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RESEARCH ARTICLE

Metronidazole Loaded Novel Microemulsion Formulation for Topical Delivery and Characterization With Validated New UPLC Method

Tilbe ÇEVİKELLİ[*°], Umay Merve GÜVEN[**], Ahmet Alper ÖZTÜRK[***]

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SUMMARY

The purpose of the study is to develop metronidazole (MET) containing microemulsion systems for topical application, to overcome limitations of local antibacterial treatments. For preparation of drug loaded microemulsions pseudo-ternary phase diagram technique was applied. IPP as oil phase, Span 80 and Cremophor EL as surfactant, ethanol and propylene glycol as co-surfactant and distilled water was used as aqueous phase. Globule size ranging between 126.8±2.8 to 150.8±1.6 nm and PDI between 0.21±0.02 to 0.35±0.06 were obtained. Zeta potentials of the formulations measured as 0.48±0.08 – 0.68±0.14 mV and conductivity was between 0.5±0.0 and 0.6±0.0, implicating the formation of w/o emulsions. A UPLC method was developed and validated according to the ICH Q2 (R1) guideline, for quantification of MET, and drug content was calculated as 99.18±0.08 – 99.33±0.12%. MET release of 80.62±0.86% for S_{1MET} and 62.06±1.08% for S_{2MET} formulations at 24h, indicated the control over the MET release by the microemulsions. After 3. and 6. months, no difference observed in physicochemical properties of microemulsions, and MET release showed similar profile; implicating the good stability of formulations.

Key Words: *Metronidazole, microemulsion, release kinetics, stability, topical delivery, UPLC.*

Topikal Uygulama için Metronidazol Yüklü Yeni Mikroemülsiyon Formülasyonu ve Valide edilmiş Yeni UPLC Yöntemi ile Karakterizasyonu

ÖZ

Bu çalışmada, lokal etkili topikal antibiyotik uygulamalarında tedavi kısıtlarını aşmak amacıyla metronidazol (MET) içeren mikroemülsiyon formülasyonlarının geliştirilmesi amaçlanmaktadır. Mikroemülsiyon formülasyonları üçgen faz diyagramı tekniğiyle hazırlanmış olup; yağ fazı olarak IPP, yüzey etkin madde olarak Span 80 ve Cremophor EL, yardımcı yüzey etkin madde olarak etanol ve propilen glikol kullanılmış, ve su fazı olarak distile su ilave edilmiştir. Üretilen mikroemülsiyonların damlacık boyutu 126.8±2.8 - 150.8±1.6 nm, PDI değerleri 0.21±0.02 - 0.35±0.06 arasında kaydedilmiş olup; zeta potansiyelleri 0.48±0.08 – 0.68±0.14 mV ve iletkenlikleri 0.5±0.0 and 0.6±0.0 arasında olup yağ içerisinde su tipinde emülsiyon oluşumunu işaret etmektedir. Metronidazol miktar tayinini gerçekleştirmek amacıyla geliştirilen UPLC metodu, ICH Q2 (R1) kriterlerine göre valide edilmiş, ve mikroemülsiyonların MET içeriği %99.18±0.08 – 99.33±0.12 olarak tespit edilmiştir. S_{1MET} formülasyonunun %80.62±0.86 ve S_{2MET} formülasyonunun %62.06±1.08 salım gerçekleştirdiği belirlenerek, mikroemülsiyonların 24 saat boyunca kontrollü MET salımı sağladığı gösterilmiştir. Ayrıca, 3. ve 6. ay sonunda mikroemülsiyonların fizikokimyasal özelliklerini koruduğu ve MET salım profillerinin değişmediği tespit edilerek, formülasyonların iyi stabilite gösterdikleri tespit edilmiştir.

Anahtar Kelimeler: *Metronidazol, mikroemülsiyon, salım kinetiği, stabilite, topikal uygulama, UPLC.*

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[*] ORCID: 0000-0002-0881-0644, Department of Pharmaceutical Technology, Faculty of Pharmacy, Cukurova University, Adana, Turkey.
[**] ORCID: 0000-0003-1547-0817, Department of Pharmaceutical Technology, Faculty of Pharmacy, Cukurova University, Adana, Turkey.
[***] ORCID: 0000-0001-9596-0538, Department of Pharmaceutical Technology, Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey.

[°] Corresponding Author; Tillbe ÇEVİKELLİ
E-mail: tcevikelli@cu.edu.tr

INTRODUCTION

The major barrier for topical delivery is the complicated structure of the skin. Stratum corneum is the topmost layer of the skin, is constituted of dead cells, and this layer is a potential barrier to dermal applied drugs and limits their penetration. The stratum corneum functions as a barrier to hydrophilic drugs and macromolecules. On the contrary, lipophilic molecules can penetrate the intercellular lipids via the transcellular route. The primary obstruction is caused by stratum corneum to penetration of the drug. To overcome this obstruction, many nanosized drug delivery systems have been designed (Benson et al., 2019). Producing a novel therapeutic molecule is not only expensive and time-consuming, but it also frequently fails. However, enhancing these drugs' bioavailability, efficacy, or safety through a various methods may be a more coherent way to use them in the clinic. The researchers have thoroughly investigated several strategies, including stimulant-sensitive targeted pharmaceutical therapy, drug conjugates, therapeutic drug monitoring, and various drug delivery systems (Öztürk & Aygül, 2020; Öztürk et al., 2020). The efficiency of the topically applied antimicrobial drugs depends on the formulation's ability to overcome the stratum corneum barrier, and provide therapeutic activity in the affected area. So, nanosized drug delivery systems aiming to enhance topical penetration are essential to increase it's antimicrobial efficacy. Nanosized drug delivery is also preferred to improve solubility of the drugs, to minimize side effects and toxicity, to provide higher drug loading and to increase the bioavailability (Nagula & Wairkar, 2019).

