# CHAPTER IV

# RESULTS AND DISCUSSIONS

## 1. Synthesis of TMZ-HE

The successfully synthesise of the skin deliverable TMZ-HE, Pybrop® and DMAP were used as the coupling agents which allowed the reaction to proceed successfully yield the products (Suppasansatorn *et al.,* 2006). The optimum condition was found to be a mole ratio of alcohol: drug: Pybrop®: DMAP of 2.2: 1: 1: 2. The reaction time was around 12 hours at room temperature. After TMZ-HE was purified using siliga gel packed in the column, the percentage yield of TMZ-HE was obtained around 40-60 %. It was found that NMR spectrum of TMZ-HE product conformed to the previous standard. Aqueous solubility and log *P* value were also similar to the TMZ-HE standard.

**$^1$H NMR** ($CDCl_3$/ppm) δ 8.49 (s, 1, H-6), 4.45 (t, 2, J= 6.9 Hz, $C\underline{H}_2$-O), 4.04 (s, 3, $C\underline{H}_3$-N), 1.79 (quintet, 2, J= 7.1 Hz, C-$C\underline{H}_2$-C), 1.29-1.40 (m, 6, C-$(C\underline{H}_2)_3$-$CH_3$), 0.87 (t, 3, J= 6.9 Hz, C-$C\underline{H}_3$)

**$^{13}$CMR** ($_{d6}$-DMSO/ppm) δ 161 ($\underline{C}OO$), 139 (C-4), 137 (C-6), 129 (C-9), 127 (C-8), 64.8 ($O\underline{C}H_2$), 36.4 ($N\underline{C}H_3$), 30.9 ($OCH_2\underline{C}H_2$), 28.2 ($O(CH_2)_2\underline{C}H_2$), 25.1 ($\underline{C}H_2CH_2CH_3$), 22.1 ($\underline{C}H_2CH_3$), 13.9 ($CH_2\underline{C}H_3$)

**Solubility in 10% v/v PG in water**= 0.4 mg/mL (~ 0.04 %w/w)

**Log *P***= 2.56

## 2. *In vitro* and *in vivo* bioactivity test

### 2.1 *In vitro* tumor cell growth of temozolomide hexyl ester

The results of the *in vitro* cytotoxicity assay of TMZ-HE, TMZA and TMZ in comparison with carmustine and dacarbazine against a panel of human and murine glioma and melanoma cells were shown in Table 7. Carmustine and dacarbazine are the most commonly used drugs to treat brain and skin cancer respectively

**Table 7.** *In vitro* cytotoxicity of TMZA, TMZ-HE and TMZ, in comparison with dacarbazine and carmustine against cancer cells (Mean±s.d. ;n=3)

| Compound | $IC_{50}$ ($\mu$M) | | | | | | |
|---|---|---|---|---|---|---|---|
| | MV3 | M14 | B16 | B16-BL6 | TJ899 | SGH-44 | TJ905 |
| TMZ-HE | 89.51±4.29 | >358±11.76 | 101.18±4.23 | >358±10.74 | >358±8.75 | 33.87±2.23 | >358±12.33 |
| TMZA | 184.72±7.35 | >500±13.98 | 185.35±6.85 | 222.92±5.52 | >500±12.82 | 36.21±3.11 | >500±14.55 |
| TMZ | 40.48±3.06 | >500±12.09 | 318.51±10.25 | >500±11.46 | >500±11.64 | 46.58±2.67 | >500±13.64 |
| Dacarbazine | 3.55±0.78 | 3.06±0.67 | 54.89±10.76 | 339.01±9.65 | 30.74±2.48 | 6.92±1.16 | 37.65±3.64 |
| Camustine | 29.68±1.22 | 134.06±5.67 | 36.22±1.14 | 61.25±3.44 | 253.26±5.79 | 33.62±1.88 | 314.45±9.38 |

Within the panel of the tumor cells, the groups of cells susceptible and not susceptible to TMZ, TMZA and TMZ-HE were identical: MV3, B16 and SHG-44 cell lines were sensitive, while M14, B16-BL6, TJ899 and TJ905 were not. There was a degree of variation in the range of sensitivity of the tumor cells to TMZ, TMZA and TMZ-HE. More precisely and interesting to note was that the TMZ-HE was more active than TMZA against MV3 and less than TMZ with $IC_{50}$ varying 2-fold

respectively; while against B16, TMZ-HE again was more active than TMZA and surprisingly more active than TMZ with $IC_{50}$ varying 2-fold and 3-fold respectively; against SHG-44, all three compound were almost equally active.

Alteration of the alkyl groups (Valia *et al.*, 1985; Beall and Sloan, 1996) has significantly improved the physicochemical properties of TMZ esters by balancing an adequate aqueous and lipid solubility (Barry, 2001). The esters were effectively metabolised by the esterase enzymes *in vitro* and in the skin suggesting that the prodrug approach was valid for this system.

In the past structure activity studies of imidazotetrazine derivatives have mainly concentrated on modification of an alkyl group on N3 and an alkyl group on N of 8-carbamoyl (Lunt *et al.*, 1987), because there is an assumption that an active derivative should have at least one hydrogen-bond donor connected to the C8-substituent, and the hydrogen bonding between a hydrogen of 8-carbamoyl and N1 in the tetrazine ring is important for TMZ bioactivity (Denny *et al.*, 1994). The hydrogen of the C8-carboxylic acid group in TMZA is a strong hydrogen-bond donor and can form a hydrogen bond with N1 of the tetrazine ring. This is probably the reason for its equal cytotoxicity against the same panel of tumor cells as TMZ. The equal cytotoxicity of the hexyl ester to TMZ and TMZA may be due to its rapid hydrolysis by esterases exosmosed from the cells into the culture medium and the enzymes within the cells, converting it into its parent acid. An attempt to identify the active species of the hexyl ester in the culture medium during the *in vitro* cytotoxicity assay was made without success, due to rapid disappearance of the parental imidazotetrazine nucleus. However, ready hydrolysis into TMZA *in vitro* by pig

esterase and the immediate conversion into TMZA within skin during the permeation experiments indicated TMZA could be the active drug of the ester prodrugs.

