Preparation and Evaluation of Topical Microemulsion System Containing Metronidazole for Remission in Rosacea

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The aim of this study was to prepare a topical water-in-oil type microemulsion containing metronidazole and to compare its effectiveness with a commercial gel product in the treatment of rosacea. A pseudo-ternary phase diagram (Kw=2:1) was constructed using lecithin/butanol/isopropyl myristate/water. The microemulsion was chosen from the microemulsion region in the phase diagram. The formulation was a water-in-oil type microemulsion (droplet size: 11.6 nm, viscosity: 457.3 mPa·s, conductivity: 1.5 µs/cm, turbidity: 6.89 NTU) and the addition of the metronidazole did not alter the properties of the system. The release experiment showed that the release rate of metronidazole from the commercial gel product was higher than that of the microemulsion. Stability experiments showed that the metronidazole microemulsion remained stable for at least 6 months; none of the characteristic properties of the microemulsion had changed, the system retained its clarity and there was no sign that crystallization of metronidazole has occurred. Microemulsion was compared to a gel product in a randomized, double-blind, baseline-controlled, split-face clinical trial for the treatment of patients. After the 6-week treatment period there was a statistically significant difference in reduction of the main symptoms of rosacea. Of the patients treated with the microemulsion, 17% experienced complete relief from inflammatory lesions, and 50% from erythema. The microemulsion resulted in complete relief in 38% of the patients with telangiectasia while the commercial product did not provide any relief of telangiectasia symptoms. In conclusion, the microemulsion containing metronidazole was found to be more effective in reducing the symptoms of rosacea compared to the commercial gel product.

Key words microemulsion; metronidazole; rosacea

Rosacea is a chronic skin disorder of unknown etiology that is more common in women than men.1) It is characterized by inflammatory lesions (papules and pustules), erythema and telangiectasia. Rosacea appears most often in fair skinned individuals and occurs primarily in middle-aged adults, peaking between the ages of 30 and 50 years. It primarily affects the convexities of the central face (check, nose, and chin) and central forehead.2) Rosacea requires long-term treatment similar to other chronic inflammatory diseases, thus treatment should be safe and convenient for use over long periods. Presently, there is no cure for rosacea; management and treatment may provide only a method of suppressing signs and symptoms; the main goals of treatment are to reduce the number and severity of inflammatory lesions, reduce erythema and reduce telangiectasia.3) A prolonged course of oral antibiotics, including tetracyclines, especially doxycycline and minocycline, and clarithromycin, is generally required for effective treatment. However, long-term oral antibiotic therapy is unacceptable for many patients due to significant systemic side effects, so their long-term use is limited. Therefore, current therapy has focused on topical administration of drugs. Three topical medications are currently used for the treatment of rosacea: metronidazole 0.75% and 1%; azelaic acid 15%; and sodium sulfacetamide 10% with sulfur 5%.4)

Metronidazole, which is a synthetic nitroimidazole derivative with antimicrobial and anti-inflammatory properties, has been reported to be effective in the treatment of rosacea, through not only topical application but also systemic administration.5) As the first topical therapy approved for rosacea, metronidazole has remained a cornerstone of rosacea management.6,7) Topical application of metronidazole was shown to be as effective as systemic antibiotic therapy.8,9) Metronidazole is particularly effective against papules and pustules and is a well-tolerated alternative to oral antibacterials. The exact mechanism by which topical metronidazole reduces inflammatory lesions and erythema in rosacea is unknown; it is suggested that its anti-inflammatory effect may be due to its antioxidant action.10,11)

In the clinical setting, burning, stinging and dry appearance have been noted in patients with rosacea due to barrier deficiency.12) Therefore, the use of moisturizers is an important part of treatment. Although cream and gel formulations of metronidazole have been used in the treatment of rosacea, therapeutic and cosmetic benefit is variable or insufficient with these vehicles. Topical metronidazole products should be able to restore epidermal barrier function to maintain skin integrity and to prevent transepidermal water loss.13) Therefore, selection of an appropriate vehicle is very important for a more effective treatment.

Microemulsions are defined as thermodynamically stable dispersions composed of aqueous phase, oil phase, surfactant/s and co-surfactant, which are single, optically isotropic solutions.14,15) A co-surfactant is required to lower interfacial tension between the aqueous and oily phases to less than 1 mN/m (ca. 10−2–10−3 mN/m),16) thus they spontaneously form without requiring any additional external energy and the formed droplets range in size from a few to two-hundred nanometers (<200 nm).17) Microemulsion structure can vary from spherical droplets to bicontinuous structure, depending

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on the nature of components and composition of the system. They can be a water-in-oil or oil-in-water type, whereas approximately equal percentages of oil phase and aqueous phase form a bicontinuous structure. Microemulsions are attractive delivery systems due to advantages including enhanced drug solubility, good physicochemical stability and ease of manufacture. Many researchers working on increasing the solubility of low-soluble drugs have focused on the formulation of lecithin-based microemulsions and studies have shown that microemulsions can be formulated using lecithin and a co-surfactant such as short-, medium- or long-chain alcohols. Microemulsions were also used to stabilize active substances, and to improve oral availability of drugs. Microemulsions were shown to be effective dermal delivery mechanism for several active ingredients for pharmaceutical and cosmetic applications.

Topical microemulsions allow rapid penetration of active molecules due to the large surface area of the internal phase, and their components reduce the barrier property of stratum corneum. Microemulsions thereby enhance dermal absorption compared with conventional formulations and are therefore a promising vehicle due to their potential for transdermal drug delivery.