Metronidazole (MET) is a commonly used antibacterial agent in the nitroimidazole class. MET can be used in oral, intravenous and topical dosage forms, and its cutaneous application focuses on the rosacea (Dallo et al., 2023).

Although MET provides antibacterial and antiprotozoal activity, it's molecular weight and low lipophilicity limits it's activity in topical applications (Dwipayanti et al., 2022). Also, topical antibacterial applications include several disadvantages including drug associated contact dermatisis as a common side effect, minimal depth of penetration resulting in efficacy in only superficial infections, and concerning wound impairment (Bandyopadhyay, 2021).

To overcome these limitations, drug delivery systems for topical application have evolved from simple solutions and creams; to multiphase nanotechnologies, in recent years. They include microemulsions, nanoemulsions, liposomes, niosomes, solid lipid nanoparticles, and dendrimers. Microemulsions are thermodynamically stable liquids; simple and optically isotropic systems constituted by oil, surfactants and water (Tiwari & Sivakumar, 2022). Due to content of surfactants and oil components, these drug delivery systems act as penetration enhancers, and increase the transdermal absorption of the active agent. Use of surfactant, cosurfactant and surfactant mixtures to prepare microemulsions designed for topical administration (Erdal et al., 2020), comprises them in both aqueous and lipid phases; and would be able to penetrate the skin.

Since microemulsions hold significant advantages for topical drug delivery, application of antibacterial agents by the microemulsion systems are well studied in the literature. Pandey et al. (2014) established a microemulsion based hydrogel system that provides 5 hours of MET release for treatment of periodontitis, while Tırnaksız et al. (2012) developed a microemulsion system providing 6 hours of MET release for remission of rosaceae. The purpose of the current study was to formulate and evaluate *in vitro* characterization and stability of microemulsion formulations maintaining MET release over 12 hours, as an antibacterial agent.

MATERIALS AND METHODS

Method validation of MET by UPLC

In this study, a new method was developed according to the literature and ICH criteria (Guideline, 2005; Öztürk et al., 2018; Öztürk et al., 2017).

Agilent Technology 1290 Infinity UPLC device was used with reversed-phase Zorbax® Eclipse Plus C18 gravity column (column length: 50 mm, column diameter: 2.1 mm, particle diameter: 1.8 µm). Mobile phase was consisted of 50:50 (v/v/v) acetonitrile: methanol with 0.01 M $KHPO_4$. Flow rate of the mobile phase was 0.2 mL/min and volume of injection was 0.5 µL. The temperature of the column was set to 40°C while a fluorescent detector was used at 318 nm.

Linearity

Analytes from a standard stock solution of 100 µg/mL of MET were prepared at nine different concentrations between 1-20 µg/mL as six different sets. Absorbance values of analytes were measured to calculate MET concentrations. Calibration curve was acquired by plotting concentration (x) versus peak area (y); regression equation and the correlation coefficient were calculated (n=6).

Limit of detection and limit of quantification (sensitivity)

Detection and quantification limits of an analyte with specified conditions, represent the methods sensitivity. The calibration curve method was applied to calculate the Limit of Detection (LoD) and Limit of Quantitation (LoQ) values of the developed UPLC method for MET quantification. By calculating the standart deviation of y-intercept and slope of curve as recommended by the ICH Q2 (R1) guideline, following equations were used to determine LoD and LoQ (Eq. 1, Eq. 2).

$$LOD = 3.3 \times \sigma/S \qquad \text{(Equation 1)}$$

$$LOQ = 10 \times \sigma/S \qquad \text{(Equation 2)}$$

σ = the standard deviation of the response and S = slope of the calibration curve.

Accuracy

Accuracy was determined by calculating the recoveries of known concentrations of MET. Analytes of 10 µg/mL, 30 µg/mL, 50 µg/mL of MET solutions were analyzed and accuracy was determined as the standard deviation of mean from the nominal concentration (n=6).

Precision

Precision is the variance, standard deviation, or coefficient of variation of a set of measurements in the ICH Q2 (R1) guideline. Precision criterion was verified by the repeated absorbance measurements of 10 µg/mL, 25 µg/mL, 50 µg/mL concentrations of MET, and expressed as the RSD% of the results (n=6).

Construction of pseudo-ternary phase diagram

Microemulsion systems must be consisted of specific ratio of three constituents namely: oil, water and mixture of surfactant and cosurfactant (S_{mix}). To establish the exact ratio of these microemulsion components, a pseudo-ternary phase diagram must be constructed. The pseudo-ternary phase diagrams were obtained by using water titration method at the room temperature (Puri et al., 2017).

Isopropyl palmitate (IPP) was selected as an oil component in the microemulsion systems (Alkholifi et al., 2023). Span® 80 and Cremophor EL were used as surfactants (S), while ethanol and propylene glycol were selected as the cosurfactant (CoS) in S/CoS weight ratios of 1:1, 1:2, 1:3, 1:4, 1:5 and 1:6. As surfactants Span 80 and Cremophor EL were used separately, and as cosurfactants, ethanol and propylene glycol were used in 1:1 ratio as a mixture. For fabrication of pseudo-ternary phase diagram, the weight ratio of oil to mixture of surfactant and cosurfactant at each S_{mix} was varied as 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1, respectively for development of 10 g microemulsion. The mixtures were examined visually and classified as

microemulsions, crude emulsions, or emulgels after being equilibrated (Bharti & Kesavan, 2017; Chen et al., 2007; Nandi et al., 2003).

Based on the microemulsion areas determined from the constructed pseudo-ternary phase diagrams, six formulations containing different proportions of oil, water, and S_{mix} were developed (Çevikelli et al., 2020). Based on these diagrams, suitable concentrations of constituents were selected and utilised in preparation of microemulsions. The phase diagrams have been created utilizing a computer program; the area covered by these points was assumed as microemulsion area and all trials were done in triplicate.

