Transdermal delivery of the anti-rheumatic agent methotrexate using a solid-in-oil nanocarrier

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Abstract

Transdermal delivery of methotrexate (MTX) was investigated by using the solid-in-oil (S/O) technique. Because MTX was coated with nonionic surfactant molecules, the resulting complex was easy to dissolve in various organic solvents and provided a transparent solution in isopropyl myristate (IPM). The stability of MTX–surfactant complexes is enhanced by the addition of a basic amino acid such as L-Arginine (L-Arg) or L-Lysine (L-Lys). The average size of the dispersed complex of MTX and amino acid was reduced to below 100 nm and gave a uniform distribution. A transdermal delivery experiment was conducted using the S/O nanocarrier, and the permeation behavior of MTX through Yucatan micropig (YMP) skin was evaluated with a Franz diffusion cell. The permeation efficiency for the S/O nanocarrier (not urea addition) was two- to threefold increased compared to that of the control aqueous solution because the oil-based nanocarrier is effective for penetrating the stratum corneum. Furthermore, addition of urea has dramatically improved the release property of MTX from the S/O nanocarrier, and the S/O nanocarrier containing urea showed an optimal permeation efficiency of approximately 8.8-fold increased compared to that of the control aqueous solution after 24 h (p < 0.01).

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1. Introduction

Methotrexate (MTX) is an effective folic acid antagonist with an anti-neoplastic activity and an anti-inflammatory effect, which is most widely used for the treatment of psoriasis, skin cancer and rheumatoid arthritis (RA) [1–3]. The tablet and injection dosing of MTX was approved by the USA Food and Drug Administration (FDA) for marketing from the early 1980s. Unfortunately, some patients who have been injected or orally inoculated with large amounts of MTX for the therapy of cutaneous and subcutaneous lesions for a long period show undesirable side effects such as stomatitis, gastric disturbance, anemia, hepatic toxicity and pulmonary toxicity [4,5]. As one of the strategies for avoiding these serious gastrointestinal side effects, the transdermal delivery for the systemic or local administration cannot only avoid a digestive system, but also effectively reduce the applied dose because it was not the first pass effect [6,7]. In the development of MTX's clinical pharmacology, new pharmacological actions of MTX have been reported, including its important roles in a variety of anti-inflammatory functions in human keratinocytes or epidermal cytokines for the treatment of RA or skin cancer [8–10]. The low-dose transdermal delivery of MTX for the treatment of psoriasis and rheumatoid arthritis diseases has been attracting significant attention. However, MTX is a highly polar molecule (log p = −1.85) and has an average molecular weight of 454.44 g/mol (molecular weight >500 Dalton drugs are cut off by the stratum corneum), so that it is considered to be difficult to deliver through the lipophilic stratum corneum [11,12]. At present, the permitted clinically useful transdermal application of MTX is only used with physical enhancement technologies such as iontophoresis [13–15], low-fluence erbium YAG laser [16] or electroporation [17]. In contrast, there are relatively few successful cases of a chemically enhanced permeation transdermal drug delivery system (TDDS) for MTX. In a major breakthrough, deformable liposomes [18], and a novel liposome carrier, the ethanolic liposome, have been invented as a carrier with high potential for delivering MTX into human dermal tissue [19]. Furthermore, novel temperature stimuli-responsive and biocompatible nanogels have been recommended as promising nanocarriers that can be applied to transdermal MTX delivery [20,21]. In the
future, it is expected that a novel chemically enhanced permeation TDDS for MTX will be promising.

A novel solid-in-oil (S/O) nanosuspension was first introduced by our group as a new pharmaceutical drug nanocarrier for a TDDS [22–24]. The S/O suspension-type nanocarrier is typical reverse micellar system, in which the hydrophilic drug is directly encapsulated into a nanocarrier that consists of a layer formed with a non-ionic sugar ester surfactant such as ER290 (sucrose erucate ester, Hydrophile–Lipophile Balance (HLB) = 2), ER190 (sucrose erucate ester, HLB = 1) or L-195 (sucrose laurate ester, HLB = 1) and well-dispersed into an oil phase. In comparison with conventional water-in-oil (W/O) microemulsions, the S/O nanosuspension has a better dispersibility (diameter of 50–300 nm), excellent stability and is only slightly affected by the storage temperature. In addition, the S/O nanocarrier is capable of maintaining a better primary pharmacological activity for hydrophilic drugs than emulsion and liposome carriers, because it does not contain a “water medium” and this anhydrous environment can decrease the risk of oxidation and biochemical degradation.

