Effect of Limonene on Permeation Enhancement of Ketoprofen in Palm Oil Esters Nanoemulsion

Sakeena M.H.F.¹, Elrashid S.M.¹, Muthanna F.A.¹, Ghassan Z.A.¹, Kanakal M.M.¹, Lia Laila¹, Munavvar A.S.²* and Azmin M.N.¹

¹ Department of Pharmaceutical Technology, School of Pharmaceutical Sciences, Universiti Sains Malaysia (11800 Minden, Pulau Pinang, MALAYSIA)
² Department of Physiology, School of Pharmaceutical Sciences, Universiti Sains Malaysia (11800 Minden, Pulau Pinang, MALAYSIA)

Abstract: This study sets out to investigate the in vitro permeation of ketoprofen from the formulated nanoemulsions through excised rat skin. In vitro permeation of ketoprofen nanoemulsion through rat skin was evaluated in Franz diffusion cells and compared with marketed product (Fastum gel®). Limonene which has been reported to be a good enhancer for ketoprofen was selected. Moreover the effects of limonene which was added to the nanoemulsion formulations at levels of 1%, 2%, 3% and on rat skin permeation of ketoprofen were also evaluated. The selected optimized formulation was further studied for skin irritation. Utilization of limonene as a penetration enhancer increased the permeation of ketoprofen from the formulated nanoemulsion with increasing concentrations of limonene. The results obtained showed that nanoemulsion with 3% limonene produced similar and comparable skin permeation of ketoprofen with marketed formulation and the skin irritation study on rats showed the optimized formulation prepared was safe.

Key words: palm oil esters, ketoprofen, permeation enhancement

1 INTRODUCTION
Ketoprofen is a potent non-steroidal anti-inflammatory drug (NSAID) that is widely used in the treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and acute gouty arthritis. However, ketoprofen has to be administered in three to four doses per day orally. Its significant adverse effects, includes gastrointestinal side effects. Therefore is important to develop an alternative dosage form which is easier to administer, painless, noninvasive, easy to comply and avoids first-pass metabolism. The transdermal route meet all the above advantages and ketoprofen is suitable for this dosage form, as it has a low molecular weight, low melting point and high lipophilicity and is reported to be an excellent candidate amongst various NSAIDs.

The effectiveness of transdermal drug delivery depends on the drug’s ability to penetrate the skin sufficiently to reach therapeutic level. The stratum corneum is the outermost epidermal layer that is composed of corneocytes surrounded by a multilamellar lipid matrix is a rate-limiting factor for many drugs. Corneocytes are keratin filled dead cells containing an insoluble layer, which reduces absorption of drugs into the cells. To overcome this skin barrier problem, the most widely implemented approach is the use of chemical penetration enhancers. These penetration enhancers ideally alter the physicochemical nature of the stratum corneum safely and reversibly to facilitate the drug’s delivery through the skin.

In many attempts terpenes are used as skin permeation enhancers. Terpenes have been used in transdermal research since 1960s. These are reported to be very safe and effective class of penetration enhancers that have been classified by the FDA as generally regarded as safe (GRAS). Terpenes are series of naturally occurring compounds which consist of isoprene (C₅H₈) units. These are constituents of essential oils, which are generally used in flavourings, perfumes and medicines. Limonene is a hydrocarbon lipophilic terpene, obtained from the lemon peel of Citrus lemon. Limonene enhances the skin permeation of lipophilic and amphiphilic compounds, but is ineffective for hydrophilic drugs.

*Correspondence to: Munavvar A. Sattar, Department of Physiology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, MALAYSIA
E-mail: munavvar@usm.my
Accepted March 27, 2010 (received for review February 11, 2010)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/
drophilic compounds, such as mannitol\textsuperscript{13}.

Nanoemulsion was chosen as the transdermal dosage form because of its promising novel dosage form and suitability for efficient delivery of active ingredients through the skin. The large surface area of this emulsion system allows rapid penetration of actives\textsuperscript{14}. In this study, Palm oil Esters (POEs) was selected as the oil phase of nanoemulsion. Palm oil is derived from the fruit of the palm tree \textit{Elaesis guineenis} and consists of triglycerides, a combination of glycerol and different fatty acids\textsuperscript{15}. Alcoholyis of triglycerides from palm oil produces palm oil esters that uses lipase as a catalyst in a relatively simple process\textsuperscript{14, 15}. This modified or synthesised oil (POEs) is a new ingredient for pharmaceutical industry\textsuperscript{16, 17}. The oil is non irritation on human skin\textsuperscript{18}, increases the skin hydration due to its moisturizing properties\textsuperscript{19}, shows high thermal stability\textsuperscript{13} and could be used in cosmetic or medicinal formulations to deliver poorly water-soluble lipophilic actives or drugs\textsuperscript{14}.