Preparation of MET-loaded microemulsions

Pseudo-ternary phase diagrams were obtained to decide suitable constituents, and their concentration series that arose in a large microemulsion area were chosen. Blank and MET-loaded microemulsions with varied compositions were prepared after identification of microemulsion area from the pseudo-ternary phase diagrams.

The system was stirred using a magnetic stirrer to ensure a thoroughly mix at 25°C. Oil and S_{mix} mixtures were titrated, drop-by-drop, with double distilled water while stirring until the mixture became transparent. The microemulsions were protected from the by storing in dark-brown bottles covered with aluminum foil (Öztürk & Güven, 2019).

Physicochemical characterization of MET-loaded microemulsions

The chosen formulations were taken under the thermodynamic stability tests, in terms of the centrifugation test and heating–cooling cycles. Thermodynamic stability was assessed by the procedure of three cycles between 4°C and 40°C with storage at each temperature for 48 h were studied. Also, the formulations were centrifuged at 3500 rpm for 30 min.

Globule sizes and zeta potential of the formulations were measured using Zetasizer nano ZS (Malvern, UK) at 25°C and polydispersity index (PDI) was reported (Ekinci et al., 2022; Tilki et al., 2023).

The pH value of prepared microemulsion formulations was determined at 25°C with a digital pH meter. All measurements were done in triplicate (Chavhan et al., 2013; Ramasahayam et al., 2015; Zhu et al., 2008).

Drug content

To obtain a clear solution, MET-loaded microemulsions were mixed with methanol and sonicated respectively. Following the sonication, formulations were centrifuged at 1500 rpm for 5 min, to yield a clear supernatant, which were injected to the UPLC for quantification of MET.

***In vitro* release study**

In vitro release studies of MET-containing microemulsion formulations were conducted by the dialysis bag method. The medium used in the release study was phosphate buffer having 7.4 pH. All sets were incubated at 37°C and were shaken at 100 rpm by using a magnetic stirrer. The release medium volume was taken for the study as 50 mL. The formulation (1 mL) was placed in a dialysis bag for the research, and the drug release was evaluated for 24 h. At 0.5, 1, 2, 3, 6, 9 and 12, 24 h, 1 mL of samples were collected. After samples being withdrawn at predetermined time interval, release medium was replaced with an equivalent amount of the fresh medium. The obtained samples were then analyzed for MET quantification by the UPLC. Three replicates were performed for each formulation (Kumbhar et al., 2020; Talaat et al., 2019).

Evaluation of release kinetics

Data were transmitted to the DDSolver program after obtaining the MET release profiles to determine the four most important criteria: coefficient of determination (R^2), adjusted coefficient

of determination ($R^2_{adjusted}$), Akaike information criterion (AIC), and model selection criterion (MSC). To compare various kinetic models, the lowest AIC values, maximum R^2, $R^2_{adjusted}$, and MSC values were utilized (Öztürk et al., 2021; Öztürk et al., 2020).

Stability of microemulsion formulations

The optimized microemulsion formulations were kept at ambient temperature for six months, and then the clarity, phase separation, globule sizes, PDI, *in vitro* release, and concentration of MET were investigated. Microemulsion samples were analysed at 0, 3 and 6 months, respectively.

Statistical analysis

The collected data (n=3) were presented as mean±S.D. The Student's t-test was used to analyze statistical data at the level of $p \leq 0.05$.

RESULTS and DISCUSSION

UPLC method and validations

Linearity

Linearity of MET between 1-20 µg/mL was studied and the regression equation was found to be *y=1,0232x - 0,3237* by plotting concentration (x) versus peak area (y). The correlation coefficient (R^2) was determined as 0.9996 and found to be highly significant and suitable (Çağlar et al., 2022). Linearity test results are given in Table 1 and regression curve is shown in Figure 1.

Table 1. Sets and Area/RT values prepared for the linearity study

Conc (µg/mL)	AREA/Rt								
	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6	Mean	SD	SE
1.0	0.798	0.906	0.976	0.908	0.904	0.821	0.885	0.064	0.026
2.5	2.224	2.354	2.405	2.323	2.230	2.194	2.288	0.084	0.034
5.0	4.401	4.433	4.702	4.691	4.504	4.417	4.525	0.137	0.056
7.5	7.286	7.522	7.587	7.131	7.107	7.064	7.283	0.224	0.091
10.0	9.910	9.565	9.979	9.990	9.982	9.613	9.840	0.197	0.080
12.5	12.922	12.735	12.446	12.299	12.754	12.277	12.572	0.268	0.109
15.0	15.233	15.293	15.107	14.886	15.509	14.700	15.121	0.291	0.119
17.5	17.631	17.655	17.265	17.263	17.453	17.581	17.475	0.177	0.072
20.0	20.580	20.362	19.394	20.549	20.720	19.611	20.202	0.558	0.228

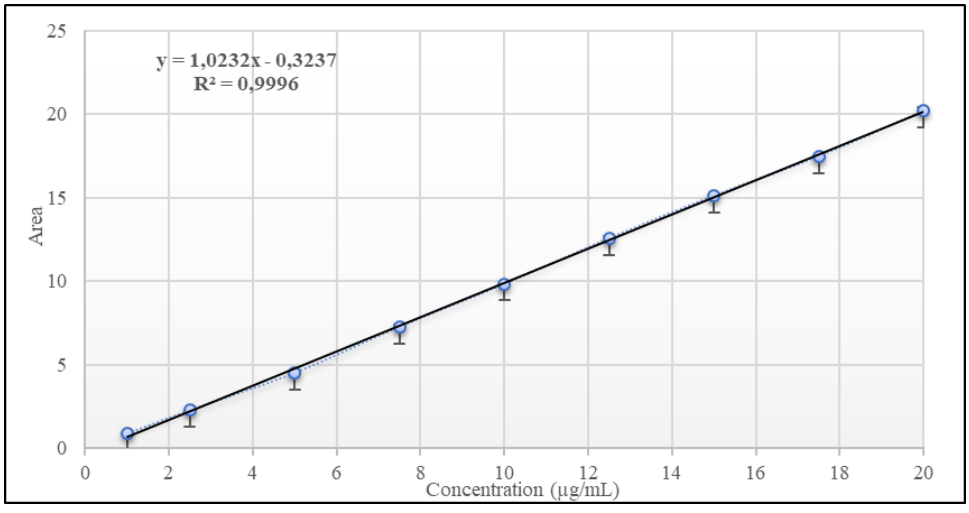

Figure 1. Regression profile of MET.