This study investigated whether a microemulsion formulation containing 0.75% metronidazole was as effective as a commercial gel product containing the same amount of drug. To date, there are no published data concerning either the formulation or the clinical efficacy of a topical microemulsion containing metronidazole in the treatment of rosacea. Therefore, the study aimed to a) develop an appropriate microemulsion formulation, b) determine whether metronidazole microemulsion is effective and safe in treatment of rosacea, and c) compare the efficacy and safety of microemulsion formulation with that of a commercial topical gel product in the treatment of patients with rosacea.

**Experimental**

**Materials** The metronidazole used during the experiments was kindly provided by I. E. Ulagay, Istanbul, Turkey. Lecithin (Phospholipon® 90G, Natterman Phospholipid GmbH, Germany), butanol (Merck, Germany), isopropyl myristate (IPM, Sigma, U.S.A.) and distilled water were used as the surfactant, co-surfactant, oil phase and hydrophilic phase, respectively. Freshly distilled water was used in the preparation of solutions. All other chemicals were of analytical grade.

The commercial product (Roza Jel, ORVA, Izmir, Turkey) containing 0.75% metronidazole used in this study was a topically hydrophilic gel.

**In-Vitro Studies. Solubility of Metronidazole** The solubility of metronidazole was determined in pH 5.2 buffer solution and also in distilled water at 32°C and 25°C, respectively. An excess amount of the drug was added to distilled water or buffer solution and the resulting suspension was mixed for 24 h to achieve equilibrium. The mixture was filtered through a membrane filter (0.45 µm) and a portion was diluted with an appropriate amount of related aqueous phase, and then spectrophotometrically analyzed for metronidazole content at 320 nm. All the UV measurements were performed on a Beckman DU 650 spectrophotometer.

**Preparation of Microemulsions** In order to determine the concentration range of components for the microemulsion, a pseudo-ternary phase diagram was constructed using water titration method at room temperature. The phase diagram was prepared with a ratio of 2:1 lecithin to butanol (K_m) by weight at different ratios of oil to the mixture of surfactant and co-surfactant. Oil and surfactant ratios were varied from 9:1 to 1:9. In order to establish the microemulsion region border, the mixture of oil, surfactant and co-surfactant were diluted with water dropwise under moderate magnetic stirring and then the mixture was visually examined for transparency. The optical changes were observed from turbid to transparent and inversely. The transparent region consisting of single-phase was determined. For further studies, one microemulsion formulation was selected from this area. The formation of a selected microemulsion took 10 min. This formulation was also examined for lack of birefringence using a polarized light microscope (Leica DMEP, Germany). A simple mixing procedure was used to prepare the microemulsion. A drug-loaded microemulsion was formulated by admixing and stirring appropriate quantities of the components and drug in a well-sealed flask until a clear solution was obtained (Table 1). This process took approximately 45 min and, during this stage, metronidazole was completely dissolved.

**Characterization of Microemulsion** The physical characteristics (viscosity, droplet size, pH, density, conductivity, turbidity and release) of the microemulsion containing metronidazole were measured at room temperature (25±0.1°C). All experiments were carried out in triplicate and the results are presented as arithmetic mean±standard deviation (S.D.). The microemulsion was also visually observed for its color, clarity and homogeneity. The centrifuge test, which is a basic and simple accelerated stability technique, was carried out and the microemulsion was centrifuged (Hettich, EBA 12) for 5 h at 1200×g. The density of the microemulsion was measured using a pycnometer. Viscosity and the pH value of sample were determined with a calibrated Ubbelohde viscometer and using a pH meter (CG840, Schott-Gerate GmbH, Mainz). The electrical conductivity of the microemulsion was measured using a conductometer (Hanna HI 9033). Turbidity was measured using a Velp turbidimeter (Velp Scientifica, Italy). The droplet size of the microemulsion was measured using a Zeta-Sizer Nano ZS particle size analyzer (Malvern Inst., England). The optical properties of the microemulsion were determined using a light microscope (Soif, Anka, Istanbul).

**Release Study** In-vitro release studies were carried out using a simple mixing procedure. The unloaded and drug-loaded microemulsions were examined by analyzing the suspension after a predetermined interval using a light microscope.

| Table 1. Composition of Microemulsion Containing Metronidazole |
|--------------------------|---------------|
| Components of microemulsion | % |
| Lecithin                  | 35.73         |
| Butanol                   | 17.86         |
| Isopropyl myristate       | 26.80         |
| Distilled water           | 18.86         |
| Metronidazole             | 0.75          |
using dialysis technique to evaluate the amount of metronidazole released from the microemulsion and commercial gel product. The surface of the dialyzing tubing, sealed at each end, was arranged to be 12 cm². The dialysis bag was then immersed in the release medium (500 mL of pH 5.2 buffer solution, which simulates the pH of skin surface); this volume of buffer solution maintained the sink condition for metronidazole. A magnetic stirrer (600 rpm) was used to mix the release medium to minimize any stagnant layer. The temperature was accurately controlled at 32°C to mimic human skin. The samples were withdrawn from the release medium at suitable time intervals and analyzed at 320 nm using a UV spectrophotometer. The release study was conducted 2 h after the microemulsion was prepared. The same study was performed after storing the microemulsion at 25°C for 6 months.

