CHAPTER III

MATERIALS AND METHODS

Materials

Chemicals

TMZ free drug (synthesised)

Sodium nitrite (AnalaR BDH Chemicals Ltd. Poole, UK)

Concentrated sulphuric acid (Fisher Scientific Ltd., UK)

Anhydrous hexanol, (Sigma-Aldrich Co. Ltd., UK)

Pybrop® (Calbiochem-Novabiochem Corp., UK)

4-dimethylaminopyridine, DMAP, (Avocado, Research chemicals Ltd. UK)

Dried dimethylformamide, DMF, (Fisher Scientific Ltd., UK)

Dried tetrahydrofuran, THF, (Fisher Scientific Ltd., UK)

Sodium phosphate monobasic anhydrous (Sigma-Aldrich Co. Ltd., UK)

Dimethylsulfoxide (DMSO) (Fisher Scientific Ltd., UK)

Isopropyl myristate (IPM) and Oleic acid (OA) (Sigma-Aldrich Inc., UK)

Vitamin E-TPGS NF grade (Peboc division of Eastman Company, limited UK)

Isopropyl alcohol (IPA) (Sigma-Aldrich Co., UK)

Skin and membrane

Silicone membrane (Advance Bio-technology, Inc, Silverdale, USA)

Hairless mouse skin (Medical research unit, Aston University, UK)

Cancer cell

Human colon carcinoma; HCT-8,

Human non small lung carcinoma; A549,

Human breast cancer; MCF-7,

Human hepatocellular carcinoma; Bel-7402,

Human stomach adenocarcinoma; BGC-823,

Two human melanoma cell lines; MV3 and M14,

Two mouse melanoma cell lines; B16 and B16-BL6,

Three human glioma cell lines; TJ899, SHG-44 and TJ905,

All tumor cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Materials Co. Ltd., China),

Instrumentation and materials

Bruker NMR AC 250 spectrometer

Rotary evaporator

Jacketed Franz-type diffusion cell (Tianjin Beifang Sic-Tech, China)

HPLC (Water 712 WISP autoinjection, Supelco, INC.)

Spectrophotometer and Bench mark microliter plate reader (Bio-Rad, Bio-Rad Laboratories, CA, USA)

3 M Microspore®

Animal

BALB/c-nude mice were provided by Medical Animal Institute of Chinese Academy of Medical Sciences, and were housed in autoclaved filter-capped cages. Animals had free access to a sterilized pellet diet and autoclaved water.

Methods

1. Synthesis of TMZ-HE

1.1 Synthesis of TMZA

8-Carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazine-4(3H)-one (TMZ) (2.577 mmol) was stirred in 4 mL of conc. H_2SO_4. Sodium nitrite (9.4 mmol) was dissolved in 2.6 mL water and added dropwise into the mixture on an ice bath at controlled temperatures below 15°C and then the mixture was kept stirring overnight at room temperature. Ice (10 mL) was added into the reaction mixture and cooled on ice bath for 1 h. The precipitated solid TMZA was collected with filtration and dried under vacuum (98.6% yields).

1.2 Coupling reaction

Anhydrous DMF (2 mL), and THF (3 mL) were injected into a flask containing TMZA (1 mmol) and Pybrop® (1 mmol), and the mixture was stirred until the solids were completely dissolved. On an ice bath, the anhydrous hexanol (2.2 mmol) was injected into the mixture followed by the addition of DMAP (2 mmol). The reaction was allowed to continue on an ice bath for 30 min, and then continueously stirred at room temperature overnight.

1.3 Separation and purification

1.3.1 Separation

After completion of the reaction, the suspension containing the excess solids of by products was filtered through an Ace Buchner funnel. The

obtained clear solution was evaporated under a normal and high rotary evaporator to remove THF and DMF respectively. Ice was added to the residue and the product was extracted from the suspension using 10 ml of ethyl acetate in a separating funnel. The extraction was repeated three times. The ethyl acetate layers were collected, and a drying agent, magnesium sulphate, was added. The clear solution was separated and then it was evaporated using normal rotary evaporator.