Limit of detection and limit of quantification (sensitivity)

While LoD and LoQ parameters are interrelated, they cover different properties for the analytic method development and validation. As there are different definitions to describe LoD and LoQ, generally LoD refers to the minimum concentration in a sample under the specified test conditions, but is not found to be quantifiable. The term "LoQ" refers to the minimum concentration of an analyte that can be accurately and precisely measured under the required test conditions. The detection and quantification limits are typically determined using linear regression, signal-to-noise, limit of blank, and precision-based techniques, as well as blank determination (Guideline, 2005). Linear regression method was applied to calculate LoD and LoQ values for this study and found to be 0.9073 µg/mL and 2.7494 µg/mL, accordingly.

Accuracy

As given in Table 2, recoveries of MET at different concentrations were obtained between 100.4673 – 106.3123%, and relative standard deviation (RSD%) values <2% were calculated for all concentrations studied, which is the acceptance criteria, implicating suitable accuracy for the UPLC method developed for the MET (Çağlar et al., 2022; Guideline, 2005).

Table 2. Accuracy results calculated for the 10 µg/mL, 30 µg/mL and 50 µg/mL of the MET

Area/RT			**Concentration**		
10 µg/mL	**30 µg/mL**	**50 µg/mL**	**10 µg/mL**	**30 µg/mL**	**50 µg/mL**
10.710	30.566	50.747	10.784	30.190	49.914
10.306	30.712	51.079	10.389	30.332	50.238
10.618	30.288	51.863	10.693	29.918	51.005
10.764	30.075	52.125	10.837	29.710	51.261
10.574	30.853	50.785	10.651	30.471	49.951
10.349	30.593	51.290	10.431	30.217	50.444
			Recovery (%)		
			10 µg/mL	**30 µg/mL**	**50 µg/mL**
			107.840	100.635	99.828
			103.898	101.109	100.477
			106.938	99.728	102.011
			108.372	99.035	102.522
			106.512	101.570	99.902
			104.312	100.724	100.889
Recovery Mean (%)			**106.312**	**100.467**	**100.938**
Difference (%)			**6.312**	**0.467**	**0.938**
RSD			**1.726**	**0.927**	**1.101**
95% Confidence Interval			**1.925**	**0.975**	**1.166**

Precision

Intermediate precision and repeatability results were calculated for concentrations of 10 µg/mL, 25 µg/mL, 50 µg/mL of the MET to evaluate precision parameter as recommended by the ICH to cover low, middle and high concentrations of the range, and given in Table 3. RSD% values < 2%, were found to be suitable for ICH Q2(R1) guideline, and method was foud to be precise (Guideline, 2005).

Table 3. Precision results of the 10 µg/mL, 25 µg/mL, and the 50 µg/mL of MET

Area/RT			**Concentration (10 µg/mL)**		
1st Day	**2nd Day**	**3rd Day**	**1st Day**	**2nd Day**	**3rd Day**
10.820	10.710	10.053	10.891	10.784	10.142
10.678	10.306	10.585	10.752	10.389	10.661
10.302	10.618	10.417	10.385	10.693	10.497
10.521	10.764	10.716	10.599	10.837	10.789
10.397	10.574	10.643	10.477	10.651	10.718
10.728	10.349	10.792	10.802	10.431	10.864
		Mean	**10.651**	**10.631**	**10.612**
		Standard Deviation (SD)	**0.197**	**0.183**	**0.261**
		Coefficient of Variation (RSD)	**1.850**	**1.726**	**2.467**
		95% Confidence Interval	**0.206**	**0.192**	**0.274**
Area/RT			**Concentration (25 µg/mL)**		
1st Day	**2nd Day**	**3rd Day**	**1st Day**	**2nd Day**	**3rd Day**
25.476	26.158	25.801	25.215	25.882	25.533
26.010	25.825	26.231	25.738	25.556	25.953
26.296	26.425	26.638	26.017	26.143	26.351
26.412	26.157	26.321	26.130	25.881	26.041
25.684	25.464	26.124	25.419	25.204	25.848
26.240	26.732	26.978	25.962	26.443	26.684
		Mean	**24.747**	**25.851**	**26.068**
		Standard Deviation (SD)	**0.362**	**0.434**	**0.402**
		Coefficient of Variation (RSD)	**1.406**	**1.680**	**1.541**
		95% Confidence Interval	**0.380**	**0.455**	**0.421**
Area/RT			**Concentration (50 µg/mL)**		
1st Day	**2nd Day**	**3rd Day**	**1st Day**	**2nd Day**	**3rd Day**
52.263	50.600	50.747	51.396	49.770	49.914
50.401	52.667	51.079	49.575	51.790	50.238
50.816	51.403	51.863	49.982	50.555	51.005
49.574	50.606	52.125	48.767	49.776	51.261
51.225	52.373	50.785	50.381	51.503	49.951
49.433	53.412	51.290	48.630	52.518	50.444
		Mean	**49.789**	**50.985**	**50.469**
		Standard Deviation (SD)	**1.039**	**1.130**	**0.555**
		Coefficient of Variation (RSD)	**2.087**	**2.217**	**1.101**
		95% Confidence Interval	**1.090**	**1.186**	**0.583**