In-Vivo Studies. Patients This study was carried out in accordance with ‘The Code of Ethics’ of the ‘World Medical Association (Declaration of Helsinki).’ The Ethical Committee Board of Gazi University gave prior approval for the clinic study (No. 13/06/2001–2001/3). The trial was conducted in a single center, at Gazi University School of Medicine, Department of Dermatology. Clinical assessments were performed by the same observer (E.A.) throughout the study. Subjects were informed about the study and each signed an informed consent form. Patients were instructed to report possible side effects whenever they occurred. All patients commenced the study during winter and were assessed on entry to the study (# 13/06/2001–2001/3). The trial was conducted in a 30 day for double-blind, bilateral split-face paired comparison between 0.75% metronidazole released from the microemulsion and commercial gel product, for periods up to 72 h according to the method recommended by Walker et al.37) At the end of the clinical study, all patients were also investigated for topical side effects of microemulsion and commercial gel formulations using the method recommended by the International Contact Dermatitis Research Group.

Some signs of rosacea, such as stinging, burning, itching, and dryness, were also assessed after 6 weeks, according to patient feedback and the researcher’s observations. Patients’ opinions were also sought on the cosmetic acceptability, degree of absorption (i.e., required too much rubbing, speed of absorption) and skin feel (i.e., moisturized, oily, sticky etc.) after using the microemulsion. The number of papules and pustules on both sides of the face were counted and recorded. Erythema was graded on a 4-point scale as follows: 0, no perceptible erythema; 1, mild-slight erythema; 2, moderate-pronounced erythema; and 3, severe-erythema or purple hue. The degree of telangiectasia was also assessed using a 4-point scale as follows: 0, absent; 1, mild-fine vessels covering less than 10% of the face; 2, moderate-several fine vessels and/or a few large vessels covering between 10–30% of the face; and 3, severe-many fine vessels and large vessels covering more than 30% of the face.31)

Statistics: Data evaluation and statistics were performed using the INSTAD computer program. The percentage of changes and comparisons of changes from baseline were analyzed using the t-test. The statistical comparisons for lesion counts, erythema index and telangiectasia score were made by means of Wilcoxon’s test, paired t-tests and analysis of variance (with α: 0.05). Descriptive statistics are reported as mean±S.E.M. (standard error of the mean). The McNemar test was used to determine the skin status prior to treatment. At baseline, none of the subjects showed a significant difference between each half of the face for any of the assessed variables.

Results

In-Vitro Studies A pseudo ternary phase diagram of the investigated system water/lecithin/butanol/IPM is presented in Fig. 1. Phase behavior investigation of this system demonstrated that a transparent single-phase low-viscous system was formed. The region that was outside the clear section of the phase diagram was blurred. Solubility in water was observed to increase with higher lecithin content in the formulation in the transparent region. The water solubilizing capacity of the system increased slowly below 39.4% lecithin content; a sharp increase in solubilizing capacity was observed within a narrow range of surfactant concentration (39.4–42%) (Fig. 2) as a consequence of the liquid crystalline phase.38) Therefore, the surfactant-rich (above 39.4%) and oil-poor (below 25.3%) section of the transparent region was determined to be the liquid crystalline phase. Therefore, a microemulsion formulation was chosen from the transparent region outside this

<table>
<thead>
<tr>
<th>Patients</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>35.4</td>
<td>44.2</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>29–42</td>
<td>33–63</td>
</tr>
<tr>
<td>Mean duration (years)</td>
<td>1.86</td>
<td>2.80</td>
</tr>
</tbody>
</table>
range, consisting of 36% lecithin, 27% oil and 19% water. A 0.75% metronidazole was added to this formulation to obtain the formulation used in the present study (Table 1). No liquid crystalline structure was observed using the cross polarizer in the selected microemulsion formulation.

Characteristic properties of microemulsion and the release profiles of both microemulsion and the commercial gel product are presented in Table 3 and Fig. 3. The solubility values of the metronidazole in water and pH 5.2 buffer solution were found to be 10.2 (± 0.1) mg/mL (n=6) at 25°C and 13.9 (± 0.1) mg/mL (n=3) at 32°C, respectively. The microemulsion containing metronidazole was stable at 4°C and 25°C. The study conducted at 40°C was terminated after blurring and phase separation was not observed at the 4th month. One of the main reasons for choosing butanol rather than ethanol as the co-surfactant was butanol’s lower vapor pressure (approximately 59 mmHg vs. 6.1 mmHg) at 25°C; unfortunately, at 40°C phase separation occurred due to the evaporation of butanol despite the use of a well-sealed glass vial. It is likely that evaporation led to changes in the composition and therefore subsequent destabilization. This indicated that the microemulsions formulated using co-surfactants including short, medium or long-chain alcohols should either be kept below 25°C or stored in hermetically sealed and disposable packaging.

No significant change was observed in the appearance of the microemulsion; its color, clarity and homogeneity did not change. The centrifuge test also showed that the microemulsion had good physical stability. There were no signs of metronidazole crystallization at 4°C.

In-Vivo Studies A total of 12 patients participated in the study (5 male, 7 female). At initial evaluation, each patient was investigated in terms of rosacea symptoms to determine baseline values. The results of the mean baseline values (final assessment) for microemulsion-treated sides and gel-treated sides are shown in Table 5. At baseline, no statistically significant difference was found between each half of the face in terms of inflammatory lesion counts (p=0.092), erythema scores (p=0.109) and telangiectasia (p=0.112), indicating that the two sides of the face for every patient had similar severity and symptoms of rosacea.