It was interesting to note that *in vitro* cytotoxicity assay of TMZ-HE showed more activity than TMZA against MV3 cells with a 2-fold difference in $IC_{50}$ values, while the activity against the other cancer cells was approximately equal. Although there is no evidence suggesting there are significant differences between MV3 cell membranes and other cancer cells, these results may indicate the hexyl group of the TMZ-HE promotes permeation through MV3 cell membranes due to its increased hydrophobicity. Not surprisingly, this study showed *in vitro* cytotoxicity of TMZ, TMZA and TMZ-HE could not match that of dacarbazine and carmustine. A number of independent investigations of TMZ have obtained similar results. For example, a recent study revealed TMZ inhibited metastatic melanoma cell proliferation only at very high concentrations, median $IC_{50}$ 228 $\mu$M (Prignano *et al.*, 2002). However, in a randomized phase III trial involving patients with advanced malignant melanoma, TMZ produced an objective response rate of 13.5% compared with 12.1% in the dacarbazine group (Darkes *et al.,* 2002).

## 2.2 *In vivo* antitumor of TMZ-HE on BALB/c nude mice inoculation with MV3 melanoma

With topical application at a dose of 20mg/mouse/day for two weeks, TMZ-HE effectively inhibited the tumor growth in the mice with % inhibition of over 80% (table 8). At the end of the experiments, both tumor weight and tumor volume of the treatment group showed significant statistical differences compared to the control group.

**Table 8.** *In vivo* inhibition activity of the TMZ-HE against MV3 melanoma in mice (20 mg of TMZ-HE/mouse/day)

| Group | No. of animals | | Body weight (g)[a] | | Tumor volume ($mm^3$)[a] | | RTV | T/C (%) | Tumor weight (g)[a] | Inhibition (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | start | end | Start | End | Start | End | | | | |
| Control | 8 | 8 | 23.0±1.50 | 25.0±0.54 | 105±79.9 | 2381±980 | 8.92±8.97 | - | 2.10±0.80 | - |
| Treatment | 8 | 8 | 24.0±1.53 | 22.9±2.23 | 116±27.0 | 622±545* | 3.01±1.95 | 33.7 | 0.41±0.30* | 80.5 |

*P<0.05 vs control

[a]Body weight (g), tumour volume (mm) and tumour weight (g) were expressed by mean±s.d.; n=8

The hairless mouse has been used more than other breeds in studies of topical administration of drugs because shaving was not required. DMSO was used in preparation of the solution of the TMZ-HE, because DMSO was one of limited solvents in which the drug could be dissolved to an effective concentration and remains stable for the period of experiments. Furthermore DMSO was a known skin permeation enhancer and was tolerated well by animal skins however it is anticipated to replace DMSO in the future *in vivo* bioactivity and clinical studies of the drug. The

significant inhibition of tumor growth in mice by TMZ-HE *via* a topical application indicated the active drug has been adequately delivered into tumor tissues. To achieve this goal, the TMZ-HE has to permeate through the mouse skin, and then be converted into the active form locally. As discussed above, only those compounds which possessed an adequate balance of aqueous and lipid solubility can achieve a skin delivery. Therefore, this result warrants the TMZ-HE to be a good candidate as a skin deliverable anti-cancer prodrug. Currently, the most effective treatment for malignant skin cancer was surgery (Braud *et al.*, 2003) because it was immediate and is effective in removing cancer tissues. However, surgery cannot guarantee 100% removal particularly in late diagnosed cases where variable degrees of metastasis can occur cutaneously. Therefore a topical application to follow-up treatment after surgery could improve the therapeutic efficiency of surgery and prevent further cutaneous metastasis. Furthermore, treatment of early stages of skin cancer certainly was benefited by a locally deliverable active drug on preventing cancer metastasis further and avoiding unnecessary surgery. There was no such treatment available currently, therefore the prodrug, the TMZ-HE, may fulfil this task.



## 3. Delivery of TMZ-HE through skin from VE TPGS microemulsion systems



Regarding the previous studies, it was found that TMZ-HE, with the same *in vitro* bioactivity as TMZ and TMZA as described in previous section exhibited the promising permeability coefficient ($K_p$) and Flux ($J_{ss}$) value through rat and human skin. This ester derivative was therefore chosen to incorporate in topically applied formulation. Microemulsions (MEs) were proposed to use in this study as they are

promising vehicles for skin delivery of drugs demonstrating high drug loading capacity and a penetration enhancer effect (Kreilgaard, 2002).

The choice of components for a pharmaceutical ME is often a balance between compounds, which are able to form MEs, are nontoxic and are able to fulfill the requirements of a good vehicle for optimal absorption (*i.e.,* high solubility of drug of interest). Vitamin E-TPGS NF is a new excipient whose monograph was recently adopted by the United States Pharmacopoeia (Wu and Hopskin, 1999). Structurally, it is amphipathic and hydrophilic, exhibiting the characteristics of typical surface-active agent. It can also be used as an emulsifier, solubilizer, absorption enhancer, and the vehicle for lipid-based drug delivery formulations (Ke *et al.*, 2005). In addition, skin and eye irritation studies of vitamin E-TPGS indicated that it was safe (Wu and Hopskin, 1999). It is thus interesting to employ this agent as a surfactant in ME system. Several oil like-substances in ME systems have been reported to be used as skin enhancers (Peltola *et al.*, 2003). In this study, oleic acid (OA) and isopropyl myristate (IPM) were used as the oil phase. It is known that OA can interact with SC lipids and disrupt their structures, increasing their fluidity and consequently increasing the flux (Larrucea *et al.,* 2001). IPM has been used in several transdermal formulations as a skin penetration enhancer, however, its mechanism of action is poorly understood (Peltola *et al.*, 2003).