Hence, in this study, the effects of limonene as a penetration enhancer for ketoprofen from nanoemulsion were evaluated. Furthermore this study evaluated the effects of the limonene containing nanoemulsions produced by formulating the POEs as the oil phase on the permeation, through rat skin (\textit{in-vitro}) and compared to a product available in market.

2 EXPERIMENTAL

2.1 Materials

Tween 80 was chosen as the surfactant (Sigma pharmaceauticals, USA). Ketoprofen was purchased from Eurochem Asia limited, Shanghai, China. Palm Oil Esters (POEs) were provided by co-researchers in Universiti Putra Malaysia. Limonene was purchased from Sigma-Aldrich chemie, Germany (Fluka). Fastum\textsuperscript{®} gel (A.Menarini industrie Farmaceutiche Riunite, Italy) was bought from a retail pharmacy in Penang, Malaysia. Water used in this study was distilled water and all other solvents and chemicals were either of analytical reagent or high performance liquid chromatography (HPLC) grades.

2.2 Preparation of ketoprofen loaded nanoemulsion

Nanoemulsions were prepared by spontaneous emulsification process (titration method)\textsuperscript{18}. Palm Oil Esters and Tween 80 were stirred for 30 min under magnetic stirring 600 rpm at 25°C to mix thoroughly. Following a weighed amount of ketoprofen was added into the solution and mixed thoroughly, until a clear dispersion was formed. This indicated that the drug solubilisation was completed. To the resulting mixture, water was added drop by drop while mixing with the aid of magnetic stirrer at 600 rpm and temperature of 25°C. Limonene was further added to the nanoemulsion formulation at concentration levels of 1, 2 and 3% w/w. The effects of these concentrations of limonene on the permeation of ketoprofen from formulations were evaluated by \textit{in-vitro} through the rat skin and compared with the marketed product (Fastum\textsuperscript{®} gel).

2.3 \textit{In vitro} transdermal diffusion studies

2.3.1 Preparation of rat skin

Male Sprague-Dawley rats (240 ± 20 g) were anaesthetized with intraperitoneal sodium pentobarbitone (60 mg/kg). The fur of the rats was shaved by using a hair clipper from the dorsal surface of rat’s peritoneal region. A \(5 \times 5\) cm patch of skin from the dorsal surface in peritoneal region was excised carefully. Subcutaneous fat and other extraneous debris was removed carefully and the excised skin was placed in a Petri dish containing normal saline at room temperature for 2 h before storage at \(-20°C\). The animals were euthanized by cervical dislocation. Their carcasses were disposed off in accordance to the USM animal ethical regulations.

2.3.2 Diffusion of ketoprofen through rat skin in Franz diffusion Cell

The diffusion of ketoprofen across excised rat skin was investigated using Franz diffusion cells (LG-1083-PC; Erweka). The effective area available for diffusion was 0.636 cm\(^2\) (d = 9 mm). The receptor compartments were filled with pH 7.4 phosphate buffer which was stirred by a magnetic bar and the temperature maintained at 37 ± 0.1°C. The skin was mounted between the donor and receptor compartment of Franz diffusion cells, where the dermal side faced the donor compartment and stratum corneum side faced the receptor compartment and in contact with pH 7.4 phosphate buffer. Ketoprofen formulations were placed on the skin surface in the donor compartment which was occluded with parafilm. From the receptor medium 400 μL was withdrawn and replaced immediately with an equal volume of fresh phosphate buffer at every hour up to the \(8\)th h. These collected samples were analyzed by HPLC for ketoprofen content. Each set of experiments were carried out in triplicate.

2.4 Analysis of ketoprofen

The quantities of ketoprofen in samples collected from Franz Diffusion Cells were determined by HPLC method which was developed and validated\textsuperscript{20}. The HPLC system consisted of an isocratic pump (Shimadzu Co., Japan, Model LC-10-ATVP), a degasser, an auto-sampler (Shimadzu Co., Japan, Model SIL-10 ADVP) and a UV visible detector (Shimadzu Co., Japan, Model SPD 10 A). Samples were chromatographed into a column, 250 mm long and internal diameter 4.65 mm containing 5 μm C\(_18\) ODS hypersil as stationary phase. The samples were eluted by the mobile phase consisting of a mixture of 52% acetonitrile: 10% methanol (HPLC grade): 38% phosphate buffer (0.005 M Na\(_2\)HPO\(_4\) adjusted to pH 3.0 with orthophosphoric acid).
which was filtered through 0.45 µm membrane filter. Detection wavelength was set at 260 nm and the run time was 10 min. The mobile phase flow rate was 0.9 mL/min and the injection volume was 10 μL.