Table 3. Characteristics of Microemulsion (Mean±S.D., n=3)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.37±0.01</td>
</tr>
<tr>
<td>Density (g/cm³)</td>
<td>0.902±0.000</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>6.89±0.14</td>
</tr>
<tr>
<td>Conductivity (µS/cm)</td>
<td>1.5±0.01</td>
</tr>
<tr>
<td>Droplet size for metronidazole-loaded microemulsion (nm) (polydispersity index: 0.155±0.200)</td>
<td>11.6±1.2</td>
</tr>
<tr>
<td>Droplet size for drug-free microemulsion (nm) (polydispersity index: 0.201±0.127)</td>
<td>10.3±1.10</td>
</tr>
<tr>
<td>Viscosity (mPa·s)</td>
<td>457.30±1.17</td>
</tr>
<tr>
<td>Release rate (released %/h)</td>
<td>7.150±0.230</td>
</tr>
<tr>
<td></td>
<td>(r²=0.983)</td>
</tr>
<tr>
<td>Release rate (released %/h)</td>
<td>7.210±0.110</td>
</tr>
<tr>
<td></td>
<td>(r²=0.989)</td>
</tr>
</tbody>
</table>

a) Calculated from release data 2h after preparation of microemulsion. b) Calculated from release data 6 months after preparation of microemulsion.

Fig. 1. Pseudo-Ternary Phase Diagram with Microemulsion Region of Lecithin/Butanol/Isopropyl Myristate/Water at K_m: 2:1 at Room Temperature

Fig. 2. Solubilization Capability of Water in the Oil Phase (IPM) and Surfactant Mixture (Surfactant/Co-surfactant) (Lecithin/Butanol)

Fig. 3. Release Profiles for Microemulsion and Commercial Gel Product (Mean±S.D., n=3)

Fig. 4. Stability Profiles of Microemulsion Containing Metronidazole at 25°C (Mean±S.D.)
The patch test performed at the beginning of the study caused no allergic or irritant reaction in the application area in patients. Moreover, at the end of the therapy, none of the patients reported side effects on the skin of their faces due to the microemulsion or gel product containing the same amount of metronidazole.

The formal protocol did not include a cosmetic acceptability control of the microemulsion and commercial product; therefore, this trial was not subjected to statistical analysis. The microemulsion was determined as an appropriate formulation according to the comments from all patients, it was quickly absorbed into the skin, and it moisturized the skin much better than the commercial product. Stinging, burning, itching and dryness, which are local tolerance variables, were improved by the microemulsion formulation. Also, the microemulsion did not leave any residue, which could disturb the patients, on the surface of the skin. Patients’ tolerance for both products was statistically significant from baseline to final assessment (Table 5). Moreover, at the end of the therapy, none of the inflammatory lesion counts decreased in both microemulsion and commercial gel. Mean inflammatory lesion counts decreased from 3.75±0.74 at baseline to 1.57±0.13 for the microemulsion. On the side of the face treated with commercial gel, mean lesion counts decreased from 3.01±0.59 at baseline to 1.83±0.53. The reduction of inflammatory lesion counts in both products was statistically significant from baseline to final assessment (Table 5). Moreover, there was a statistically significant difference between products with respect to the mean papule/pustule counts at the end of the study (p=0.03). The mean percentage reduction of inflammatory lesion from baseline to the end of the study achieved with microemulsion and commercial gel were 52.3% and 39.2%, respectively. Papules/pustules also disappeared from the faces in 17% of patients for both microemulsion and commercial gel preparations.

**Erythema** Both products produced a clinically appreciable and statistically significant reduction in erythema symptoms between baseline and final assessment (Table 5) and the microemulsion produced a significantly greater reduction in erythema compared to the commercial gel (p=0.0269). The mean percentage reductions achieved with microemulsion and commercial gel were 70% and 43.8%, respectively. Erythema disappeared in 50% of patients on the microemulsion-treated side compared with only 16.7% of patients for the commercial gel (p=0.001).

**Telangiectasia** Telangiectasia reduced in both products from baseline to final assessment, but the reduction was not statistically significant for the commercial gel product (Table 5). The clinical assessment showed a significant difference between the products, with the microemulsion showing a significantly greater mean decrease from baseline compared with the gel product (p=0.01). The mean percentage reduction achieved with the microemulsion and commercial gel was 85% and 38%, respectively. Telangiectasia symptoms disappeared in 38% of patients with microemulsion treatment.

**Discussion**

**Determining Components, Composition and Physical Characteristics of Microemulsion** The first stage in formulating a topical microemulsion is to determine the appropriate proportions of surfactant, co-surfactant and oil components. The lecithin/IPM/butanol/water combination is widely used when developing a water-in-oil or oil-in-water type microemulsion.38-42 The lecithin used in this study consists of 93% phosphatidylcholine, which is slightly too lipophilic to form a balanced microemulsion when used as the sole surfactant.43 Lecithin tends to form lamellar structures/bilayers, since it has a high critical packing parameter (CPP, approximately 0.8).44 45 In order to achieve a zero mean curvature lipid layer and ultra-low interfacial tension, i.e. in order to spontaneously form a microemulsion, the CPP and the curvature of the lecithin should be reduced; this is achieved by the addition of co-surfactant molecules. Short- and medium-chain co-surfactants particularly reduce the tendency of lecithin to form a non-elastic film layer, by infiltrating between the surfactant molecules located between the oil/water interface, i.e. they increase the fluidity of the surfactant layer. Furthermore, these molecules reduce the hydrophilicity of the aqueous phase by dissolving in water. In the pseudo-ternary phase diagram, the presence and extent of the microemulsion region depends on the partition coefficient of co-surfactant and the surfactant/co-surfactant ratio.39,40,42,46 The butanol used in this study has medium level water solubility; its affinity to the IPM used in

Table 4. Stability Data of Microemulsion Containing Metronidazole at 25°C (Mean±S.D.)