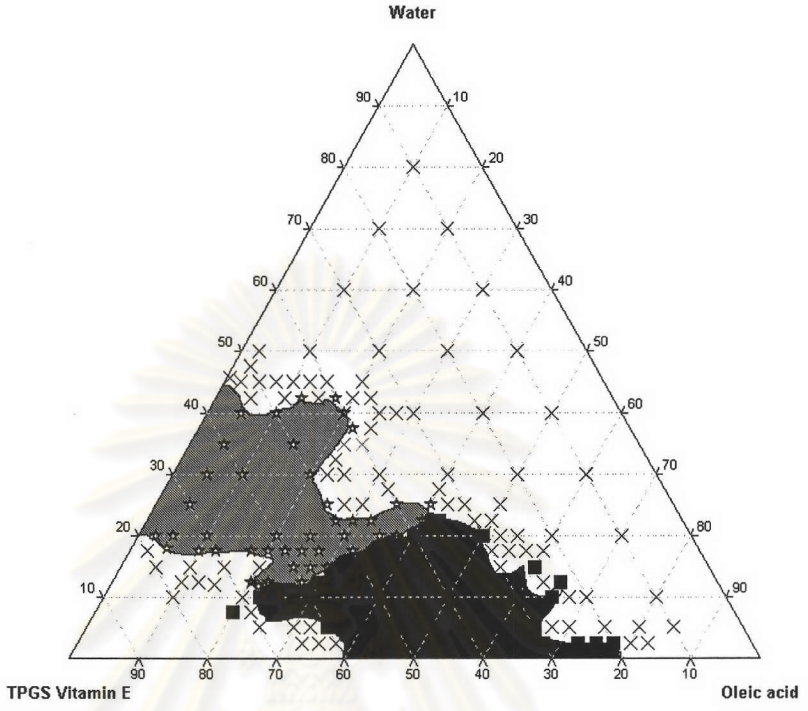
This study is thus to investigate the potential of several ME formulations using vitamin E-TPGS as a surfactant, OA and IPM as an oil phase for increasing transdermal delivery of TMZ-HE. The effect of different oils used and the effect of co-surfactant on skin permeation were also investigated.

## 3.1 Construction of phase diagrams

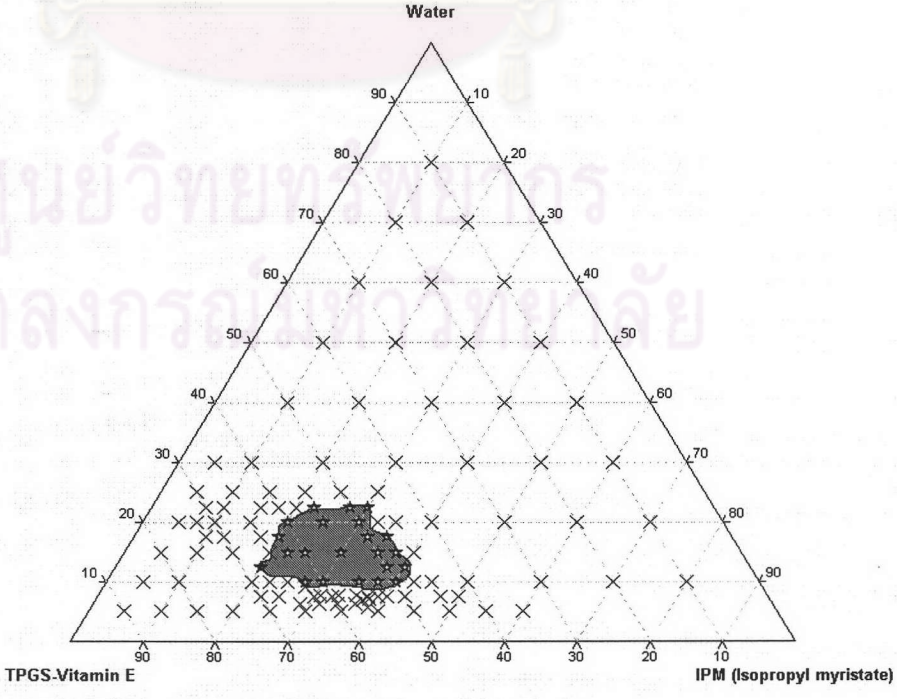
The area of phase behavior was mapped on phase diagrams (figure 22) with the top apex representing water and the other apices showing the oil like substance (OA or IPM) and surfactant (Vitamin E- TPGS) or the combination of surfactant and co-surfactant (Vitamin E-TPGS and isopropyl alcohol). The transparent, low viscosity ME domains were shown in the dark areas while high viscosity ME gel (non-flowable) domains were represented by grey region. The rest of the regions on the phase diagrams represent turbid and conventional emulsions based on visual observation. As shown in figure 22(A), vitamin E –TPGS used as a surfactant can form the ME system *a*, without introducing any co-surfactant. However, when IPM was used instead of OA in ME system *b*, only a narrow ME gel domain resulted (figure 22(B)). This may because OA has carboxylic acid group that can intervene the polar head group (hydroxyl group) of surfactant leading to the increase of the flexibility of the surfactant film. Conversely, IPM is an ester compound and it may reside only in the hydrocarbon chain of VE TPGS (figure 23). The shot chain alcohol (isopropyl alcohol, IPA) was thus employed as a co-surfactant. The surfactant and co-surfactant weight ratio was fixed at 4:1.

As illustrated in figure 22(C), the ME gel region is broadened, and a liquid ME can be formed. This is probably due to lowering of the interfacial tension of the surfactant film by insertion of IPA in the polar head group of surfactant resulting in a more flexible and dynamic layer (Kreilgaard, 2002; Sintov and Shapiro, 2004).