1.3.2 Purification

A small amount of ethyl acetate was added to dissolved the residue obtained from 1.3.1 and then the concentrated liquid mixture was passed through a chromatographic column packed with silica gel using ethyl acetate as an eluent.The eluate was evaporated, and the pure solid product was obtained.

1.3.3 Characterization

The obtained product from section 1.3.2 was characterized using NMR spectroscopy.

1.3.4 Physical properties analysis

1.3.4.1 Solubility study

The solubility of TMZ-HE was determined in 10% v/v propylene glycol (PG) in water by stirring an excess amount (1 mg/mL) of this agent at room temperature (25 °C) for 24 h. The mixture was then filtered, diluted with sample solvent and analysed using HPLC (see section 3.6 for HPLC condition).

1.3.4.2 Log *P* (octanol/water partition coefficient) measurement

The equal volumes of two solvents (octanol and water), 4 mL, with TMZ-HE dissolved in aqueous phase (0.3 mg/mL) were placed in screw-capped glass tube. To obtain equilibration, the sample was vertexed in water bath at 37 °C for 1 h. The sample tube was left overnight in water bath and allow the phase to separate. After removing the organic phase, TMZ-HE in aqueous water phase was determined. The octanol/water partition coefficient (Log P) was calculated using; Log P= $(C_0-C)/C$, Where C_0 is the initial concentration in the aqueous phase and C is the concentration after partition (Hatanaka *et al.,* 1998).

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

2.1 *In vitro* tumor cell growth inhibition

For the estimation of *in vitro* tumor cell growth inhibition, MV3 (human melanoma), M14 (human melanoma), B16 and B16-BL6 (mouse melanoma), SHG-44, TJ899 and TJ905 (human glioma), MCF-7 (human breast), HCT-8 (human colon), A549 (human non small lung), Bel7402 (human hepatocellulor), and BGC-823 (human stomach) were used.

All tumor cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 incubator. After being harvested from culture flasks, a cell suspension of 1 x 10^4/mL was made by using 1640 RPMI medium supplemented with 10% fetal bovine serum. One hundred µl (contains 1000 tumor cells) of the cell suspension was seeded per well of 96-well flat-

bottom Costar culture plates at 37°C in a humidified 5% CO_2 incubator for 24 h before introducing drugs (TMZ, TMZA, and TMZ-HE). Each tested compound was dissolved in DMSO and made for five different concentrations and 100μl of each concentration was introduced into parallel three wells; control wells were added with 100 μl of plain medium alone. Cells were then incubated for 96 h. Proliferative response and cell death were determined using MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide) assay.

MTT assay: Following incubation of cells with the tested compounds for 96 h, 100μl of 0.4 mg/mL MTT in RPMI 1640 was added to each well and the plate was incubated at 37 °C for 4 h. The plate was then centrifuged and followed by removal of medium. One hundred and fitty μl of DMSO was then added to dissolve formazan crystal. DMSO-added plates were placed on a plate shaker for 5 min and absorbance was measured spectrophotometrically at 540 nm using a Benchmark microtiter plate reader (Bio-Rad 550, Bio-Rad Laboratories, CA, U.S.A.). The IC_{50} was calculated from the curve of the tested compound concentrations against the % inhibition of the tumor cells.

2.2 *In vivo* anticancer activity of the TMZ-HE on BALB/c nude mice inoculated with MV3 melanoma

The TMZ-HE was chosen to conduct an *in vivo* antitumor test. BALB/c nude mice were inoculated with MV3 melanoma (human) on their right axilla. Once the tumor volume became 100-300mm^3, the mice were divided into a control and a test group of eight with a rational distribution of the mice according to the sizes of the

tumors. The tested group was treated with 5% (w/v) dimethyl sulfoxide (DMSO) solution of the TMZ-HE twice a day *via* repeated topical application (total dosage is 20 mg/mouse/day), while the controlled group was left to grow naturally. Preparation of 5%(W/V) DMSO solution of the TMZ-HE was carried out by weighing an actual amount of the TMZ-HE, and then using DMSO to make the concentration to 50mg/ml. After TMZ-HE was completely dissolved and a clear solution was obtained, the fresh made 5% (W/V) DMSO solution of the TMZ-HE was used in the experiments.