<table>
<thead>
<tr>
<th>Month</th>
<th>Viscosity (Pa·s)</th>
<th>Density (g/cm³)</th>
<th>Conductivity (μS/cm)</th>
<th>pH</th>
<th>Turbidity (NTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.457±0.001</td>
<td>0.902±0.000</td>
<td>1.5±0.0</td>
<td>4.37±0.01</td>
<td>6.89±0.14</td>
</tr>
<tr>
<td>2</td>
<td>0.551±0.003</td>
<td>0.907±0.000</td>
<td>1.7±0.0</td>
<td>4.01±0.01</td>
<td>6.63±0.04</td>
</tr>
<tr>
<td>4</td>
<td>0.500±0.007</td>
<td>0.906±0.000</td>
<td>1.67±0.05</td>
<td>3.86±0.02</td>
<td>7.04±0.05</td>
</tr>
<tr>
<td>6</td>
<td>0.482±0.004</td>
<td>0.904±0.000</td>
<td>1.4±0.0</td>
<td>4.05±0.01</td>
<td>6.69±0.08</td>
</tr>
</tbody>
</table>

Table 5. Mean Changes in Inflammatory Lesion Counts, Erythema Scores and Telangiectasia from Baseline to Final Assessment (Mean±Standard Error of Mean)

<table>
<thead>
<tr>
<th></th>
<th>Microemulsion</th>
<th>Commercial gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory lesion counts</td>
<td>Before treatment: 3.75±0.74</td>
<td>3.01±0.59</td>
</tr>
<tr>
<td></td>
<td>After treatment: 1.57±0.13</td>
<td>1.83±0.53</td>
</tr>
<tr>
<td>Inflammatory lesion counts</td>
<td>p 0.0021</td>
<td>0.0024</td>
</tr>
<tr>
<td>Erythema scores</td>
<td>Before treatment: 2.50±0.22</td>
<td>2.08±0.26</td>
</tr>
<tr>
<td></td>
<td>After treatment: 0.75±0.12</td>
<td>1.17±0.28</td>
</tr>
<tr>
<td>Erythema scores</td>
<td>p 0.00002</td>
<td>0.00007</td>
</tr>
<tr>
<td>Telangiectasia</td>
<td>Before treatment: 1.50±0.17</td>
<td>1.08±0.31</td>
</tr>
<tr>
<td></td>
<td>After treatment: 0.217±0.110</td>
<td>0.667±0.210</td>
</tr>
<tr>
<td>p</td>
<td>0.0015</td>
<td>0.251</td>
</tr>
</tbody>
</table>

Before treatment: baseline (initial assessment). After treatment: at the end of the study (at 6th week, final assessment).
the experiment was 1.6 times higher than its affinity to water (log F/IPM/water = -0.199). Therefore, butanol was thought to have partition to the oil phase, aqueous phase and also to the surfactant film layer formed by the lecithin. The following section examined how this might have influenced the release of metronidazole from the system and the clinical effectiveness of the product.

One of the main disadvantages of microemulsions for pharmaceutical use is that they usually contain high levels of surfactants, which can cause skin irritation. Therefore, the selection and proportion of a surfactant is an important issue when developing a microemulsion formulation. Lecithin, which was chosen as a surfactant in this study, is widely used as an emulsifier for cosmetics, and in pharmaceutical applications such as total parenteral nutrition. Lecithin is an ideal surfactant for use in pharmaceutically acceptable topical microemulsions, as it does not cause skin irritation even when used at high percentages in topical formulations. Isopropyl myristate is a non-toxic ester with good local tolerance and is pharmaceutically acceptable for use as the oil component in microemulsions. The only substance in the present study that had the potential to cause skin irritation was butanol, which is a common co-surfactant used in microemulsion formulations. Therefore, patch tests were conducted on patients using a drug-free microemulsion prior to the clinical trial. Since no topical side effects were noted in patients, the use of 17.9% butanol in the microemulsion was deemed safe.

The construction of a phase diagram can help determine the appropriate proportions of surfactant, co-surfactant and oil for a microemulsion. The microemulsion region in the phase diagram is usually determined by simple visual inspection or, preferably, by using a polarized light microscope; we used both methods in our study. First, the microemulsion region in the phase diagram was determined by visual inspection. A composition was then selected within that region and was inspected using a polarized light microscope. Once it was confirmed that the composition was not in liquid crystal form, two forms of the microemulsions (metronidazole-loaded and drug-free) were prepared and their characteristics were compared. The metronidazole-loaded microemulsion took longer to prepare; the droplet size was slightly larger than the drug-free system, but this difference was not statistically significant (p = 0.173). Differences in droplet sizes between drug-loaded and drug-free microemulsions were also noted by Chen et al. Although this difference was not statistically significant in our study, the larger droplet size in the drug-loaded microemulsion shows that metronidazole affects the interface characteristics between oil phase and aqueous phase. The lower polydispersity index of the metronidazole-loaded microemulsion indicates that the droplet size distribution is monodisperse.