(A)




(B)



(C)

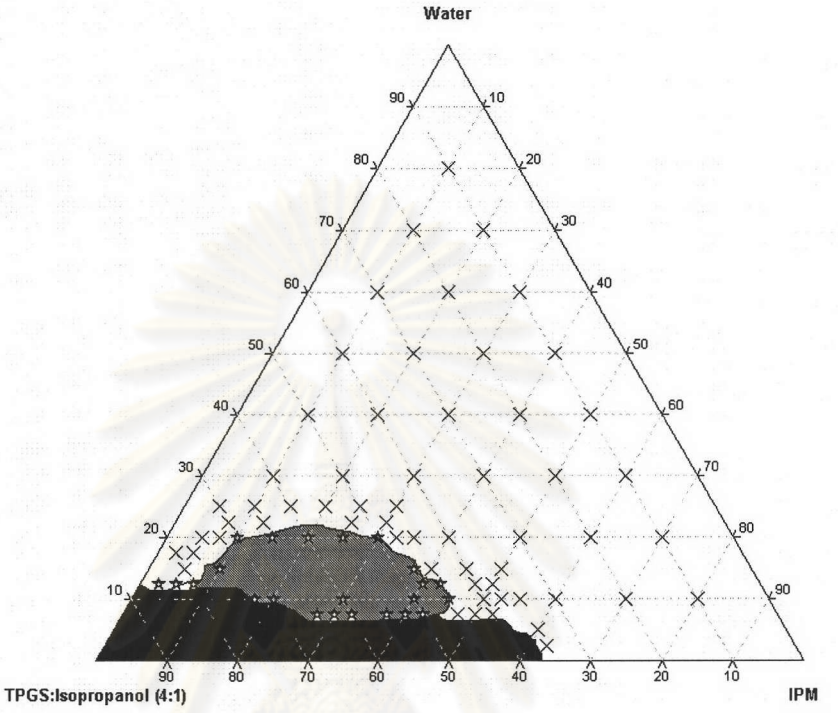
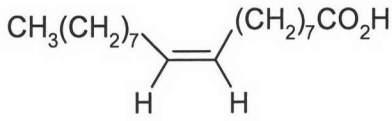
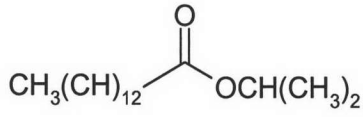


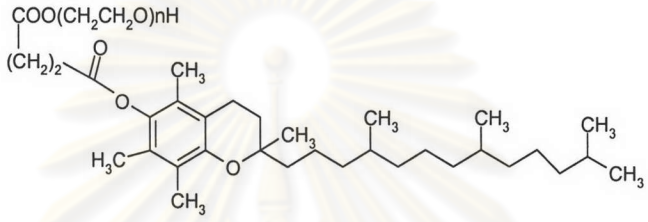

**Figure 22.** Pseudo-ternary phase diagram of (A) ME system *a* consisting of vitamin E -TPGS, water, and oleic acid; (B) ME system *b* consisting vitamin E-TPGS, water and isopropyl myristate; and (C) ME system *c* consisting vitamin E-TPGS / isopropyl alcohol




OA




IPM




VE TPGS

**Figure 23.** Chemical structures of OA, IPM and VE TPGS

## 3.2 Preparation of microemulsions

Vitamin E-TPGS, a solid waxy substance, has a melting point of 37-41°C (Wu and Hopkins, 1999). The key step in ME preparation using vitamin E-TPGS as a surfactant is that this compound should be melted before incorporation into the other ingredients. Furthermore, the preparation of the ME should be carried out at temperature higher than vitamin E-TPGS melting point in order to avoid the precipitation of this substance during the formation of ME. In this study, the temperature was maintained at 45 °C throughout ME preparation.

## 3.3 Electron microscopy study

The isotropic property of all ME preparations selected from the phase diagram were confirmed using cross polarization microscopy. ME $1^a$, ME $2^a$ and ME

3[a] present completely isotropic properties (the formulations appear dark between cross polarizers). Gel-like ME preparations (ME 4[b] and ME 5[c]) also show the complete isotropic properties. Freeze fracture electron microscopy (FFEM) pictures demonstrated the highly packed of surfactant and oil region with a small amount of water in the systems.

FFEM technique is generally used to characterise the microstructure of water rich microemulsion (Burauer *et al.,* 1999). During the freezing process, water is dehydrated and other liquid (surfactant and oil) remained in the samples are used for further visulisation under electron microscopy. In this study, only small amount of water incorporated in the formulation leads to the densely packed of oil and surfactant remained (figures 24 and 25).

**Figure 24.** FFEM image of ME 3 (x 50,000)

**Figure 25.** FFEM image of ME 5 (x 50,000)

### 3.4 Drug loading and solubility testing

Various amounts of TMZ-HE were loaded in different ME formulations selected from the phase diagrams as described in table 9. After drug loading in the formulations, the transparency of the preparations was confirmed using cross polarization microscopy. The loading dose in all ME preparations was up to 75 folds higher than that its aqueous solubility (0.4 mg/mL in 10% PG v/v in water, ~0.04 % w/w, see section 1) as a control. This confirms that ME vehicle was greatly enhanced the solubility of TMZ-HE confirming that the ME system was an effective formulation to increase the solubility of various compounds. In ME 1, ME 2, ME 3 (formulations using ME system *a*), the amount of drug that can be loaded depends on the concentration of vitamin E-TPGS used (see table 9). As the amount of vitamin E-TPGS increased, more drugs can be loaded. This indicates that the more surfactant containing in the formulation leading to the more solubilising capacity of the

formulation. The more surfactant used results in the high aggregation of the molecule (*i.e.* from micellar to hexagonal) and this causes the more space of drug can be dissolved in microemulsion system.