In a period of two-week experiment, the tumor growth in the mice was carefully recorded through body weighing and the measurement of the tumor sizes. The tumor volumes (TV) in the mice were measured for twice of a week (once on Thursday and Sunday), calculated using TV (mm^3) = 1/2(length X $width^2$), and carefully recorded. After two weeks, the mice were sacrified; tumor bulks were isolated, weighed and the growth inhibition of TMZ-HE was calculated. The antitumor activity of the TMZ-HE against MV3 melanoma (human) in BALB/c nude mice was evaluated by the relative tumor proliferation ratio (T/C %). T/C (%) was calculated by the following equation:

$$T/C\ (\%) = \frac{RTV\ (Tested\ group)}{RTV\ (Controlled\ group)}\ X\ 100 \qquad (1.8)$$

Where, RTV = Vt/Vo (Vo was the tumor volume (TV) at the time when the mice were divided into the tested group and the controlled group; Vt was the tumor volume (TV) which were measured at each time interval (Figure 16). The criteria

for effectiveness were (1) when T/C (%)>60, the compound was no effect; (2) when T/C (%) ≤60 and statistically significant P<0.05, the compound was effective.

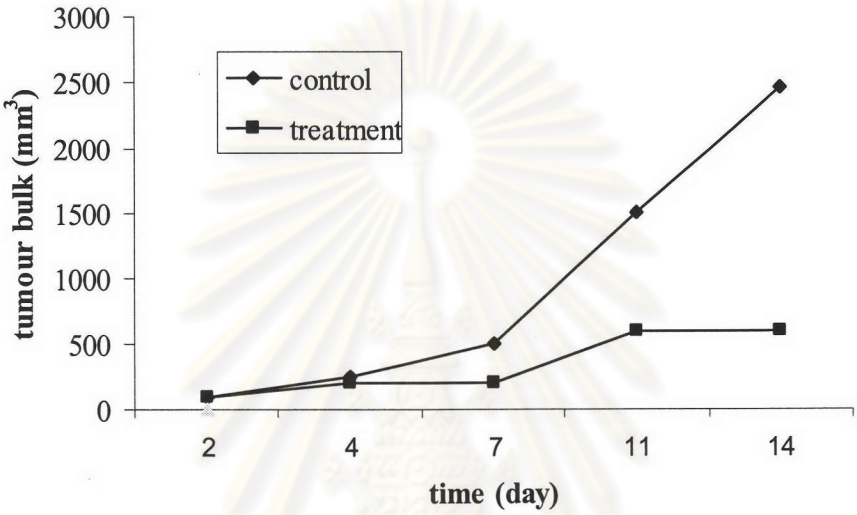

Figure 16. Changes (means) in tumor volumes of TMZ-HE treatment group *vs* the control group

2.3 Statistical analysis

Student's t test was performed to investigate significant different of tumor volume and tumor weight in mice between treated (applied with 5% TMZ-HE in DMSO) and control untreated group (p= 0.05).

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

3.1 Preparation of microemulsion

VE TPGS was melted in a controlled temperature at 45 °C ± 0.2 °C. The oil phase, Oleic acid (OA) or Isopropyl myristate, (IPM) was then added and kept stirring using magnetic stirrer until the mixture was homogeneously mixed. Finally, the mixture was titrated with water and stirred until it became clear.

3.2 Determination of ternary phase diagram

Pseudo-ternary phase diagrams of oil (OA or IPM), water and surfactant (VE TPGS) or the combination of co-surfactant (Isopropyl alcohol, IPA)/surfactants were preliminary determined by preparing various combinations of those constituents corresponding to the whole area of triangle diagram. The boundary was subsequently determined by titrating a small amount of water with the desired oil: surfactant ratio obtained from the previous preliminary studies.