Although Djordjevic et al. stated that some characteristics, such as viscosity, density, and conductivity of drug-loaded microemulsions depend on the percentage of water and characteristics of drug, we determined that the physical characteristics of both the metronidazole-loaded and drug-free microemulsions were nearly identical in our study. Fubini et al. suggested that the enthalpy of an oil-in-water type microemulsion containing water/IPM/lecithin/butanol does not change with the addition of the drug. The viscosity of a topical microemulsion, which is suggested to be a minimum of around 100 mPa·s, is an important parameter that determines the convenience of use by the patient. The microemulsion used in our study had mid-level viscosity and Newtonian flow properties. As patients found the microemulsion easy to apply during the clinical tests, the system is thought to have appropriate viscosity for topical application.

Conductivity provides information about the structure of a microemulsion. Unchanging conductivity over time at different temperatures shows that the microemulsion structure remains unchanged. The conductivity levels measured in our study showed that the outer phase of microemulsion was oil, confirmed by low conductivity level. Conductivity was similar to that of a water-in-oil microemulsion prepared by Podlogar et al. that used IPM as the oil phase. Furthermore, we concluded that the internal aqueous phase in our microemulsion was in droplet form, since the water phase was less than 20%, and the system did not present a bi-continuous structure.

The microemulsion prepared for the present study had a dermatologically acceptable pH level. The density of the system was less than 1 g/cm³, since 80% of the microemulsion was in oil phase. Its ultra-low surface tension, combined with low density and viscosity, made the microemulsion easy to apply to the skin during in-vivo trials. It was determined that packaging the system in a squeezable container or a glass bottle with dropper would allow patients apply the product more easily.

**Stability Evaluation of Metronidazole-Loaded Microemulsion** Although microemulsions are regarded as thermodynamically stable, the stability of those systems should be investigated. The characteristic properties of the microemulsion remained unchanged during long-term stability tests (6 months at 4°C and 25°C). The physical appearance of the microemulsion was unchanged during that time and no phase separation or discoloration was noted. Although the microemulsion contained 18.86% water, metronidazole remained in a soluble state in this system, despite the water solubility value indicating that 73.5 mL of distilled water was required for 0.75% metronidazole to dissolve. No signs of crystallization were observed during the stability study at room temperature (25°C) and at 4°C.

The observation that the microemulsion viscosity remained unchanged over 6 months was very important, because we were not able to measure the droplet size during the stability study due to technical limitations. The unchanging viscosity showed that the size of the inner phase droplets of the microemulsion did not change significantly.

**Release Experiments** There are generally two rate-limiting factors in dermal penetration of active molecules: release of drug from vehicle and, consecutively, penetration of the stratum corneum. Although in-vitro release experiments do not provide adequate information on the dermal permeability or clinical efficiency of a drug, they allow comparison of the release mechanisms of different drug formulations based on the structures of the vehicles or delivery system. Release experiments performed during the stability study show whether release parameters (release rate or release percentages) change over time. These results indicate any structural change in the products over time and also indicate the quality of the product.

In the present study, the release of metronidazole from both the microemulsion and the commercial gel product was...
evaluated using a dialysis membrane. The solubility of metronidazole is moderate. Mahfouz and Hassan\(^6\) determined the aqueous solubility of metronidazole at 25°C as a 10.5 mg/mL, which was supported by the solubility value that we obtained at 25°C in the present study. The solubility of metronidazole in pH 5.2 buffer solution showed that 500 mL of buffer was sufficient to provide sink conditions during release experiments. The dialysis membrane used during the release experiment was found not to affect the release of metronidazole, since approximately 8% of metronidazole was released from the commercial gel product within the first 15 min; no inhibition occurred in release of the drug from the gel.

No visible changes were observed in the appearance of the microemulsion in the dialysis bag, including clarity and transparency, following each release experiment. The release profiles were found to be exactly the same for the metronidazole-loaded microemulsion (2 h after preparation) and the stored microemulsion (25°C for 6 months), and there was no significant difference in release rate (\(p>0.05\)). This finding shows not only that the microemulsion remained structurally unchanged, but also that metronidazole remained chemically stable within the system.

As seen from the release profiles (Fig. 3), there was no release of metronidazole from the microemulsion during the first 30 min. Since the release profile was linear after the first hour (zero order kinetics), release data after this point were used to calculate the release rate as 14.8%/h (determination coefficient: 0.928). The release rate of metronidazole and the release percentage at the 6th hour were lower in the microemulsion than in the commercial gel (\(p=0.0011\)), even though the viscosity of the microemulsion was relatively low.

It was predicted that preparation of the drug-loaded microemulsion using simple mixing procedures would result in the metronidazole being distributed within both the oil and water phases due to its relatively higher partition coefficient (\(\log P_{\text{octanol-water}}=0.75, 25^\circ\text{C}\)\(^{58}\)). Furthermore, the distribution of butanol throughout both oil and water phases is thought to enhance the solubility of metronidazole in both phases.\(^{47}\) As Lee et al. suggested, it is likely that butanol acts as a partition enhancer for metronidazole between phases.\(^{59}\) The distribution of metronidazole in the phases resulted in increased drug movement in the system, as also reported by Kreilgaard et al.\(^{60}\) Furthermore, the low viscosity of the microemulsion facilitated the diffusion of metronidazole within the system, from the water phase to oil phase, and then to the dialysis membrane. Based on this finding, the microemulsion was expected to show faster and greater release of metronidazole. However, either because butanol was not present in the release medium, or because the amount of butanol diffused from the microemulsion to the release environment was very low, the partition enhancer effect was not observed during the release experiment. As a result, the release of metronidazole from the microemulsion was slower and the percentage released was less than for the commercial gel product. During the experiment, it was also observed that metronidazole release did not occur for a certain period of time, since the partition of metronidazole in the hydrophilic membrane was difficult, even though its affinity to the oil phase was 5.6 times higher than to the water phase. The reason for the constant release rate of metronidazole was that the water droplets and surrounding by oil phase acted as a membrane reservoir system. It is likely that the reduced level of metronidazole in the outer oil phase, due to diffusing into the release medium, was supplemented by the transfer of metronidazole contained within the inner aqueous phase of the microemulsion into the oil phase.