In this study, there were three objectives: the first was to confirm that a stable S/O nanocarrier containing MTX can be prepared by introduction of the surfactant coating technique. To achieve this, the MTX–basic amino acid (e.g., l-Arginine or l-Lysine) complexes were employed in the preparation, because an MTX is better dissolving in the aqueous solutions containing the basic amino acid than in pure water solution.

The MTX–basic amino acid ion-pair formulation (molar ratio: MTX/l-Arg = 1/3) has shown to have better chemical stability and higher potential applications for enhanced permeability across the rabbit nasal mucosa in vitro [25]. The second objective was to clearly demonstrate the transdermal potential of an S/O nanosuspension-type TDDS containing MTX. To meet this objective, the transdermal behavior of the designed S/O TDDS was evaluated by in vitro permeation experiments using Yucatan micropig skin and a Franz diffusion cell. The third objective was to find an effective strategy for enhancing the release of MTX from S/O nanocarriers. To meet this objective, urea a high water absorbing and widely used in the cosmetic industry and medicinal treatment [26,27] was introduced into the S/O nanosuspension, and the release experiment was conducted using a Franz diffusion cell, in which the interface was separated with a polycarbonate membrane.

2. Materials and methods

2.1. Materials

Methotrexate (MTX), l(+)-Arginine and l(+)-Lysine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Urea was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Fluorescein sodium salt was purchased from SIGMA–ALDRICH Inc., and RYOTO (R) Sugar Esters ER290 was kindly gifted from Mitsubishi-Kagaku Foods Co. (Tokyo, Japan). Isopropyl myristate (IPM) was purchased by Tokyo Kasei Co. (Tokyo, Japan). The polycarbonate membrane (PVDF) was purchased from GE, and Yucatan micropig (YMP) skin sets (5-month-old female, frozen at −80°C within 3 months) were obtained from Charles River Japan, Inc. (Tokyo, Japan).

2.2. Preparation of MTX aqueous solutions

Aqueous solutions containing MTX/amino acid l-Arg or l-Lys (pKa = 10.5, log p = −3.05) ion-pairs were prepared as follows: MTX was dissolved in 10 mL of Milli-Q water containing an amino acid (molar ratio: MTX/amino acid = 1/2–3, pH: 8.5–8.8) by ultrasound sonication for 1 min. The resulting aqueous solutions were filtered through a 0.20 μm pore size membrane (Millex (R)-LG) and used to prepare the S/O nanosuspension.

2.3. Preparation of S/O nanosuspension containing MTX

An MTX containing S/O nanosuspension was prepared by an emulsification–freeze-drying method as described previously [23]. The prepared process was as follows: the prepared MTX aqueous solution (10 mL) and the surfactant cyclohexane solution (20 mL) were poured into a round-bottomed flask (50 mL), then vigorously mixed with a homogenizer (POLYTRON (R) PT2100; Generator Shaft: EC easy clean, 12 mm) at 26,000 rpm for 2 min to form a stable W/O emulsion. The resulting emulsions were rapidly frozen in liquid nitrogen for 30 min and lyophilized (removal of the aqueous and organic solvents) using a freeze-dryer (FD5N; Eyela (R), Tokyo, Japan) for 24 h. The resulting pastes are complexes (solid phase, S) between MTX and sugar esters. Then, the S/O nanosuspensions of 1.0 mg/mL and 5.0 mg/mL (MTX content) were prepared by continuously vortex mixing the resulting solid complexes (S) in the solutions of the oily chemical penetration enhancer (oil phase, O) isopropyl myristate (IPM) using a laboratory vortex mixer (VORTEX-GENIE (R) 2, Scientific Industries, Inc.) for 3 min.

A sample containing urea and MTX/amino acid (l-Arg or l-Lys) ion-pairs as a positive control and MTX containing the salts of phosphate buffered saline solution (PBS, 0.01 M, pH 7.4) as a negative control were prepared. The compositions in step 1 are listed in Table 1.

2.4. Evaluation of particle size distribution, entrapment efficiency and stability

The particle size distribution of the prepared MTX containing the S/O suspension was characterized by a dynamic light scattering (DLS) technique using a Mastersizer nano-ZS (Malvern Instruments, Malvern, Worcestershire, UK), and the polydispersity index (PDI) was also determined as an index of homogeneity. For the measurement of entrapment efficiency (EE%), 2 mL of the resulting S/O sample was added to the 0.5 mL of Milli-Q water and constantly mixed (500 rpm) for 15 s, then centrifuged (5000 rpm) for 1 min at 20°C. The separated aqueous phase was then analyzed for MTX concentration by HPLC. Entrapment efficiency (EE%) was also evaluated. The concentration of the above-mentioned samples was adjusted to be 1.0 mg/mL, and they were kept in an opaque container, stored in a dry environment at room temperature.

