than that of microemulsions because they were increased viscosity at least 7 times. BZN-loaded M3 and M3-based hydrogels ($M3BH1_{BZN}$ and $M3BH2_{BZN}$) were higher flux values than M1 and M1-based gels. Thus $M3_{BZN}$ and $M3BH_{BZN}$ were selected for further *ex vivo* studies.

Ex vivo permeation and penetration studies

Rat abdominal skins were used for *ex vivo* permeation and penetration studies. The *ex vivo* studies from the selected formulations ($M3_{BZN}$, $M3BH1_{BZN}$ and $M3BH2_{BZN}$) were performed with diffusion cell in pH 7.4 buffer solution using the excised rat abdominal skin. BZN release profiles over time from the various developed formulations and BZN solution are shown in Figure 3.

Permeability parameters like a steady-state flux (*J*ss) and permeability coefficient (K_p) and lag time (h) as shows in Table 5. Flux and K_p values were significantly increased ($p < 0.05$) in

Table 3. Characterization of the developed BNZ-loaded MBHs (mean ± SD, $n = 5$).

Formulations	$M1BH1_{BZN}$	$M1BH2_{BZN}$	$M3BH1_{BZN}$	$M3BH2_{BZN}$
pH	6.72 ± 0.013	6.88 ± 0.024	7.73 ± 0.015	7.75 ± 0.032
Viscosity (C_P)	241 ± 0.001	387 ± 0.003	273 ± 0.002	395 ± 0.001
Drug content (%)	95.91 ± 0.26	96.83 ± 0.15	99.21 ± 0.1	97.13 ± 0.24
Spreadability (cm)	11.16 ± 0.46	10.86 ± 0.15	10.83 ± 0.90	9.13 ± 0.32

Table 4. The permeation and release parameters of the BZN loaded formulations through synthetic membrane.

Formulation	*J*ss (mg/cm^2/h)	Lag time (h)	r^2	K_P ($\times 10^{-4}$cm/h)	D ($\times 10^{-4}$cm^2/h)
$M1_{BZN}$	0.665 ± 0.011	0.361 ± 0.032	0.954 ± 0.034	332.70	0.018 ± 0.0001
$M1BH1_{BZN}$	0.636 ± 0.014	0.581 ± 0.021	0.995 ± 0.034	318.30	0.011 ± 0.0004
$M1BH2_{BZN}$	0.597 ± 0.008	0.347 ± 0.012	0.995 ± 0.013	298.80	0.019 ± 0.0001
$M3_{BZN}$	0.739 ± 0.006	0.985 ± 0.020	0.976 ± 0.035	369.55	0.0067 ± 0.007
$M3BH1_{BZN}$	0.718 ± 0.023	0.729 ± 0.022	0.948 ± 0.018	359.00	0.0091 ± 0.002
$M3BH2_{BZN}$	0.606 ± 0.030	0.567 ± 0.036	0.995 ± 0.041	303.40	0.011 ± 0.0005

*J*ss: Steady-state flux, K_p: Permeability coefficient, D: Diffusion coefficient.