In this experiment, the release rate of metronidazole and the amount released at the 6th hour, which are among the characteristic properties of a microemulsion, were evaluated as quality control parameters. The results indicated that the prepared microemulsion system, which contained lecithin, butanol and IPM, was physically stable for at least 6 months at 4°C and 25°C; that metronidazole remain unchanged within that system; and that it was an appropriate delivery system for use in topical treatment of rosacea. Following this stage, the clinical effectiveness of the product was evaluated.

**Evaluation of in-Vivo Data** Topical metronidazole has long been used in the treatment of moderate to severe rosacea.\(^{61}\) Metronidazole is currently available in a variety of formulations, including gels, creams, and lotions.\(^{5}\) In several clinical trials, these formulations have been found to be effective in reducing or completely relieving the symptoms of rosacea (inflammatory lesions and erythema), when compared with placebo vehicles or baseline values of patients, except for telangiectasia.\(^{52-60}\) Our study was not a vehicle-controlled study. Instead, the efficacy of both a commercial product and the microemulsion formulation were compared, both with each other and with the patients’ baseline values. Although a different experimental design was used in the present study, the rate of reduction in inflammatory lesions and erythema using the commercial gel were similar to the results of other studies.\(^{64}\) The microemulsion formulation used in this study resulted in significantly reduced rosacea symptoms compared to both the patients’ baselines and to the commercial product. The microemulsion formulation’s effects on erythema and telangiectasia are particularly significant.

Lowe et al. reported 53% reduction in erythema at the 8th week (19 subjects) compared with the baseline using 0.75% metronidazole gel.\(^{66}\) Our study showed a 43.8% reduction in erythema at the 6th week in subjects using a commercial gel and 70% reduction in subjects using the microemulsion formulation, with complete disappearance of erythema observed in half of the patients. Same research group\(^{66}\) reported that 21% of subjects showed complete elimination of lesions, compared with 17% for both microemulsion and commercial gel in our study. Although some studies\(^{56,64,65}\) found that topical metronidazole was not effective on telangiectasia (which is the vascular stage of rosacea), a study conducted in 2001 reported that 1% metronidazole cream containing sunscreen, applied over 12 weeks, resulted in 17% improvement in facial telangiectasia compared with a vehicle containing only sunscreen.\(^{67}\) While our 6-week study showed no improvement with the commercial gel product, the microemulsion formulation resulted in statistically significant improvements compared to both patient baselines and the commercial gel during the same period. None of the subjects using the commercial product experienced complete relief of telangiectasia symptoms, compared with approximately one third of the patients using the microemulsion. This reduction in telangiectasia compared to the baseline is important, since the number of patients enrolled was low and patients whose symptoms alleviated had the minimum level of telangiectasia.

Topical metronidazole is generally applied at two different
doses (0.75% or 1%) once or twice daily. Previous studies reported a median \( t_{\text{max}} \) value of 5.98 h after 0.75% topical metronidazole gel\(^{64}\) and 7.93 h (range, 5.92–10 h) to reach maximum plasma concentration with 1% topical metronidazole gel\(^{68}\), suggesting that the use of a product containing 0.75% metronidazole twice daily or 1% metronidazole once daily would be sufficient. In our study, patients used products containing 0.75% metronidazole twice daily.

We used a split-face study design in our study, as patients had symmetrical facial distribution of rosacea symptoms \((p>0.05)\). The use of a split-face comparative study offered the important advantage that each patient served as their own control; and also minimized extraneous sources of variation.\(^{64,65}\) Since the microemulsion and the gel product used in our study contained the same percentage of metronidazole, resulting differences between treatment sides can be safely ascribed to the effect of formulation type. Patients were instructed in correct application of the medication, thereby avoiding potential crossover contamination between products, which is a risk in such study designs.

The transdermal absorption of metronidazole is minimal, so the level in the blood is insignificant.\(^{5,61,63,64,69}\) Maximum serum concentration of a topically applied cream containing 1% metronidazole is reported to be roughly equivalent to 1% of that of 250 mg oral metronidazole.\(^{70}\) It is therefore considered that the potential for drug related systemic side effects following topical application of metronidazole is extremely low.\(^{5,61,64}\) In our study, we did not observe any systemic side effects in any of patients following 6 weeks of treatment, indicating that the metronidazole was not absorbed through the skin sufficient to cause systemic side effects, which is a desired condition for topically applied drugs.

Topical medication of metronidazole is generally well tolerated,\(^{70}\) but may occasionally induce sensation and even worsening of rosacea; different local reactions (burning, stinging, redness, dryness and itching) caused by metronidazole have been reported in up to 2% of patients.\(^{6}\) However, true allergic contact dermatitis from topical use of metronidazole is rare.\(^{6,72}\) Also, the 9-month study that established the safety of metronidazole reported that no patients withdrew due to adverse effects of topical application.\(^{73}\) However, patients who suffer from rosacea can be susceptible to adverse reactions caused by topical formulations.\(^{72}\) Therefore, the excipient used in the formulation should not aggravate the symptoms of rosacea. This makes the choice of vehicle excipients critical to the development of topical rosacea formulations.