**Table 9.** Composition of the microemulsions (w/w, %)

| Formulation | % drug loading (w/w) | Ingredient (w/w, %) | | | | |
|---|---|---|---|---|---|---|
| | | Water | OA | IPM | Vitamin E-TPGS | IPA |
| 1 ME 1[a] | 1.5 | 20 | 50 | - | 30 | - |
| 2 ME 2[a] | 2.0 | 10 | 50 | - | 40 | - |
| 3 ME 3[a] | 3.0 | 11 | 33 | - | 56 | - |
| 4 ME 4[b] | 2.0 | 8 | - | 35 | 57 | - |
| 5 ME 5[c] | 2.0 | 10 | - | 40 | 40 | 10 |

[a] = chosen from ME system *a*

[b] = chosen from ME system *b*

[c] = chosen from ME system *c*

Using similar levels of vitamin E TPGS in ME systems *a* and *b* (ME 3 and ME 4 respectively) shown in table 9, the amount of drug that can be incorporated in system *b* was reduced because of the greater solubility of TMZ-HE in OA than IPM (table 10). TMZ-HE has a little solubility in water confirming the high lipophilicity of this compound (lop P= 2.56). However, TMZ-HE has the greatest solubility in OA because this oil substant can also behave as emulsifying agent in topical pharmaceutical formulation (Kibbe, 1999). The carboxylic acid group in OA molecule is recognized as a polar head region and it also has a long chain hydrocarbon tail demonstrating an amphiphilic property of the molecule.

**Table 10**. The solubility of TMZ-HE in OA, IPM, and water (n=3; mean±s.d.)

| Solvent | Concentration mg/ml |
|---|---|
| OA | 17.22±0.23 |
| IPM | 13.62±0.67 |
| Water | 0.050±0.004 |

## 3.5 *In vitro* permeation studies

### 3.5.1. Silicone membrane

#### 3.5.1.1 The effect of different loading dose

*In vitro* TMZ-HE flux data through silicone membrane from ME 1, ME 2, and ME 3 were shown in table 11. In all cases, the permeation rate of TMZ-HE from microemulsion formulation was increased significantly ($p<0.05$) when compared to control solution.

Regarding to the preparations of ME 1, ME 2, and ME 3 (from ME system *a*), it was found that increasing the drug loading resulted in an increase in skin permeation rate. Similar to previous literature, drug loading effect was likely to be an effective method to improve the skin permeation rate of various compounds (Chen *et al.*, 2004). The higher concentration of drug loaded in the formulation leads to the increase of driving force of drug to pass through the membrane (figure 26).

**Table 11.** *In vitro* TMZ-HE flux data ($J_{ss}$, nmol/cm$^2$/h, n=3; mean±s.d.) through silicone membrane from ME 1, ME 2 and ME 3.

| Formulation | Ingredient (w/w %) | | | % Drug loading (w/w) | Flux ($J_{ss}$) (nmol/cm$^2$/h) |
|---|---|---|---|---|---|
| | W | OA | VE | | |
| 1 ME 1[a] | 20 | 50 | 30 | 1.5 | 67.94±2.23 |
| 2 ME 2[a] | 10 | 50 | 40 | 2.0 | 79.01±5.04 |
| 3 ME 3[a] | 11 | 33 | 50 | 3.0 | 101.00±1.83 |
| 4 Control sol$^n$ | - | - | - | 0.04 | 35.14±5.95 |

[a] = chosen from ME system *a*

W= water, OA= oleic acid, VE= VE TPGS

Control sol$^n$= 10% propylene glycol (v/v) in water

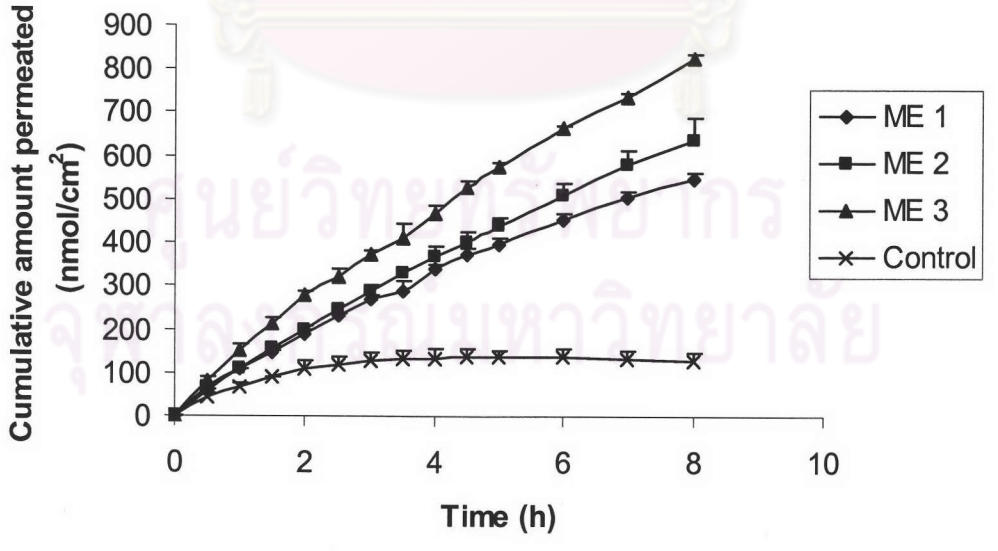



**Figure 26.** The permeation profiles of TMZ-HE from ME 1, ME 2, ME 3 and control solution.

### 3.5.1.2 The effect of co-surfactant

As the data presented in table 12, ME 5 showed a more effective vehicle in increasing TMZ-HE permeation rate through silicone membrane compared to ME 4 with the same amount of drug loading. Figure 27 also showed the higher permeation profile of TMZ-HE from ME 5 than ME 4. IPA was used as a co-surfactant together with VE TPGS in ME 5 at the weight ratio of 4:1. This resulted in the increase of permeation rate of TMZ-HE. The combination of surfactant and co-surfactant seems to has greater solubilising potency than the use of surfactant alone.