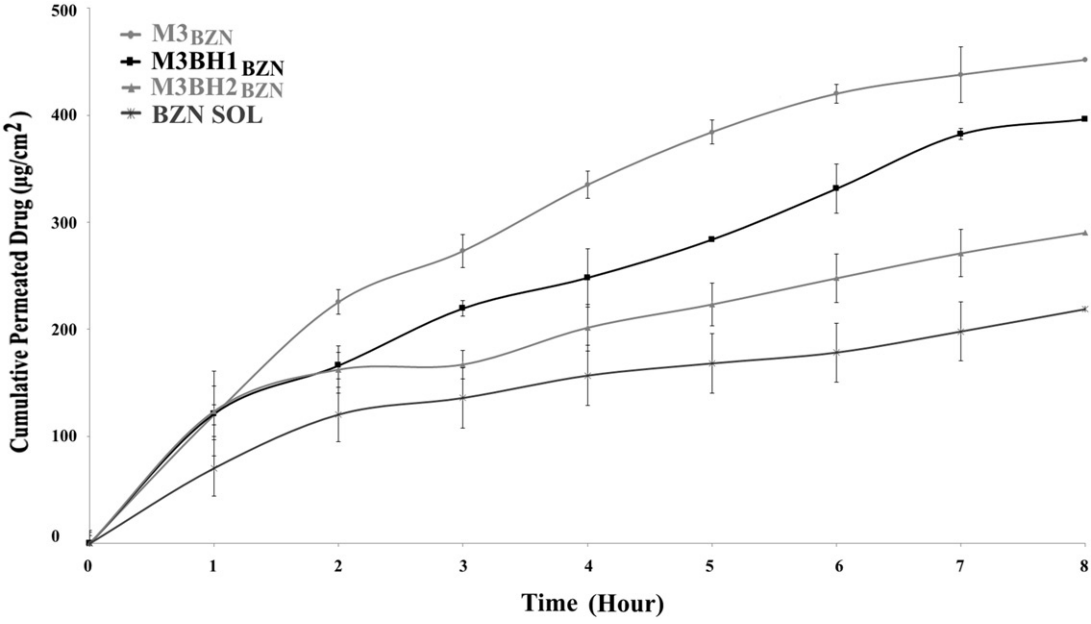

Figure 3. *Ex vivo* BZN permeation profiles through rat abdominal skin from microemulsion, microemulsion-based hydrogels and BZN solution (mean ± SD, $n = 3$).

Table 5. Permeation parameters and penetration % BZN in the rat abdominal skin after 8 h.

Formulation	*J*ss (mg/cm^2/h)	Lag time (h)	r^2	K_P ($\times 10^{-4}$cm/h)	D ($\times 10^{-4}$cm^2/h)	ER	Penetration (%)
$M3_{BZN}$	0.632 ± 0.006	0.847 ± 0.020	0.976 ± 0.035	316	0.0078 ± 0.0004	1.26	9.379 ± 0.120
$M3BH1_{BZN}$	0.584 ± 0.023	0.787 ± 0.022	0.990 ± 0.018	292	0.0084 ± 0.0001	1.16	7.931 ± 0.134
$M3BH2_{BZN}$	0.407 ± 0.030	0.798 ± 0.036	0.968 ± 0.041	203.5	0.0083 ± 0.0003	0.812	5.277 ± 0.021
BZN SOL	0.501 ± 0.021	0.608 ± 0.022	0.968 ± 0.031	250.5	0.0109 ± 0.0005	-	4.673 ± 0.064

*J*ss Steady-state flux, K_p: Permeability coefficient, D: Diffusion coefficient, ER: enhancement ratio.

$M3_{BZN}$ and $M3BH1_{BZN}$ as compared to $M3BH2_{BZN}$ and BZN solution. It means $M3BH2_{BZN}$ and BZN solution having prolonged drug release behavior as compare to $M3_{BZN}$ and $M3BH1_{BZN}$. Moreover, this can be thought that developed formulations contain permeation enhancers like surfactants and cosurfactant, which was also responsible for the increased permeation ability in comparison to the BZN solution. However, being higher viscosity, the permeation enhancers could not diffuse at the same rate as the low viscosity, which explains the lesser permeability of $M3BH2_{BZN}$ compared to $M3_{BZN}$ and $M3BH1_{BZN}$. Statistically significant difference was not detected for permeation between $M3_{BZN}$ and $M3BH1_{BZN}$ at the end of 8 h. In addition to this, enhancement ratio (ER) of $M3_{BZN}$, $M3BH1_{BZN}$ and $M3BH2_{BZN}$ was determined as 1.26, 1.16 and 0.812, respectively.

Numerous studies have reported that microemulsions increase skin permeation of incorporated drugs[35,36,37]. Skin penetration enhancers are commonly used in dermal formulations and are believed to work by transiently changing the barrier properties of