Visual inspection after stirring confirmed the formation of a microemulsion and the boundary ratios were then marked as points in the phase diagram. The phase state was classified into three categories: *i.e.* a single clear one-phase of low viscosity, a non-birefringent high viscosity gel-like phase and a multiple phase (Kawakami *et al.*, 2002; Agatonovic-kustrin *et al.*, 2003) . The areas covered by these points were termed the microemulsion, microemulsion gel, and non-microemulsion region of existence respectively.

3.3 Saturated solubility study

To determine the saturated solubility of TMZ-HE in neat IPM and OA, an excess amount of drug (0.05 g) was added to 2 mL of these solvents. The mixtures were kept stirring at room temperature for 2 days, then the contents were centrifuged at 10,000 rpm for 15 min (Schmalfuβ *et al.,* 1997; Narishetty and Panchagnula, 2004). One hundred μl aliquots of supernatant containing the saturated solutions were diluted with 20 % v/v acetonitrile in water to 100 mL and the solutions were assayed by HPLC. During the operation of permeability testing, the saturated solutions of TMZ-HE in neat IPM and OA were used as a control formulations comparing to TMZ-HE in microemulsion formulations.

3.4 Drug loading in microemulsions

According to the preliminary studies of the maximum amount of TMZ-HE loaded in microemulsion formulations, the maximum dose of TMZ-HE was thus incorporated into each formulation. The higher amount of drug loaded leaded to the precipitation of TMZ-HE. The high drug concentration behaves as the driving force to obtain the highest permeation rate of drug from the formulations. TMZ-HE was first dissolved in the oil phase (OA or IPM) and then molten VE-TPGS was added. The mixture was finally titrated with water.

3.5 Characterization of microemulsion

In order to characterize the obtained systems, polarization microscopy and electron microscopy were carried out (Schmalfuβ *et al.,* 1997). The formulations that appeared dark when viewed between crossed polarizers would exhibit non-

birefringent (isotropic) properties. The samples not showing non-berefringent (anisotropic) properties would be classified as liquid crystal (Baroli *et al.*, 2000). The promising formulations were also investigated using Freeze Fracture Electron Microscopy (FFEM) technique.

3.6 HPLC analysis of TMZ-HE and TMZA

All HPLC studies were carried out under isocratic conditions using a Waters 712 WISP autoinjector, 600E pump and 484 UV detector at 330 nm. All separations were carried out using a flow rate of 1.0 mL/min, a 200 µl injection volume on a Hypersil ODS 5µm column (100 x 4.6 mm) preceded by a guard column. TMZA were quantified using a mixture of 0.5% acetic acid/methanol (90:10) as mobile phase and mitozolomide as the internal standard. The TMZ-HE was quantified using a mixture of acetonitrile: water (80:20) as mobile phase and anthracene as the internal standard. A calibration curve of peak area ratio against drug concentration was constructed for each compound giving good linearity ($r^2 \geq 0.999$) and used to quantify the unknown samples.

3.6.1 Preparation of stock and internal standard (IS) solutions

3.6.1.1 Standard stock solutions

(a) TMZA 0.002g was dissolved in 2mL of 10% w/v of propylene glycol in water and then water was added to 50 ml.

(b) TMZ-HE was dissolved in 5mL of 10% w/v of propylene glycol in water and then 5mL of acetonitrile was added as a co-solvent, finally it was diluted with water to 50mL.

3.6.1.2 Internal standard solutions

(a) Mitozolomide internal standard solution for TMZA; Mitozolomide (0.0018g) was dissolved in 150mL of 0.5% v/v acetic acid.

(b) Anthracene internal standard solution for TMZ-HE; Anthracene (0.002g) was dissolved in 75mL acetonitrile.

Typical chromatograms of TMZ-HE and TMZA were shown in figures 17 and 18 respectively.