**Table 12.** *In vitro* TMZ-HE fluxe data ($J_{ss}$, nmol/cm$^2$/h, n=3; mean±s.d.) through silicone membrane from ME 4 and ME 5.

| Formulation | Ingredient (w/w %) | | | | % Drug loading (w/w) | Flux ($J_{ss}$) (nmol/cm$^2$/h) |
|---|---|---|---|---|---|---|
| | W | IPM | VE | IPA | | |
| 1 ME 4[b] | 8 | 35 | 57 | - | 2.0 | 162.86±19.26 |
| 2 ME 5[c] | 10 | 40 | 40 | 10 | 2.0 | 263.58±4.90 |
| 3 Control sol[n] | - | - | - | - | 0.04 | 35.14±5.95 |

[b] = chosen from ME system *b*

[c] = chosen from ME system *c*

W= water, IPM= isopropyl myristate, VE= VE TPGS, IPA= isopropyl alcohol

Control sol[n]= 10% propylene glycol (v/v) in water

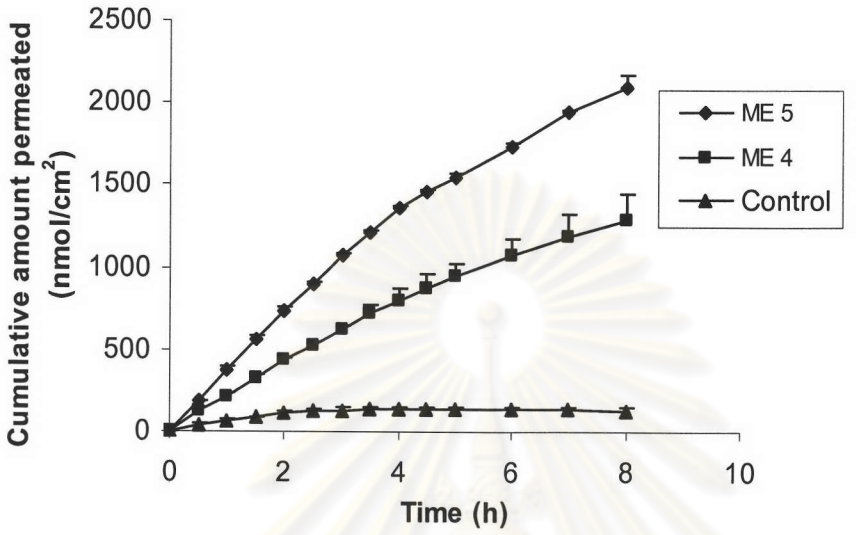



**Figure 27.** The permeation profiles of TMZ-HE from ME 4, ME 5 and control solution. ME 4= IPM containing formulation, ME 5= IPM and IPA containing formulation.

### 3.5.1.3 The effect of different oils used

Interestingly, although, TMZ-HE loading in ME 3 was higher than those in ME 4 the latter presented the greater drug permeation rates (table 13). This seems that the IPM used in ME 4 and ME 5 was more effective than OA employed in ME 3 in delivery of TMZ-HE through silicone membrane. This is also corresponded to previous paper, OA as a solvent elicited the lowest flux of hydrocortisone through silicone membrane when compare to other solvents including IPM. It was suggested that OA could form hydrogen bond with silicone membrane leading to the accumulation of the drug dissolved in this agent within the membrane (Cross *et al.*, 2001).

**Table 13.** *In vitro* TMZ-HE fluxe data ($J_{ss}$, nmol/cm$^2$/h, n=3; mean±s.d.) through silicone membrane from ME 3 and ME 4.

| Formulation | Ingredient (w/w %) | | | | % Drug loading (w/w) | Flux ($J_{ss}$) (nmol/cm$^2$/h) |
|---|---|---|---|---|---|---|
| | W | IPM | OA | VE | | |
| 1 ME 3[a] | 11 | - | 33 | 56 | 3.0 | 101.00±1.83 |
| 2 ME 4[b] | 8 | 35 | - | 57 | 2.0 | 162.86±19.26 |
| 3 Control sol[n] | - | - | - | - | 0.04 | 35.14±5.95 |

[a] = chosen from ME system *a*

[b] = chosen from ME system *b*

W= water, IPM= isopropyl myristate, OA= oleic acid, VE= VE TPGS

Control sol[n]= 10% propylene glycol (v/v) in water

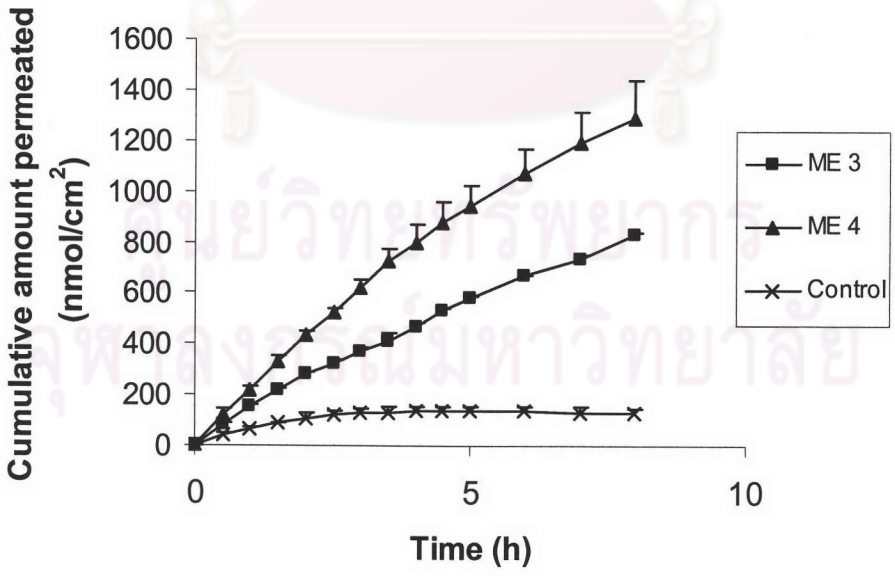



**Figure 28.** The permeation profiles of TMZ-HE from ME 3, ME 4 and control solution. ME 3=OA containing formulation, ME 4= IPM containing formulation

### 3.5.1.4 Overall permeation profiles of TMZ-HE from microemulsion formulations comparing to control solution

The overall permeation profiles of TMZ-HE in different microemulsion formulations were compared to this drug in an aqueous solution (10 % v/v propylene glycol in water) as a control preparation through synthetic silicone membrane (figure 29). The flux continues to increase for all ME preparations over 8 hours studies. On the contrary, the permeation flux from the aqueous solution trends to decrease after 4 hours. ME 5 shows the highest permeation profile when compared to other microemulsion formulation. This confirms the effectiveness of microemulsion to enhance the skin permeation. The reason for this enhancement is the high drug loading capacity of this system and it contains a large amount of surfactant. The oil constituents (OA, IPM) used in microemulsion formulations also behave as the skin enhancer to increase drug permeation. IPA which was used as co-surfactant in ME 5 could increase the permeation rate of TMZ-HE.