The commercial product used in this study has been safely used by rosacea patients in Turkey for many years without causing any side effects. The components used in the microemulsion formulations that were developed for our study were commonly-used agents for pharmaceutical and cosmetic purposes. In the present study, the drug-free microemulsion formulation was shown to be safe by using a patch test. The same experiment was repeated for metronidazole-loaded microemulsion and for the commercial product. None of those products caused any allergic or irritant effects. Furthermore, the microemulsion formulations used in the study were deemed safe after no topical side effects were observed following 6 weeks of application to the same part of the body twice daily.

Skin barrier integrity is an important issue in the treatment of rosacea. Damage to the stratum corneum leads to dryness, stinging, burning or itching in patients with rosacea. Topical formulations for rosacea are expected to be free of side effects and also to reduce the symptoms by treating the skin. Some ingredients, such as emollients, can be helpful in restoring the epidermal barrier.\(^{12,70}\) In a previous study, 0.75% metronidazole topical gel used with a twice-a-day moisturizing product was found to improve symptoms (skin discomfort, dryness, roughness) compared to a gel-only group.\(^{72}\) Lecithin, used in the microemulsion formulation in our study, improves the barrier properties of the stratum corneum.\(^{75}\) The IPM used as oil phase is an emollient with partially occlusive property that reduces transepidermal water loss through the skin.\(^{70}\) The presence of IPM within the outer phase of the microemulsion shows that water loss will be prevented better than with the gel product. Patients’ own evaluations of the reduction in skin dryness and other local effects after 6 weeks of use is likely to be influenced by the use of lecithin and IPM in the formulation. In addition, the water droplets making up the inner phase are thought to enhance moisturizing of the skin. The water droplets have an average diameter of 11.6 nm, and are likely to enter the lipid region between the cells of the stratum corneum, increasing the water content there.

In order to produce a clinical response in treatment of rosacea, metronidazole must reach the live epidermis and dermis, as the pathology of rosacea is seen predominantly in the dermis. This indicates that greater percutaneous absorption may increase the clinical effectiveness of metronidazole. The most important factor in enabling metronidazole to penetrate the skin is to choose appropriate components or the carrier system. The capability of the components to increase penetration of the drug through the skin is one of the most critical parameters in the delivery of metronidazole. Trials indicate that the dosage form (gel or cream) and the solubility of metronidazole in the system affect dermal penetration.\(^{68,77–79}\) In our study, both the microemulsion and the commercial gel product contained 0.75% solubilized metronidazole, so therefore the superior clinical effectiveness of the microemulsion formulation results from its interaction with the skin.

Microemulsions spread over the skin and penetrate skin indents due to their low viscosity and very low surface tension.\(^{71}\) In this study, patients stated that the microemulsion was easier to apply compared to the commercial gel product and that it spread easily on the skin surface. The ability to spread without further rubbing is an important consideration in order not to irritate the skin, which is sensitive to external stimuli in patients with rosacea.

Microemulsions are known to have very high dynamic structures.\(^{17}\) This shows that, within the system, every component of the microemulsion (surfactant, oil or co-surfactant) can be presented independently in phases. One of these components, short or medium-chain alcohol, which are also used as co-surfactants, is known to enhance dermal permeability.\(^{28}\) Butanol, which is used as a co-surfactant in the present study, could enhance permeation of the metronidazole. Butanol is located between the lecithin molecules during the formation of the microemulsion system, and can be distributed in both the oil and water phases of the microemulsion.\(^{59}\) The preference of butanol for the outer oil phase, which will be the first contact with the skin, facilitates the permeation of metronidazole through the skin. Isopropyl myristate, which is used as
the oil phase, is also reported to have penetration-enhancing effects.\textsuperscript{81,82} Lecithin is also thought to facilitate permeation of metronidazole through the stratum corneum, by increasing lipid fluidity after mixing with the lipid components in the stratum corneum.\textsuperscript{28,33,83} In addition, the water nano-droplets comprising the first phase of the microemulsion enter the interlamellar region of the stratum corneum and cause disorder of the lipid bilayer.\textsuperscript{50} The penetration-enhancing effect can also be seen due to the outer oil phase, which provides a occlusive layer on the skin, and thus increases the moisture content of the stratum corneum compared to the non-occlusive hydrophilic gel structure of the commercial product. Metronidazole can penetrate the stratum corneum and then diffuse into the epidermis after topical application due to its small molecule size (molecular weight: 171.16) and a relatively high partition coefficient.\textsuperscript{80} In the present study, it is estimated that metronidazole distributed in the outer oil phase of the system during preparation of the microemulsion easily penetrated the stratum corneum immediately after contact with skin. The components also had penetration enhancing effects after the microemulsion was applied to the skin. Furthermore, the water droplets could directly enter the stratum corneum (without droplet fusion), thereby transporting metronidazole.\textsuperscript{84,85} Although we were not able to determine the individual effects, all of the above factors are thought to play a role in metronidazole reaching the live epidermis and dermis by increasing diffusion of the drug through the stratum corneum. The clinical effectiveness of the microemulsion was found to be greater than that of the gel product for this reason.

**Conclusion**

In this study, a stable water-in-oil type microemulsion was prepared, consisting of lecithin/butanol/IPM/water and metronidazole. The system was found to be appropriate for topical application and more effective than a commercial gel in reducing the clinical symptoms of rosacea. Both in-vitro experimental findings and in-vivo clinical results show that the prepared microemulsion formulation could be a safe, effective and efficient delivery system for metronidazole.

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