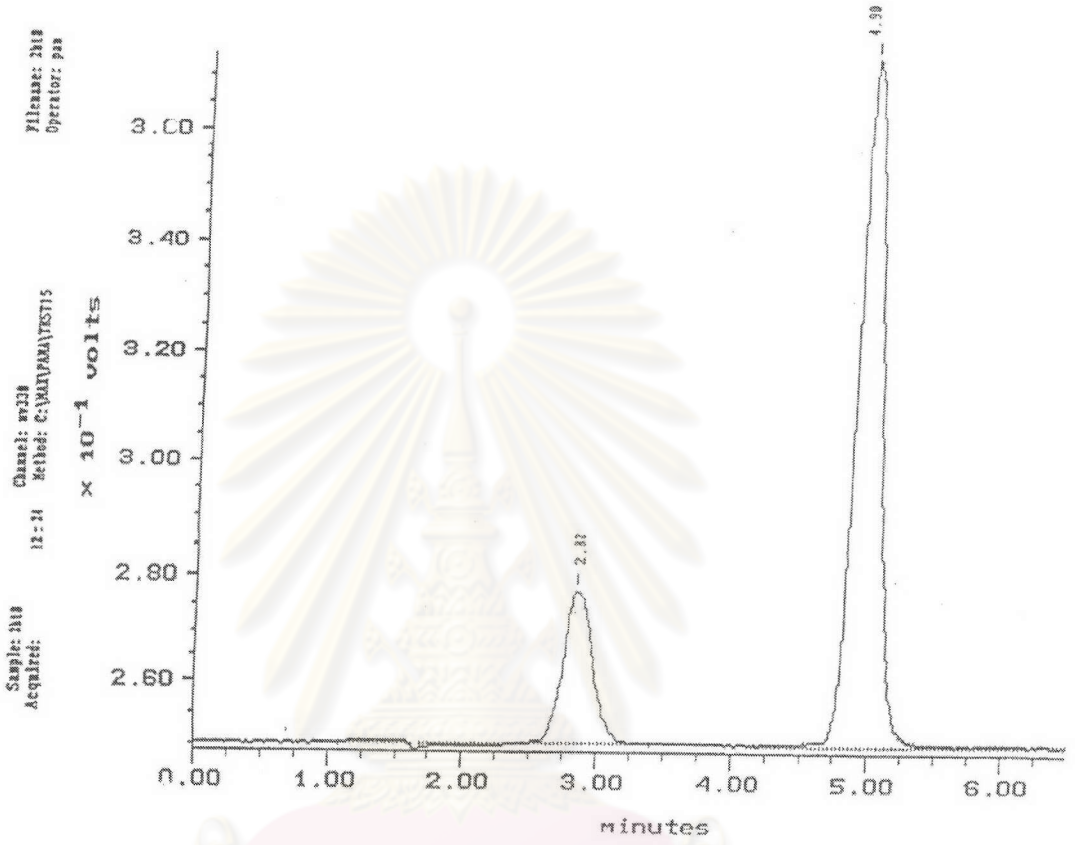

Figure 17. A typical chromatogram of TMZ-HE (2.82) and anthracene as internal standard (4.90)