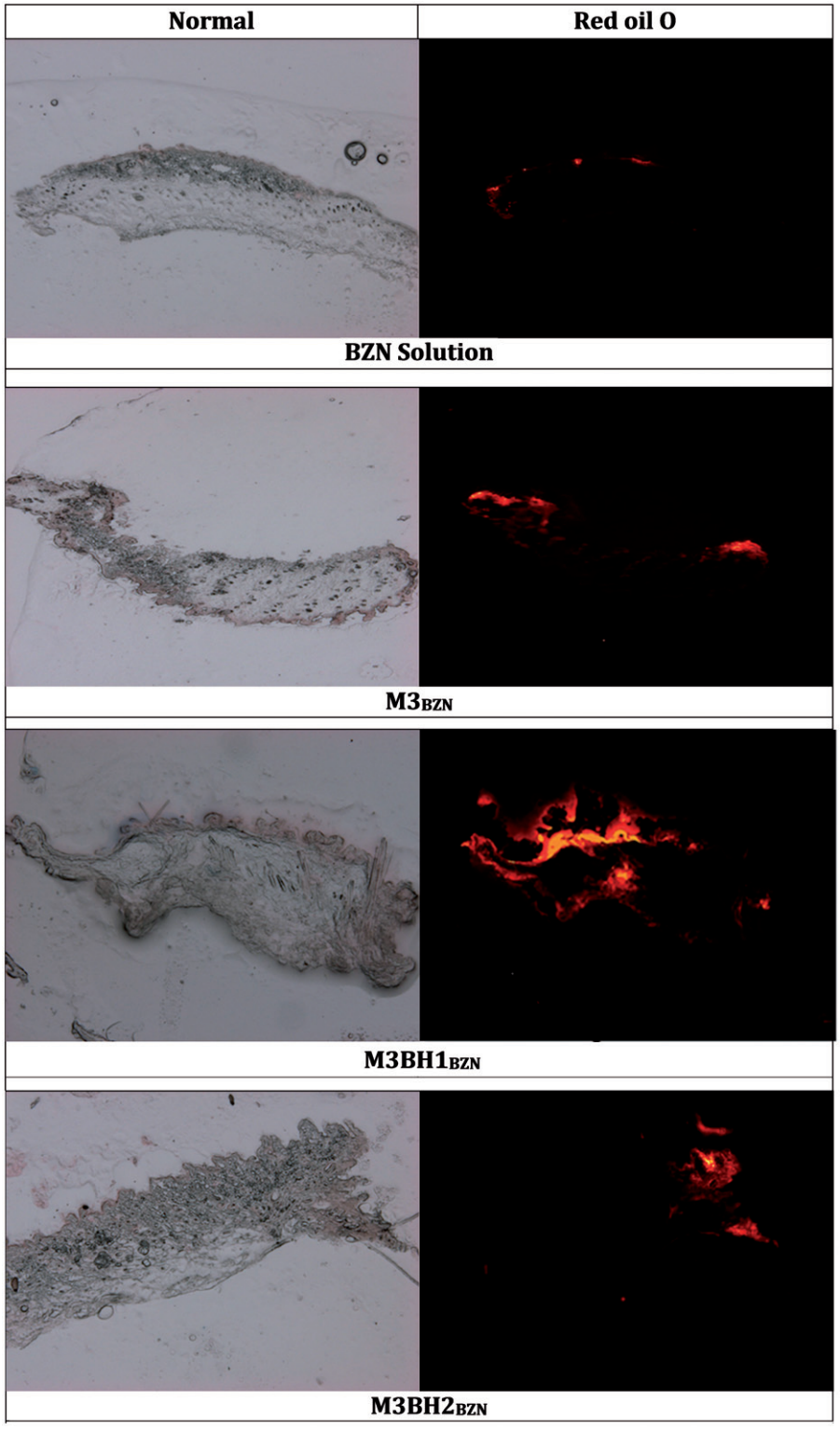

Figure 4. Penetration of red oil O-labeled formulations into rat skin after 1-h exposure.

the skin resulting in enhanced drug diffusion or partitioning into the stratum corneum[38,39]. Moreover, ethanol is used as cosurfactant at microemulsions and it shows promising results as penetration enhancer for dermal formulations[14]. Especially, M3 and M3BHs are containing surfactant and cosurfactant, which was also responsible for the increased permeation ability in comparison to the solution. The aptness of microemulsion to increase the concentration gradient and thermodynamic activity toward skin along with permeation enhancement activity of its components makes the system expedient for dermal delivery[40]. Similar to our study, El Maghraby et al. developed indomethacin and BZN-loaded eucalyptus oil-based microemulsion was used with Tween 80 and ethanol. Application of microemulsion enhanced the flux compared to saturated aqueous control[13].