2.5 Skin irritation study

Nine male Sprague-Dawley rats weighing 280-300 g were obtained from Animal House of Universiti Sains Malaysia. Animals were divided into 3 groups (n = 3) as follows: Group 1: Blank formulation (Control), Group 2: Marketed formulation (Fastum® gel), Group 3: Nanoemulsion contain 3% limonene. The dorsal abdominal area 2 cm² patch (both side) was made by removal of fur that were shaved on the previous day before the commencement of experiment. One side of this skin was abraded, while the other left normal. Formulations were applied on both sides (abraded and normal). These groups were observed for 24, 48 and 72 h, for any signs of erythema. The degree of erythema to be observed was as follows: No erythema = 0, barely perceptible-light pink = 1, moderate erythema-dark pink = 2, moderate to severe erythema - light red = 3 and severe erythema-extreme redness = 4.

2.6 Statistical analysis

The results were expressed as mean value ± S.D. Statistical analyses were carried out using SPSS software and one way analysis of variance (ANOVA) followed by post hoc test was performed to see any significance difference. Data with $p<0.05$ were considered to be significant.

3 RESULTS AND DISCUSSION

Figures 1 (A) and (B) are examples of HPLC chromatograms showing the peaks of ketoprofen resulting from analysis of sample collected 1 and 8 h after application of the product onto the rat skin respectively. The chromatogram showed that the retention of ketoprofen peaks at 5.85 min. An increase in height of the peaks from 1 to 8 h (Fig. 1) indicated that ketoprofen release increases with increasing time. In Fig. 1 (A) the height of peak is 0.0010, the amount permeated for the first hour and the height of in Fig. 1 (B) is 0.05, the amount permeated for 8 h. It is also noted in Fig. 1 (A), there were other peaks besides ketoprofen in 2-4 min retention time period. These are interferences arising from the endogenous sources of excised rat

Fig. 1 Example of Chromatogram Resulting from HPLC Analysis of Sample Collected from the Receiver Cell after a Topical Formulation Applied to the Rat Skin (A) after 1 h (B) after 8 h.

skin. However, in Fig. 1(B), when ketoprofen concentration increases, the interferences almost disappear from the chromatograph.

As mentioned in the introduction, the objective of the work was to evaluate the effect of limonene in POEs nanoemulsion for topical delivery of ketoprofen and to compare with an established marketed product. To improve the skin permeation rate of ketoprofen from formulations, different percentages (1, 2 and 3%) of limonene were added to the nanoemulsions. The flow property of nanoemulsion was not changed upon addition of limonene up to 3%.

The amount permeated through the excised rat skin calculated from the profiles are presented in Table 1. It is noted the drug permeation increased with the increasing concentrations of limonene. It was also noted that the drug permeation through rat skin is time dependant, i.e. the amount of ketoprofen permeated increases with increasing time. The 3% limonene nanoemulsion mimics the skin permeation profiles of the marketed product (Fastum® gel). There is no significant difference in the amount of ketoprofen permeation through rat skin at 8 h between the marketed formulation and the formulation with 3% of limonene (p<0.05). It is also worthy to note that at 8 h, limonene increased the permeation rate of ketoprofen by ~2 fold as compared to the formulation containing no limonene.

In this study, the use of penetration enhancer, i.e. limonene is shown to be a valuable and important ingredient for achieving better results on in-vitro skin permeation for ketoprofen and also in obtaining similar permeation profiles to that of marketed product. This observation further supports an earlier study by Rhee et al. (2001) in which they have reported the enhancing effect of terpenes on the permeation of ketoprofen through excised rat skin. In their study they have also shown that the permeation of ketoprofen through rat skin was enhanced by limonene. This finding suggested that limonene is a potential chemical enhancer and a more effective for ketoprofen in comparison to menthol, cineole and camphor. Moreover in several earlier reports, limonene is claimed to be a better penetration enhancer for ketoprofen. The effectiveness of limonene had also been demonstrated for other lipophilic drugs such as indomethacin, and estradiol.