Microemulsions development

A pseudo-ternary phase diagram for the determination of microemulsion region can be constructed by titration method. Constructing a pseudo-ternary phase diagram is important to establish the concentration range of ingredients for the existence range of microemulsion. Microemulsion system is formed when the interfacial tension between water and oil interface is occured at deficient level. This condition, resulting in a spontaneous dispersion of water phase into oil phase. The pseudo-ternary phase diagram is usually provided by an appropriate selection of surfactants and cosurfactants and their ideal proportions (Lawrence & Rees, 2012). Four different S_{mix} proportions were chosen (1:2, 1:3, 1:4, 1:5) using Span® 80, ethanol and propylene glycol. And then, four different S_{mix} proportions were chosen (1:1, 1:2, 1:3, 1:4) using Cremophor EL, ethanol and propylene glycol. These proportions were created to generate a pseudo-ternary phase diagram, shown in Figure 2. It was observed that the area of the microemulsion region increased as the surfactant/cosurfactant mixture increased. This is probably due to decreased interfacial tension and increased mobility of the system. It was monitored that percentage area of microemulsion area in the most of phase diagrams was most wide-ranging at S/CoS weight ratio of 1:4 compared to others. After determining the Figure 2/c and Figure 2/h shows the larger microemulsion area, formulations used in the next experiments (Table 4) were selected from the weight center of these diagrams (Öztürk & Güven, 2019). Weight center of these pseudo-ternary phase diagrams were coded as S_1 and S_2 formulations, and MET-loaded microemulsions were produced based on these formulations (S_{1MET} and S_{2MET}, respectively).

Span 80 is a non-ionic surfactant that is biodegradable and nontoxic with low irritant properties. A surfactant for topical administration must decrease the interfacial tension between the oil–water interfaces and have convenient solubilizing capacity for drug (Kogan & Garti, 2006). Cremophor EL is a non-ionic surfactant with a high HLB value; due its less hydrophylic nature, providing a high solubilizing capacity of hydrophobic components with strong emulsifying capacity (Zhang et al., 2020). Due to their advantegous properties and common applications in microemulsion formation, Span 80 and Cremophor EL were chosen as the surfactans in this study.

Cosurfactant is a necessary constituent in the microemulsion development. This component reduces the interfacial tension between oil and water phase and provide a small internal globule size (Laothaweerungsawat et al., 2020). In our study, it has been reported that lipophilic surfactants promote water in oil (w/o) microemulsion formulations. Propylene glycol was used as a vehicle for penetration enhancement. And then, ethanol was chosen as cosurfactant, because of short to medium chain length alcohols are frequently used as co-surfactants to improve the fluidity of interface (Okur et al., 2020).