2.5. Transdermal studies in vitro

2.5.1. Franz static diffusion cell and pig skin membrane

An in vitro transdermal experiment for MTX containing S/O nanosuspensions was conducted using a Franz diffusion cell. The effective permeation area of the diffusion cell was 0.785 cm². The receiving compartment (5 mL PBS; 0.01 M, pH 7.4) was maintained at 32 ± 1°C and stirred at 600 rpm [24]. In these transdermal permeation studies, YMP skin was used because its characteristics are known to be very similar to that of human skin [28]. Before the transdermal study, the frozen YMP skin sample was allowed to thaw at room temperature for approximately 30 min. The full-thickness skin was carefully prepared by removing the subcutaneous fat while keeping the stratum corneum undamaged and...
was set at 0.8 mL/min. The sample injection volume was 20 μL. A polycarbonate membrane (pore size: 0.4 μm, K04SH04700), one side of which has a modified hydrophobic surface (the other being hydrophilic), was applied to evaluate the MTX release capabilities of S/O nanocarriers. The hydrophobic surface faced the donor compartment, and the hydrophilic side faced the receptor compartment. MTX (500 μL) containing S/O formulations (MTX: 5.0 mg/mL) was applied to the donor cell, and samples were taken from the sampling port at predetermined time intervals and the MTX concentrations analyzed in the receiving phase by HPLC. The MTX release behavior of S/O nanocarriers and the effect of urea addition on enhancing release of MTX were evaluated.

Table 1
Composition of S/O formulations.

<table>
<thead>
<tr>
<th>Formula no.</th>
<th>MTX (mg)</th>
<th>Amino acid</th>
<th>Urea (mg)</th>
<th>ER290 (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA-1</td>
<td>10</td>
<td>10</td>
<td>–</td>
<td>1000</td>
</tr>
<tr>
<td>MU-1</td>
<td>10</td>
<td>–</td>
<td>10</td>
<td>1000</td>
</tr>
<tr>
<td>MA-2</td>
<td>50</td>
<td>50</td>
<td>–</td>
<td>1000</td>
</tr>
<tr>
<td>ML-2</td>
<td>50</td>
<td>–</td>
<td>50</td>
<td>1000</td>
</tr>
<tr>
<td>MU-1</td>
<td>10</td>
<td>10</td>
<td>–</td>
<td>5000</td>
</tr>
<tr>
<td>MU-2</td>
<td>45</td>
<td>45</td>
<td>–</td>
<td>1000</td>
</tr>
<tr>
<td>MP-1</td>
<td>10</td>
<td>Without addition</td>
<td>–</td>
<td>1000</td>
</tr>
</tbody>
</table>

MTX was detected by UV (302 nm) with a retention time of 14.2 min.

2.7. Statistical analysis

The data obtained were expressed as a mean ± standard deviation (SD). Statistical analysis of release test (Fig. 4) and transdermal test (Fig. 5) was performed employing the one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons using the PRISM-5 (Graph Pad) software. Confidence intervals were set at 95%, and a value of p < 0.05 was defined as statistically significant.

3. Results and discussion

3.1. Characteristics of S/O nanosuspension containing MTX

Photographic images of the resulting S/O nanosuspension and UV–Vis absorption spectra for MTX in the aqueous solution and the S/O dispersed environment are shown in Fig. 1. It is revealed here that the highly hydrophilic complexes MTX–amino acid (L-Arg or L-Lys) and urea are well-dispersed into the chemical penetration enhancer IPM (oil solvent) by the S/O suspension technique. And the S/O formulations that are prepared with ion-pairs of MTX and L-Arg (curves of MU-2, MA-2) where MTX is highly dispersed in IPM showed stronger UV absorption of MTX than that of the S/O formulation that is prepared with MTX alone (curve of MP-1) in the wavelength range from 275 to 330 nm. Also, the maximum absorption of MTX was detected by UV (302 nm) with a retention time of 14.2 min.