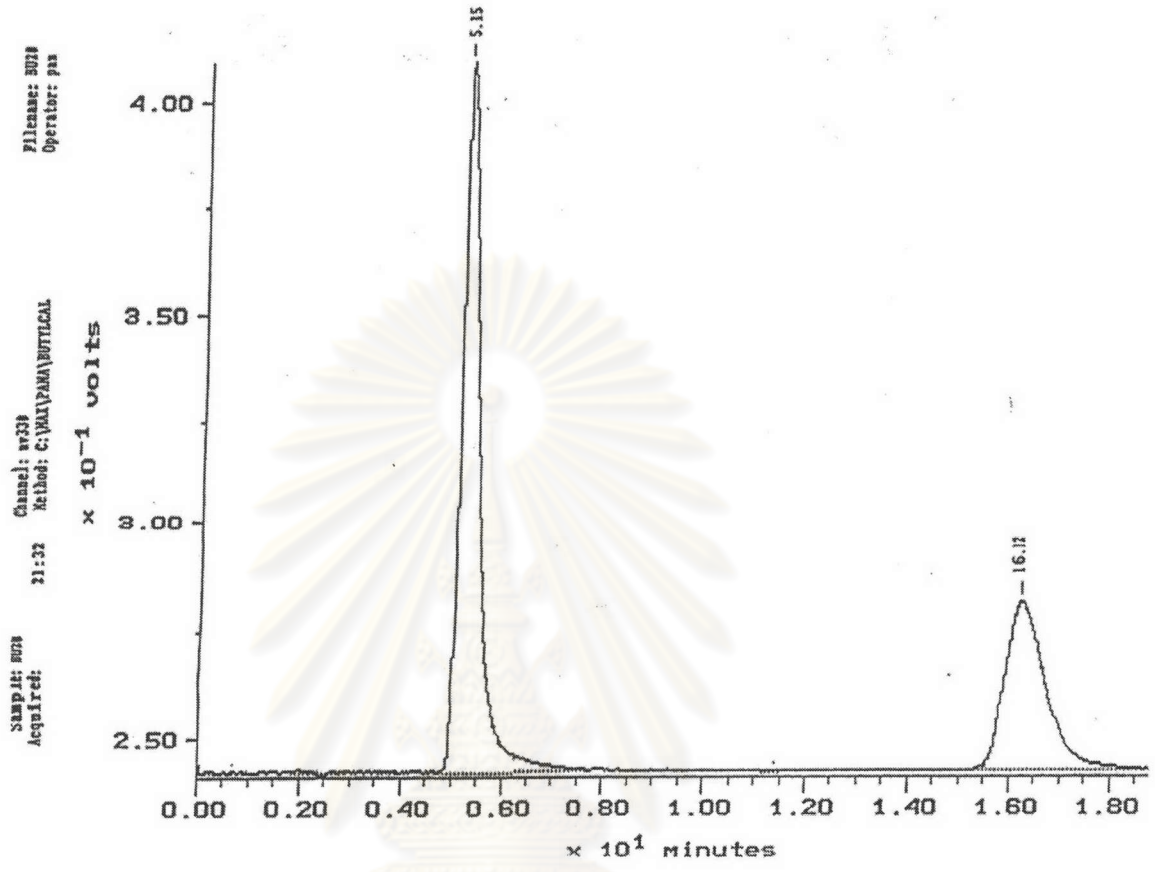

Figure 18. A typical chromatogram of TMZA (5.15) and mitozolomide as internal standard (16.12)

3.6.2 Preparation of calibration solutions

Standard stock solutions (40μg/mL) of TMZ-HE and TMZA were diluted to various concentrations, which were generally 5-6 standards (20, 8, 4, 2, 0.04 μg/mL). Standard working solutions (1mL) were added to an injection vial and then internal standard solution (1mL) was added to it. The solution mixture (200 μl) was injected onto the column.Typical calibration curves of TMZ-HE and TMZA were shown in figure 19 and 20 respectively.