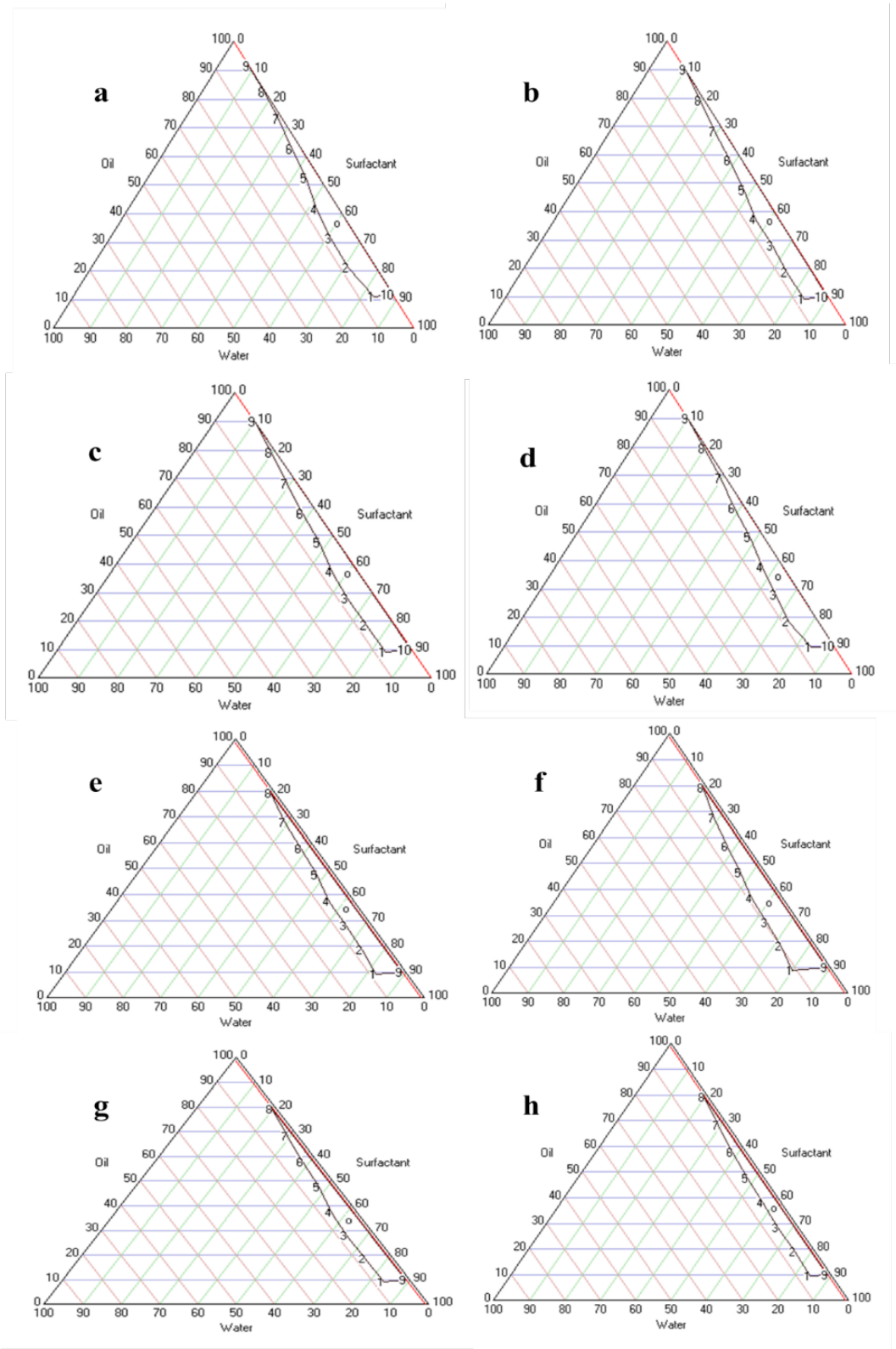

Figure 2. Pseudo-ternary phase diagram of microemulsions composed of oil, surfactant, cosurfactant and water. (a, b, c, d; 1:2, 1:3, 1:4, 1:5 using Span® 80, ethanol and propylene glycol) (e, f, g, h; 1:1, 1:2, 1:3, 1:4 using Cremophor EL, ethanol and propylene glycol)

Table 4. Composition of microemulsion formulations (%, w/w)

Code / Formulation	IPP	Water	Cremophor EL	Span 80	Ethanol	Propylene Glycol	MET
S_1	37.20	3.00	-	11.96	23.92	23.92	-
S_{1MET}	35.34	2.85	-	11.36	22.72	22.72	5
S_2	35.10	4.60	20.10	-	20.10	20.10	-
S_{2MET}	33.34	4.37	19.09	-	19.09	19.09	5

Physicochemical characterization of metronidazole loaded microemulsions

Physicochemical characterization was required to evaluate the effect of drug loading on microemulsion properties. Visual evaluations of phase separation, undissolved MET, transparency, and clarity of microemulsion formulations were performed (Üstündağ Okur et al., 2019). The prepared blank and MET-loaded microemulsions were clear, transparent, liquid, single phase, no drug precipitation and with homogeneous appearance (Lin et al., 2018; Ryu et al., 2020).

Emulsions usually are thermodynamically unstable systems and may separate when exposed to physical tension. Although microemulsions are visually appear to be homogeneous as a single phase system, in fact they are two phased systems (Hashem et al., 2011). Microemulsions were subjected to centrifugation and heating–cooling cycles to support the absence of no separation. After being subjected to centrifugation, blank and drug-loaded formulations did not show any sign of phase separation, which implicates the physical stability of the microemulsion (Zhu et al., 2008).

It is well known that one crucial factor for drug delivery systems is the vehicle's globule size. Nano globule size provides a larger surface region to interact with the skin, leading to improved permeation of active agents (Sita & Vavia, 2020). The globule sizes and PDI of the prepared microemulsions were determined by using the Dynamic Light Scattering technique. In this technique, laser light hits the globules in the solution and gets dispersed according to the size of the globules (Altaani et al., 2019). The results of the characterization are summarized in Table 5. Globule size of blank microemulsion formulations was found to be ranged 126.8±2.8 nm to 150.8±1.6 nm. The globule size for $S1_{MET}$ is 142.4±6.4 nm, while $S2_{MET}$ is 162.4±4.6 nm. Globule sizes of MET-loaded formulations were slightly larger than the blank formulations, yet the difference was statistically insignificant ($p > 0.05$).

For monodispersed systems, the PDI index, which measures the distribution of microemulsion globules, is a dimensional number ranging from 0 to 1. Lower value expresses a size distribution of more homogenous for microemulsions (Chavhan et al., 2013; Ramasahayam et al., 2015). All formulations showed a PDI ranging from 0.21±0.02 to 0.35±0.06, suggesting that they are monodispersed.

The pH was found to vary between 4.0 and 7.0 range, optimum for the skin treatment. It is clear from Table 5 that microemulsion systems are within the required physiological pH range accepted. This pH close to skin pH, allows safe and nonirritating use of this formulations as topical application (Hashem et al., 2011).

The zeta potentials of microemulsions were obtained 0.48±0.08 - 0.68±0.14 mV that were being towards neutral. It is established that the stability of lipid based microemulsions containing nonionic surfactants does not depend on the zeta potential (Kumbhar et al., 2020).

Conductivity provides information about the structure of a microemulsion. The literature reported that the w/o type microemulsions stabilized by a

nonionic surfactant has unimportant charge which results in low electrical conductivity. The formulations had conductivity values of 0.5±0.0 – 0.6±0.0 µS/cm, near zero, which confirms the formation of w/o type of emulsions (Tirnaksiz et al., 2012). Analysis for drug content determination was taken by UPLC method and the (%) drug content for microemulsions were found within the suitable limits (99.18±0.08 - 99.33±0.12 %).

Table 5. Characterization of microemulsion formulations (mean±SD, n=3)