The enhancement by limonene suggests that there are possibly multiple mechanisms that could have resulted in a more permeable intercellular pathway for ketoprofen. They include increased ketoprofen solubility within the skin and according to the lipid - protein partitioning theory they may increase the partitioning of the drug in the stratum corneum membranes. It is also possible that the penetration enhancers may increase the permeability of a drug by affecting the intercellular lipids of the stratum corneum through extraction or fluidization by changing confirmations within the keratinized protein component.

The results of skin irritation studies based on the visual observation score are shown in Table 2. The results score 0 means that no erythema was formed after the application of the formulations. This results suggests that all the tested formulations were safe to be applied on the skin. Okabe et al. (1990) suggested some terpenes are considered to be skin irritants, although they did not cause lasting erythema.

Table 1 In vitro Permeation Profile of Ketoprofen through the Excised Rat Skin from the Nanoemulsions (containing 0, 1, 2 and 3% limonene) and Product in the Market. (Mean ± SD, n=3)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>1st hour</th>
<th>2nd hour</th>
<th>3rd hour</th>
<th>4th hour</th>
<th>5th hour</th>
<th>6th hour</th>
<th>7th hour</th>
<th>8th hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% limonene</td>
<td>1.44±0.04</td>
<td>3.65±0.03</td>
<td>11.61±0.24</td>
<td>16.41±0.31</td>
<td>23.64±0.58</td>
<td>31.58±0.57</td>
<td>53.59±0.60</td>
<td>63.15±0.76</td>
</tr>
<tr>
<td>1% limonene</td>
<td>2.87±0.08</td>
<td>10.01±0.20</td>
<td>15.06±0.29</td>
<td>20.10±0.24</td>
<td>29.57±0.48</td>
<td>41.59±0.32</td>
<td>74.56±0.32</td>
<td>84.40±0.18</td>
</tr>
<tr>
<td>2% limonene</td>
<td>3.58±0.06</td>
<td>12.21±0.27</td>
<td>17.37±0.35</td>
<td>24.84±0.34</td>
<td>38.30±0.87</td>
<td>58.44±0.65</td>
<td>92.80±0.76</td>
<td>107.32±0.71</td>
</tr>
<tr>
<td>3% limonene</td>
<td>4.26±0.08</td>
<td>15.50±0.50</td>
<td>30.29±0.76</td>
<td>47.37±0.46</td>
<td>62.00±0.54</td>
<td>80.02±0.65</td>
<td>94.38±0.19</td>
<td>128.10±1.0</td>
</tr>
<tr>
<td>Fastum® gel</td>
<td>1.34±0.02</td>
<td>5.39±0.02</td>
<td>16.68±0.39</td>
<td>34.51±0.24</td>
<td>49.94±0.66</td>
<td>78.48±0.67</td>
<td>89.22±0.62</td>
<td>127.00±0.75</td>
</tr>
</tbody>
</table>

Table 2 Evaluation Scores on the Skin Irritation of Sprague-Dawley Rats to the Topical Application of Ketoprofen Loaded and Blank Formulations.

<table>
<thead>
<tr>
<th>Visual observation (Erythema formation)</th>
<th>Scores (both normal and abraded skin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>POEs nanoemulsion with 3% limonene</td>
<td>0</td>
</tr>
<tr>
<td>Market formulation (Fastum gel®)</td>
<td>0</td>
</tr>
<tr>
<td>Blank formulation</td>
<td>0</td>
</tr>
</tbody>
</table>
The nanoemulsion with 3% limonene also showed no irritation up to 72 h post application indicated that limonene is a safe and well tolerated terpene for POEs nanoemulsion formulations.

4 CONCLUSION:
This work suggested that incorporating limonene in POEs nanoemulsion increased the skin permeation rate of ketoprofen through rat’s skin. The optimum formulation contained 3% limonene and showed similar skin permeation properties to that of a marketed formulation. The results of the skin irritation studies based on the visual observation properties to that of a marketed formulation. The results of the skin irritation studies based on the visual observation score suggested that the formulations were safe to be applied on the skin.

ACKNOWLEDGMENTS
NBD-Malaysia for the research grant allocation grant no:304/ PFARMSI/650387/K105; Universiti Putra Malaysia for preparing and supplying the POEs, Universiti Sains Malaysia for the facilities provided and for the Graduate Assistantship(GA) for Sakeena. We also would like to thank Dr. Hassan A. Rathore(Department of Physiology, School of Pharmaceutical Sciences, USM) for his valuable help and advices in preparing the rat skin.

ETHICAL CLEARANCE
All procedures on rats had the clearance from the animal ethics committee of Universiti Sains Malaysia. Approval reference no: USM/PPSF/50/067/Jld.2

References