Fig. 1. Photograph of the resulting S/O nanosuspensions containing MTX and UV–Vis spectra of MTX in an aqueous solution and in the S/O dispersed solution. MP-1, MU-2 and MA-2 were MTX in the S/O dispersed solution, and their compositions were shown in Table 1. (C) Physical mixture of MTX, L-Arg, urea, ER290 and IPM by ultrasound. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
peak of MTX had a reduced and red-shifted absorption (a maximum absorption peak at around 303–306 nm) compared to that of MTX dissolved in an aqueous PBS solution.

Based on the results of the size spectra (Fig. 2) and average particle size (Table 2), it was proven that the basic amino acid (L-Arg or L-Lys) ion-pair formulations are effective for reducing the size of MTX containing S/O suspensions in comparison with MTX containing PBS solution formulation. The representative MTX–amino acid S/O nanosuspensions showed a uniform and narrow particle size distribution (PdI < 0.2) in the sub-100 nm level and few differences in dispersibility between L-Arg and L-Lys addition. The addition of urea slightly increased the average particle size, but this effect is not marked.

Physical storage studies (dispersion stability and entrapment efficiency (EE%)) were conducted for 60 days (Table 3). The prepared sugar ester complexes of MTX–amino acid and urea containing S/O nanosuspensions showed a better physical storage stability compared to that in the absence of amino acid. With respect to the dispersion stability, the average particle size slightly decreased with the leakage of MTX–amino acids and urea during 60 days, but these had very little effect on the dispersion of the S/O suspension. On the other hand, the S/O suspension was much more sensitive to inorganic salt such as those comprising the PBS buffer (MP-1). Leaked inorganic salts have a strong influence on the dispersion of the S/O carriers, which causes the problem of coagulation settling. Furthermore, we found that the urea addition (MU-2) caused a risk of reduced dispersion stability of the S/O suspension. With respect to entrapment efficiency (EE%), it was shown that a higher EE% for an S/O suspension was obtained when the S/O nanotechnique was applied than that for MTX containing ethosomes (EE%: 37–68%) [19].

3.2. Transdermal studies in vitro

3.2.1. YMP skin permeation of fluorescent marker from S/O nanocarrier

The potential for S/O nanocarrier to deliver the moderate molecular weight hydrophilic drugs into the YMP skin was investigated using a fluorescent marker (F-Na). Based on the green color in Fig. 3, the S/O nanocarrier for FE had a better ability to penetrate the skin because the fluorescent marker (F-Na) more readily passed through the stratum corneum (S-01) and was effectively delivered to a deeper region in the skin (S-02). In contrast, a low penetration of F-Na (C-02) was observed when applied using the PBS solution as a control.

3.2.2. MTX release capabilities of S/O nanocarriers

The capabilities for MTX release from the S/O nanocarriers were investigated using a polycarbonate membrane, and the results are shown in Fig. 4. As can be seen, MTX release capability of the S/O suspension depends on the amount of ER290 added because the amount of MTX for MA-2 (MTX/ER290 = 4.5% w/w) was approximately 1.3 times higher than that of MA-1 (MTX/ER290 = 1% w/w) \((p < 0.05)\). Furthermore, urea played a significant role in increasing the capability for MTX release from S/O suspensions because the amount of MTX released for MU-1 (urea/ER290 = 0.5% w/w) was approximately 4.1 times higher than that of MA-1 (without urea addition) at 24 h \((p < 0.05)\). But after 48 h, MU-1 versus MA-1 and MU-1 versus MA-2 \((p > 0.05)\) were smaller and not significant.

3.2.3. Transdermal delivery of MTX from S/O nanosuspensions

The potential of S/O nanosuspension for the transdermal delivery of MTX was investigated over 24 and 48 h, and the results are shown in Fig. 5. We found that the transdermal delivery potential of S/O nanosuspensions is dependent on the release capability of MTX from the S/O nanocarriers. After 24 h, sample MA-1 (additive amount of ER290 was maximum and without urea addition) did not show good penetration enhancement compared with that of control sample C, in which the penetration enhancement was only 1.5-fold \((p > 0.05)\). However, as a useful supplement, the administration time

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**Table 2**

Average particle size and PdI for S/O formulations (DLS).

<table>
<thead>
<tr>
<th>Formula no.</th>
<th>MA-1</th>
<th>ML-1</th>
<th>MA-2</th>
<th>ML-2</th>
<th>MU-1</th>
<th>MU-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (nm)</td>
<td>56 ± 4</td>
<td>56 ± 3</td>
<td>77 ± 4</td>
<td>75 ± 3</td>
<td>61 ± 3</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>PdI*</td>
<td>0.09–0.16</td>
<td>0.13–0.17</td>
<td>0.13–0.17</td>
<td>0.13–0.17</td>
<td>0.13–0.17</td>
<td>0.13–0.17</td>
</tr>
</tbody>
</table>

Data are mean values ± SD \((n = 3)\).