Storage condition	Duration	Code	pH	Conductivity (µS/cm)	Zeta potential (mV)	Globule size (nm)	PDI	Drug content
Fresh	Initial	**S1**	5.42±0.00	0.5±0.0	0.52±0.17	126.8±2.8	0.23±0.03	-
		$S1_{MET}$	5.34±0.00	0.5±0.1	0.48±0.08	142.4±6.4	0.26±0.02	99.18±0.08
		S2	4.98±0.00	0.6±0.0	0.64±0.12	150.8±1.6	0.21±0.02	-
		$S2_{MET}$	4.86±0.01	0.6±0.0	0.68±0.14	162.4±4.6	0.24±0.01	99.33±0.12
Stored at 4°C	3 month	**$S1_{MET}$**	5.38±0.00	0.5±0.0	0.56±0.14	151.4±5.0	0.26±0.02	99.04±0.16
		$S2_{MET}$	4.92±0.00	0.6±0.0	0.68±0.17	174.2±4.8	0.30±0.04	99.12±0.24
	6 month	**$S1_{MET}$**	5.36±0.01	0.5±0.1	0.56±0.18	150.6±5.6	0.32±0.02	98.90±0.16
		$S2_{MET}$	4.98±0.02	0.6±0.1	0.65±0.10	170.7±5.0	0.35±0.04	98.84±0.18
Stored at 25°C	3 month	**$S1_{MET}$**	5.44±0.01	0.5±0.0	0.44±0.24	150.4±3.8	0.26±0.03	99.13±0.56
		$S2_{MET}$	4.87±0.03	0.6±0.0	0.62±0.17	158.7±6.2	0.32±0.02	99.03±0.32
	6 month	**$S1_{MET}$**	5.38±0.01	0.5±0.1	0.48±0.11	155.7±8.8	0.33±0.04	98.92±0.22
		$S2_{MET}$	5.02±0.00	0.5±0.1	0.62±0.15	162.6±8.6	0.38±0.08	97.84±0.48
Stored at 40°C	3 month	**$S1_{MET}$**	5.48±0.01	0.5±0.1	0.42±0.26	156.4±8.4	0.25±0.03	98.90±0.24
		$S2_{MET}$	4.88±0.02	0.4±0.1	0.58±0.16	176.4±10.4	0.30±0.04	98.24±0.38
	6 month	**$S1_{MET}$**	5.41±0.01	0.5±0.1	0.46±0.13	164.0±9.3	0.33±0.02	98.14±0.20
		$S2_{MET}$	5.06±0.03	0.4±0.2	0.52±0.22	182.4±3.6	0.35±0.06	97.76±0.58

***In vitro* metronidazole release study**

Release profile is a significant parameter in the development of w/o microemulsion for water soluble drugs, because of depending on the solubility of the drug (Kumbhar et al., 2020). The *in vitro* release of MET from microemulsions was studied using the dialysis bag method. The *in vitro* release profile of MET from microemulsion formulation is represented in Figure 3. As expected, two microemulsion formulations showed a decrease in the amount of MET released as well as a delay in the release rate in comparison with the drug release from pure MET solution. The *in vitro* release of pure MET was 98.19 ± 1.22%, within 4 h. Cremophor EL based microemulsion formulation $S2_{MET}$ (62.66 ± 1.08%) significantly showed the low drug release from the dialysis membrane within 24 h. The release of the $S1_{MET}$ formulation was found to be 80.62 ± 0.86% at the end of 24 h. Compared with the formulations, pure MET solution has a rapid release rate, which indicated that the release of metronidazole had been significantly controlled by the microemulsions. Drug release from the $S2_{MET}$ microemulsion was slower than that from the $S1_{MET}$ microemulsion. As the reason for this, incorporation of different surfactants was altered the release profile of the formulation (Ikeuchi-Takahashi et al., 2020). Compared with pure MET solution, there was a significant difference in the release profile of MET from both the formulations ($p \leq 0.05$). Moreover, similar release profiles were obtained for $S1_{MET}$ and $S2_{MET}$ in 3. and 6. months (Figure 3 and 4).

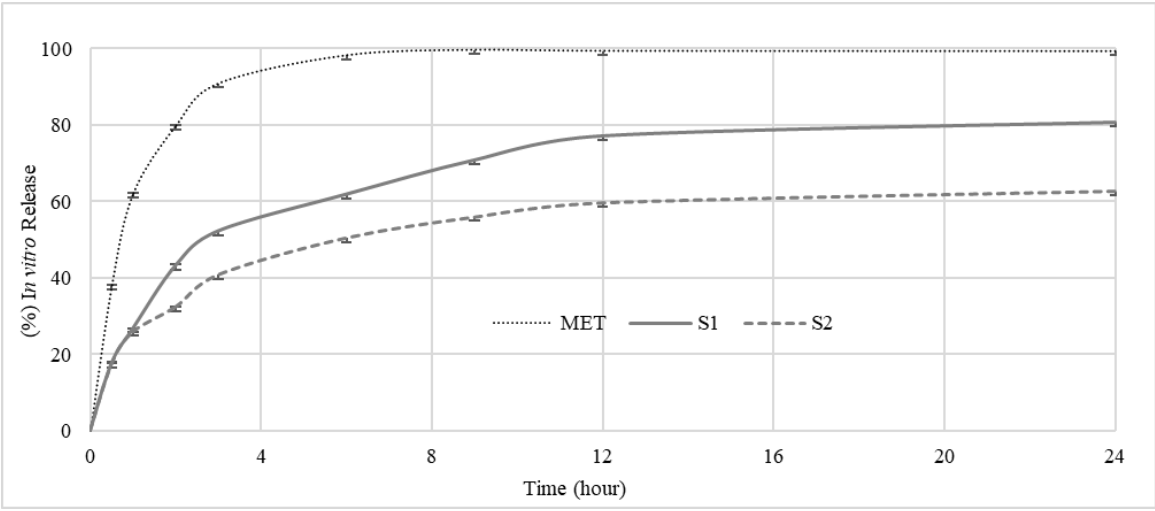

Figure 3. The *in vitro* MET release profiles of the microemulsion formulations and MET solution (n=3).

Evaluation of release kinetics

Kinetic modeling results of MET release from microemulsion formulation are given in Tables 6 and 7 for S1 and S2-coded formulations, respectively. For comparing different kinetic models, the lowest value for AIC; and the highest values of R^2, $R^2_{adjusted}$ and MSC indicate the best fitting (Kırımlıoğlu & Öztürk, 2020). Examination of the statistic parameters from Table 6 and Table 7 lead to the conclusion that in formulation, the release kinetics was best defined by the Korsmeyer–Peppas, Peppas-Sahlin and Weibull models (Scomoroscenco et al., 2023). The drug release profile from the formulation may fit more than one model, as previously reported in the literature (Baghirova et al., 2023). The best-fitting release models imply the non-Fickian diffusion mechanism of the drug (Han et al., 2022) and indicate the slow diffusion of MET into the dissolution medium (Miastkowska et al., 2016); decreasing rate of the initial release, followed by the steady release rate of the MET (Jain et al., 2015).