Junyapraserta et al. studied oil-in-water and water-in-oil Brij 97-based microemulsions in comparison to their blank counterparts and to investigate the influence of microemulsion type on *in vitro* skin permeation of model hydrophobic drugs. They were used isopropyl palmitate, water and a 2:1 w/w mixture of Brij 97 and 1-butanol. Transdermal flux of drugs was investigated *in vitro* using modified Franz diffusion cells. They were discussed that the permeation data exhibited that the nature of the microemulsions

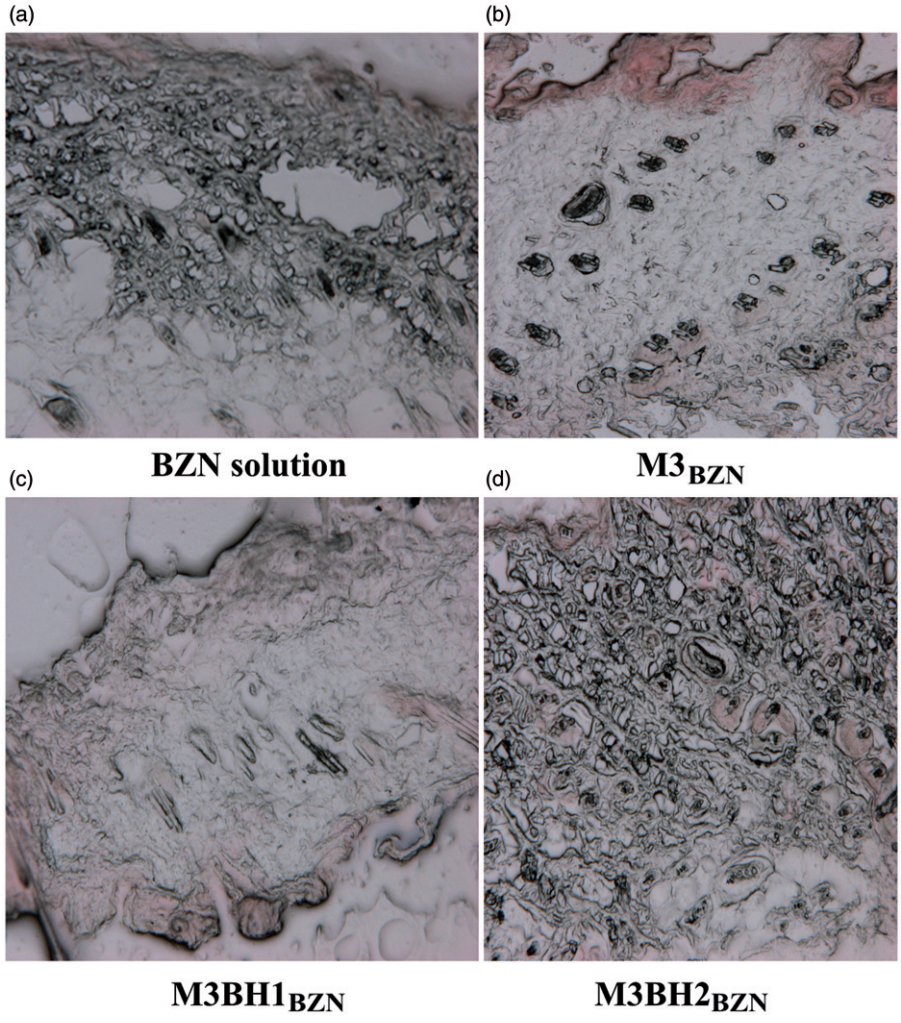

Figure 5. Microscopic images of treated rat skin with the (a) BZN solution, (b) $M3_{BZN}$ microemulsion, (c) $M3BH1_{BZN}$ and (d) $M3BH2_{BZN}$ (magnification values 125x).

was a crucial parameter for transdermal drug delivery. They stated that the oil-in-water microemulsions containing hydrophobic drugs provided the highest skin permeation enhancement[41]. In our study, oil-in-water-type microemulsions were developed to increase the BZN permeation.