* PdI: polydispersity index.

**Table 3**

Changes in particle size and entrapment efficiency (EE%) for each S/O formulations for 60 days.

<table>
<thead>
<tr>
<th>Formula no.</th>
<th>Dispersion stability (nm)</th>
<th>1 day</th>
<th>30 days</th>
<th>60 days</th>
<th>EES%</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA-1</td>
<td>56 ± 4</td>
<td>53 ± 5</td>
<td>49 ± 5</td>
<td>99.2 ± 0.2</td>
<td>96.1 ± 0.3</td>
</tr>
<tr>
<td>ML-1</td>
<td>56 ± 3</td>
<td>54 ± 4</td>
<td>47 ± 4</td>
<td>99.4 ± 0.3</td>
<td>95.2 ± 0.4</td>
</tr>
<tr>
<td>MA-2</td>
<td>77 ± 4</td>
<td>72 ± 5</td>
<td>65 ± 6</td>
<td>98.7 ± 0.5</td>
<td>93.3 ± 0.3</td>
</tr>
<tr>
<td>ML-2</td>
<td>75 ± 3</td>
<td>69 ± 5</td>
<td>63 ± 4</td>
<td>98.3 ± 0.4</td>
<td>91.5 ± 0.3</td>
</tr>
<tr>
<td>MU-1</td>
<td>61 ± 3</td>
<td>58 ± 4</td>
<td>53 ± 5</td>
<td>98.2 ± 0.3</td>
<td>88.4 ± 0.6</td>
</tr>
<tr>
<td>MU-2</td>
<td>80 ± 4</td>
<td>71 ± 6</td>
<td>51 ± 4</td>
<td>97.8 ± 0.3</td>
<td>84.3 ± 0.5</td>
</tr>
<tr>
<td>MP-1</td>
<td>478 ± 8</td>
<td>Precipitation</td>
<td></td>
<td></td>
<td>48.2 ± 4.2</td>
</tr>
</tbody>
</table>

Values represent mean ± SD \((n = 3)\).
of S/O sample was extended from 24 h to 48 h, and the penetration enhancement of MA-1 was increased to 3.4-fold ($p < 0.05$). When the molar ratio of ER290 was decreased, addition of urea was significantly effective for increasing the penetration enhancement, with a maximum of 8.8-fold for MU-2 versus C at 24 h ($p < 0.01$). This tendency is similar to that of the release behavior for S/O suspension in the membrane release (Fig. 4).

Based on in vitro permeation studies, the S/O suspension nanocarriers were found to have good potential for delivery of MTX through the stratum corneum layer. This implies that the rate-determining step for transdermal delivery using an S/O suspension is the release step in the dermis rather than transport through the stratum corneum, and as the S/O nanocarrier is a lipophilic carrier [29–31], it more easily penetrates the lipophilic stratum corneum lipid barrier but will only slowly release the hydrophilic drug into the hydrophilic tissue. Additionally, urea addition has proven effective in improving the release behavior because water absorption decreases the stability of the S/O nanocarriers and may improve the release capability of the S/O nanocarriers.

4. Conclusions

The S/O suspension nanocarrier can entrap hydrophilic multidrugs, such as MTX, amino acid and urea. The multi-complex of surfactants, MTX and a basic amino acid was well-dispersed in isopropyl myristate (IPM) and provided a uniform size distribution of 50–100 nm. The S/O nanocarrier was stable for 60 days, maintaining a well-dispersed state and good entrapment efficiency (EE%). The permeation efficiency for MTX was enhanced by using the S/O nanocarrier because the lipophilic characteristics and nanolevel size of the carriers promote the fusion with the top phase of the skin (stratum corneum). Furthermore, the addition of urea was effective in improving the release of MTX from the S/O nanocarriers because its water absorption property enhances the breakdown of the carrier shell. In summary, the prepared S/O nanocarriers showed high potential for transdermal delivery of MTX.

References


[18] N.A. Samah, N. Williams, C.M. Heard, Nanogel particulates located within diffusion cell receptor phases following topical application demonstrates uptake into and migration across skin, Int. J. Pharm. 401 (2010) 72–78.