Table 6. Release Kinetics Results for S_1 Coded Formulation

Model	Formulation Code	Evaluation Criteria			
		R^2	$R^2_{adjusted}$	AIC	MSC
Zero-order	S_1	-0.056	-0.056	81.283	-0.724
First-order	S_1	0.791	0.791	66.682	0.899
Higuchi	S_1	0.818	0.818	65.471	1.033
Korsmeyer-Peppas	S_1	**0.915**	**0.903**	**60.611**	**1.573**
Hixson-Crowell	S_1	0.691	0.691	70.233	0.504
Hopfenberg	S_1	0.692	0.648	72.201	0.285
Baker-Lonsdale	S_1	0.953	0.953	53.190	2.398
Peppas-Sahlin 1	S_1	**0.983**	**0.978**	**48.021**	**2.972**
Peppas-Sahlin 2	S_1	**0.991**	**0.989**	**40.716**	**3.784**
Quadratic	S_1	0.771	0.738	69.538	0.581
Weibull	S_1	**0.989**	**0.985**	**44.187**	**3.398**

Table 7. Release Kinetics Results for S_2 Coded Formulation

Model	Formulation Code	Evaluation Criteria			
		R^2	$R^2_{adjusted}$	AIC	MSC
Zero-order	S_2	-0.234	-0.234	77.602	-0.956
First-order	S_2	0.495	0.495	69.558	-0.063
Higuchi	S_2	0.771	0.771	62.451	0.727
Korsmeyer-Peppas	S_2	**0.950**	**0.943**	**50.770**	**2.025**
Hixson-Crowell	S_2	0.335	0.335	72.042	-0.339
Hopfenberg	S_2	0.386	0.299	73.315	-0.480
Baker-Lonsdale	S_2	0.862	0.862	57.897	1.233
Peppas-Sahlin 1	S_2	**0.996**	**0.994**	**30.967**	**4.225**
Peppas-Sahlin 2	S_2	**0.999**	**0.998**	**18.842**	**5.573**
Quadratic	S_2	0.699	0.656	66.895	0.233
Weibull	S_2	**0.986**	**0.981**	**41.272**	**3.080**

Stability study

The evaluation of the microemulsions stability is essential for indicating the physicochemical properties were preserved during the storage time, since physicochemical properties of microemulsions as drug delivery systems may affect its drug release profile (Pandey et al., 2014). The characteristic properties of the microemulsions remained unchanged during long-term stability tests. When the microemulsion formulations centrifuged and subjected to heating and cooling cycles, did not result in phase separation or turbidity; confirming that the microemulsions were physically stable. The clarity and stability of the formulations were maintained as indicated by measurements throughout the storage period. The mean globule size and the PDI of microemulsions are two critical parameters for predicting physical stability (Narala et al., 2019; Sita & Vavia, 2020). No changes of globule size, zeta potential and degradation of MET were observed during six months. There was no statistically significant change between the first and last measurements for any of the characteristics ($p > 0.05$). Furthermore, there was no significant difference ($p > 0.05$) between *in vitro* MET release from formulations and their corresponding stability studies (3 and 6 months, Figure 4). In scope of this study, the microemulsions were found to be suitable carrier systems for the administration of MET through topical application for antibacterial treatment.

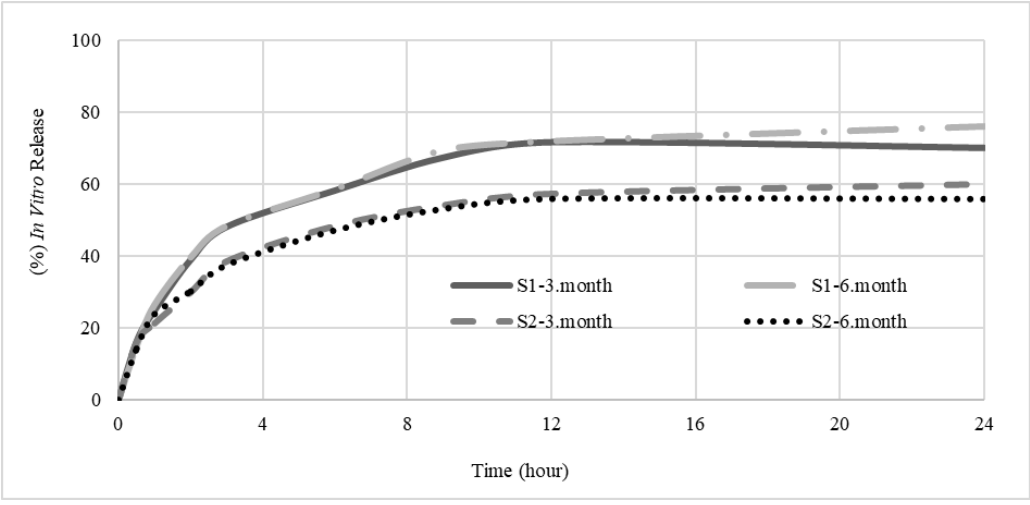

Figure 4. The *in vitro* MET release profile of the microemulsion formulations in 3. and 6. months (n=3).

CONCLUSION

In this work, MET-loaded microemulsions for topical application were developed by the pseudo-ternary phase diagram technique. Globule size, PDI, zeta potential, clarity, viscosity, conductivity, pH values, drug content, and drug release properties of the S_{1MET} and S_{2MET} formulations were satisfactory. Also, long term stability evaluation for six months, indicated good stability in terms of physicochemical properties and the drug release profiles of the formulations. In conclusion of these findings, MET-loaded microemulsions can be considered as a promising alternative for topical treatment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Concept, Experimental Design: (A.A.Ö., U.M.G., T.Ç); Data Collection, Data Processing, Data Analaysis: (A.A.Ö., U.M.G., T.Ç); Literature Research, Writing (A.A.Ö., U.M.G., T.Ç); Supervision: (A.A.Ö., U.M.G.)

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