Zhao et al. prepared ropivacaine loaded microemulsion and MBH and evaluated skin permeation of these formulations. They found that ropivacaine had a significant higher cumulative amount from microemulsion than that from MBG[42]. Similarly, the other researchers developed lornoxicam-loaded nanoemulsion and developed nanogel adding 1% w/w Carbopol 934. They have found that flux of nanoemulsion is more than nanogel[22].

When the penetration to the skin was examined, it was seen that $M3_{BZN}$, $M3BH_{BZN}$ formulations obtained higher penetrated BZN amount than BZN solution (Table 5). It can be concluded that higher permeability of BZN through the skin because of the presence of nanocarriers in the formulations. The modification of M3 with Carbopol (especially 2%) decreased the penetrated amount in a certain time. The increased viscosity value obtained by the addition of Carbopol was thought to lessen M3 motion in the formulation. Ferrari et al. have evaluated the effect of viscosity enhancer like chitosan on intestinal permeation and they have stated that the presence of chitosan in formulations provided controlled drug release and consequently, slower intestinal permeation[43].

Fluorescence observation

In order to better understand penetration behaviors of developed formulations, they were taken under further fluorescence observation. According to the skin penetration data, full-thickness rat abdominal skin was treated with oil red O-labeled formulations for 1 h. Images are obtained in normal and fluorescence mode of the same area. As shown in Figure 4, typical normal and the corresponding fluorescence images of the formulation, fluorescence emitted by oil red O-labeled $M3_{BZN}$ and $M3BH1_{BNZ}$ formulations provided direct signs of penetration, verifying the successful permeation across stratum corneum layer.

There are very few studies that have shown the skin permeation of the nanosized delivery systems into epidermal layers, where encapsulated a lipophilic fluorescent dye, was released into the deeper skin layers as observed with confocal microscope[44]. Lower intensity of fluorescence indicates a more intact barrier after exposure to formulations, while higher dye penetration signifies a more porous barrier[33]. Kong et al. developed hyaluronic acid based nanoemulsion and evaluated its penetration by fluorescence microscopy. They found that nanoemulsion indicated desirable percutaneous potency of lipophilic active ingredients for drug delivery[45]. An effective percutaneous drug delivery should be able to transport drugs not just across stratum corneum and perform good partitioning capacity into deeper tissue structures. When the penetration studies considering, $M3_{BZN}$ and $M3BH1_{BZN}$ formulations were showed highest penetration than BZN solution and $M3BH1_{BZN.}$ Fluorescent microscopy images were consistent with the results of skin penetration. It can be concluded that microemulsions can increase skin permeation of incorporated drugs, at the same time to further increase the permeation; designing a new formulation like MBH, which has high viscosity and composed of microemulsion ingredients is better than microemulsion (Figure 4).

Histological examination of skin

To determine the safety of the developed formulations, the abdominal skin was investigated after the application of the formulations. Figure 5 shows microscopic images of rat abdominal skin treated with developed formulations ($M3_{BZN}$, $M3BH1_{BZN}$ and $M3BH2_{BZN}$) and BZN solution.

The skins did not show any sign of inflammation (Figure 5) and no apparent signs of skin irritation (erythema and edema) were observed through visual examination of the skin sample treated with developed formulations. The results indicated BZN-loaded developed formulations could improve drug penetration without causing skin irritation and bring benefit to skin care.

Conclusion

In this study, microemulsions and MBHs were prepared, characterized and *in vitro* and *ex vivo* evaluated. The pseudoternary-phase diagram was used to optimize the microemulsion formulations. The present study showed that microemulsions of BZN can successfully be prepared with titration method with narrow particle size and PDI range. According to the results of the characterization, stability and *in vitro* permeation studies, the most desirable formulations for the topical delivery of BZN were considered the $M3BH1_{BZN}$. The formulations displayed high permeability through the skin. Moreover, no irritation was found in dermis and skin surface. The present study can open up a window for dermal application of microemulsions and MBHs loaded with BNZ, they would be a better alternative to conventional gels or creams in the treatment of various dermatological disorders (alleviate unpleasant sensation) with less systemic side effects.

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Declaration of interest

The authors declare no conflict of interest.

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