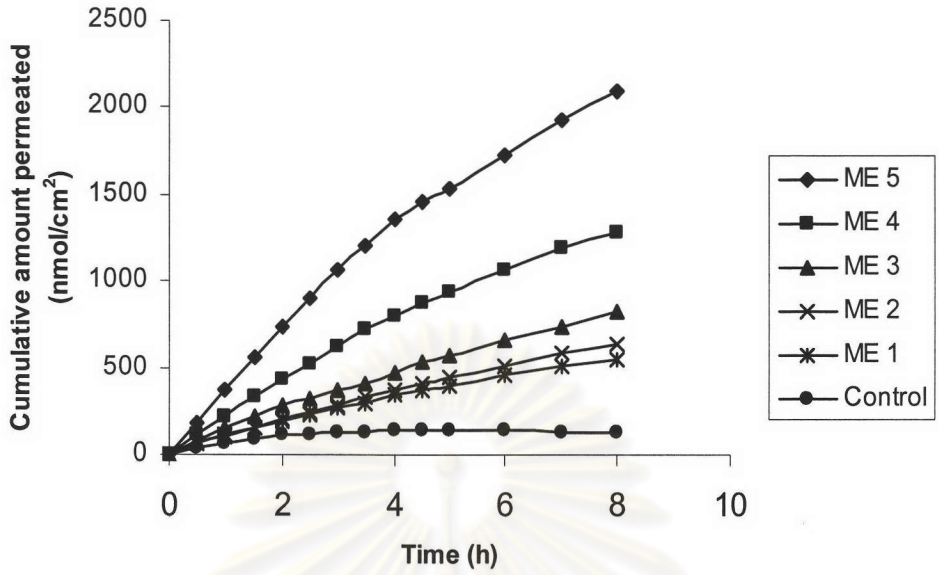



**Figure 29.** The overall permeation profiles of TMZ-HE from ME 1 to ME 5 and control aqueous solution (10 % v/v propylene glycol in water) through silicone membrane

### 3.5.2 *In vitro* permeation through full-thickness hairless mice skin

ME 5 which exhibited the greatest increase in TMZ-HE permeation rate and $K_p$ value through silicone membrane was selected to test on hairless mouse skin (table 14). Moreover, an effect of different oil constituents on the permeation of drug through viable skin was also investigated. In this study, OA and IPM were used as the oil components in ME systems and expected to act as skin enhancers. OA was used in ME 3 instead of IPM which was used in ME 5 were chosen to comparatively study the permeability through hairless mice skin. Additionally, ME 3 demonstrated the significantly ($P<0.05$) higher improve of TMZ-HE permeation rate compared to other OA-containing ME.

Similar to a previous paper (Suppasansatorn *et al.*, 2006), TMZ-HE was hydrolysed by an esterase enzyme within the skin generating TMZA and a small amount of TMZ-HE was also detected (up to 10 % of total amount permeated, data not shown). Both TMZ-HE and TMZA were detected in the receptor fluid using their individual HPLC conditions. The concentration of each compound was calculated in nanomole and then the total amount of drugs permeated was obtained by the addition of each concentration.

**Table 14**. *In vitro* permeability data through hairless mice skin

| Formulation | % Drug loading (mg/mL) | Flux ($nmol/cm^2/h$) | Permeability coefficients ($k_p$) ($cm/h \times 10^{-3}$) |
|---|---|---|---|
| 1 ME 3 | 31.8 | 78.41±6.16 | 6.88±0.54 |
| 2 Neat $OA^{Sat}$ | 17 | 10.80±5.38 | 1.77±0.88 |
| 3 ME 5 | 21.6 | 102.35±5.10 | 12.75±0.90 |
| 4 Neat $IPM^{Sat}$ | 13 | 47.97±6.62 | 10.0±1.42 |

Neat $OA^{Sat}$= Saturated TMZ-HE in OA

Neat $IPM^{Sat}$= Saturated TMZ-HE in IPM

Figure 30 showed the permeation profile of total drugs through full-thickness hairless mice skin. As can be seen in table 14, TMZ-HE fluxes and $K_p$ values from ME formulations were significantly ($p<0.05$) higher than that from their neat oil (IPM, OA) constituents using as the control preparations up to 7 folds. The flux from neat IPM is higher than that from neat OA, therefore IPM is more effective than OA in increasing drug permeation rate through the skin. As a consequence, ME 5 containing IPM in the preparation showed the higher permeation rate than ME 3 containing OA in the formulation. This may because of IPA introduced in the ME 5 system. This compound was reported to be used in combination with IPM to have a synergistic effect on microstructure of stratum corneum resulting in the increase of permeation rate (Brickman and Muller-Goymann, 2003)

The data from different scanning calorimetry as well as wide and small angle x-ray diffraction shows that IPM incorporation into SC results in densly packed bilayer lipids and a loss of order of corneocyte-bonded lipids resulting in the increase of drug permeation (Brinkmann, and Muller Goymann, 2003). However, OA effectively disrupts intercellular lipid matrix when synergistically work with polyol vehicles such as PG (William A.C. and Barry B.W., 2004).

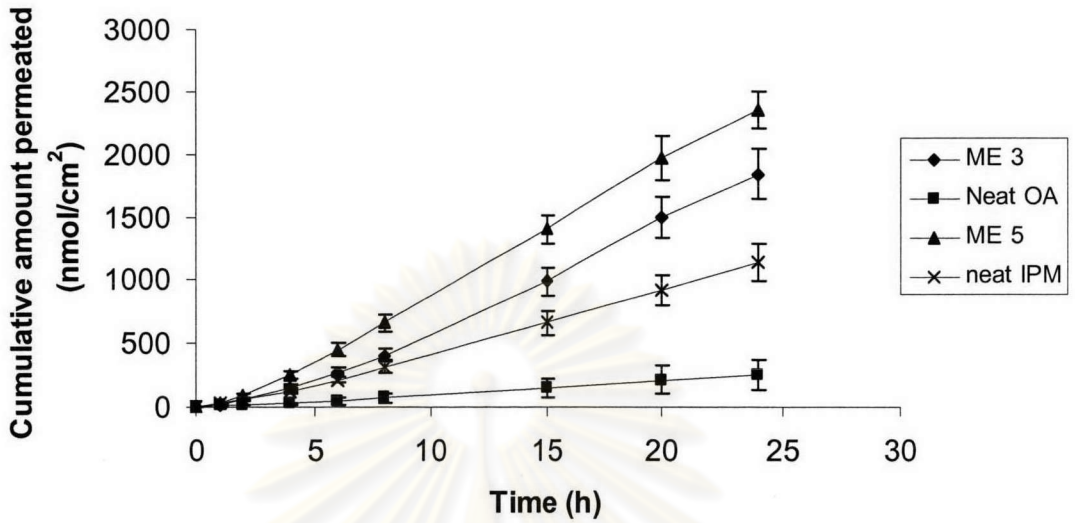



**Figure 30.** The permeation profiles of total drugs permeated (combination amount of TMZ-HE and TMZA) from ME 3, ME 5, neat OA and IPM through hairless mice skin.

## 3.6 *In vitro* skin retention studies

Both TMZ-HE and TMZA were detected during the skin extraction study. In contrast with the permeation experiment, very small amount of TMZA was detected (data not shown). This indicated that esterase enzyme might also exist in stratum corneum layer. The combination amount of drugs retained was calculated in nanomole.

As can be seen in figure 31, neat OA showed the highest drugs retention in SC (3.31 % ±0.99 of drugs permeated, table 15). Conversely, the lowest retention was found in neat IPM (0.18 %±0.06). It also demonstrated that some amount of drugs from both ME formulations was retained in SC (0.36%±0.05 and 0.22%±0.09 for ME 3 and ME 5 respectively).

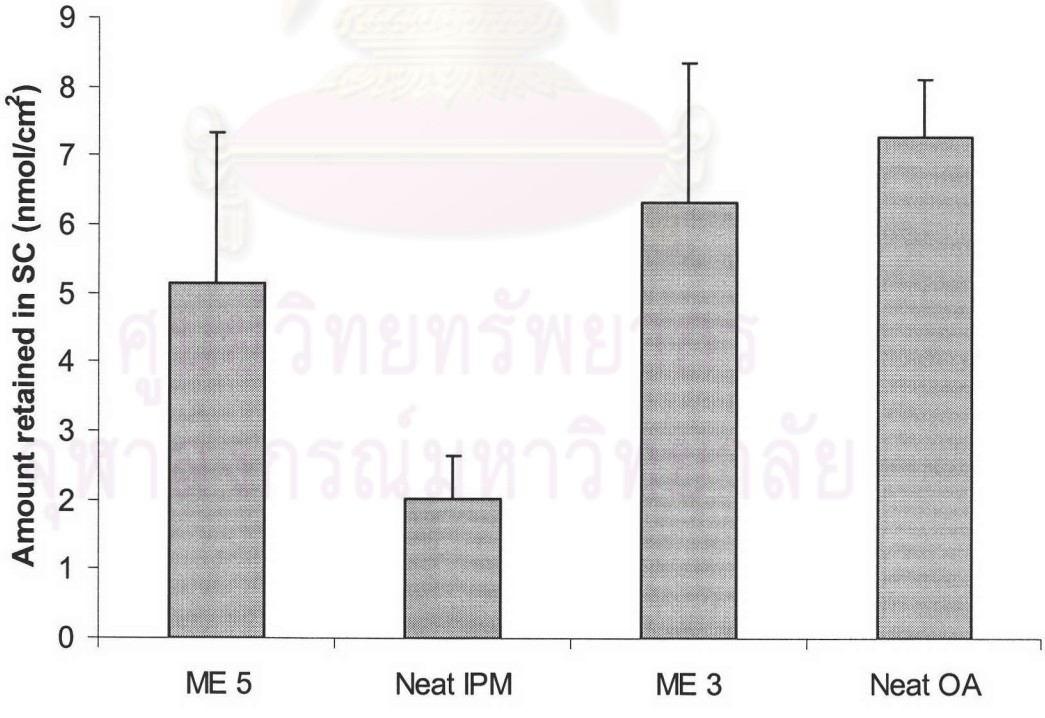



**Figure 31.** *In vitro* retained amount of drugs into SC from ME 3, ME 5, and control oil (IPM, OA) (nmol/cm$^2$, n=4; mean±s.d.)

ME 5 and ME 3 were likely to be able to deliver drug from the preparations to be retained in the skin and also to release drug from the skin to the receptor solution as their fluxes were relatively high (table 15). This result suggested that these ME formulations can behave as both topical and transdermal skin delivery. Although, neat OA presented the lowest TMZ-HE flux through hairless mice skin (table 15), it can extensively deliver drug to accumulate in the skin. Therefore, OA microemulsion is likely to be a promising vehicle for topical delivery.

**Table 15.** The amount of drugs retained in SC and permeated through skin over 24 h ($nmol/cm^2$, n=4; mean±s.d.)

| Formulations | Cumulative amount of drugs permeated ($nmol/cm^2$) | Amount of drugs retained ($nmol/cm^2$) | % drugs retained of that permeated |
|---|---|---|---|
| ME 3 | 1782.75±197.48 | 6.33±0.20 | 0.36±0.05 |
| Neat OA | 245.67±151.37 | 7.29±0.62 | 3.31±0.99 |
| ME 5 | 2320.32±179.91 | 5.15±0.22 | 0.22±0.09 |
| Neat IPM | 1135.41±119.79 | 2.01±0.81 | 0.18±0.06 |