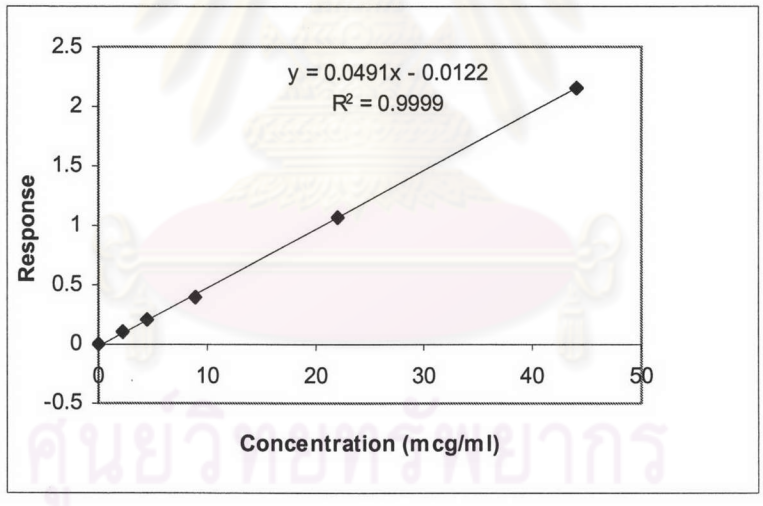

Figure 19. A typical calibration curve for HPLC detection of TMZ-HE product

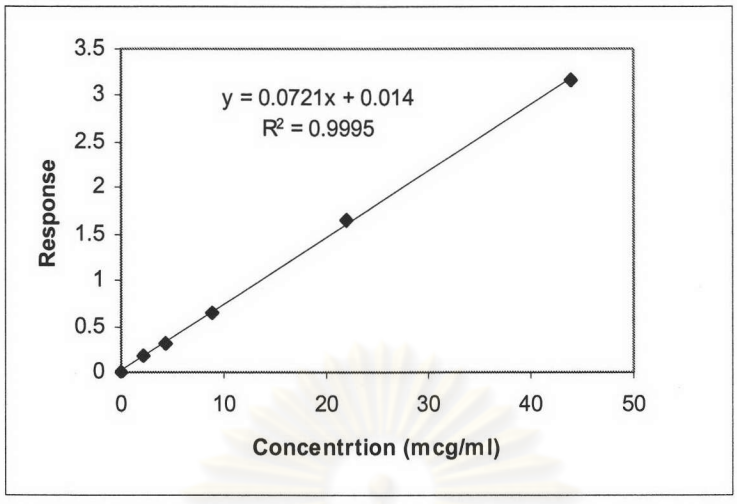

Figure 20. A typical calibration curve for HPLC detection of TMZA

3.7 *In vitro* permeation studies

3.7.1 Preparation of membrane

3.7.1.1 Silicone membrane

Synthetic silicone membrane, which was 0.05 inches thick, was cut to fit to the size of the diffusion cell cap, and was thoroughly washed before used with distilled water.

3.7.1.2 Hairless mouse skin

Full-thickness mice skin was obtained from hairless mice (HsdO1a:MF1, Harlan UK Limited, Bicester, Oxon England) aged 9 to 10 weeks and weighing 30-40 g. The animals were sacrificed by cervical dislocation and the abdominal region of each rat was carefully shaved using electric clippers. The whole-thickness intact skin was excised using a sharp pair of scissors and then the underlying connective tissue and fat tissue were removed from the undersurface. The

skin was cut into a small piece fitting to the size of diffusion cell cap, and it was used immediately.

3.7.2 Preparation of receptor phase solution

Double distilled water was boiled to get rid of the air bubbles and then it was allowed to cool down to room temperature. Finally, it was sonicated to completely de-gas.

3.7.3 Preparation of diffusion cell

The membrane (as detailed above) was mounted on the jacketed Franz-type diffusion cell, on which the surface area available for diffusion of each cell was about 2.8-3.4 cm^2. The cell cap was placed on the membrane, and it was sealed with Parafilm® connecting the cell cap, membrane, and receptor cell body (see figure 21). The capacity of each receptor cell was around 25-26 mL, which was filled with receptor solution, which was completely in contact with the membrane sheet. During the course of experiment, the solution was continuously stirred by a teflon-coated magnetic bar placed in the cell. The temperature was maintained at 37°C by a thermostatic water pump that circulated the water through the jacket surrounding the cell body. The system was allowed to equilibrate under these conditions for half an hour. Finally, the prepared sample formulation (3g) was added into the donor cap cell, and it was sealed with Parafilm® to minimise the evaporation of donor solution. 1mL of receptor solution was removed periodically for 8 h for the experiment performed on silicone membrane and 24 h on full-thickness hairless mice skin. The volumes withdrawn were always replaced with equal volumes of fresh receptor solution to maintain constant volume. The concentration of each sample solution was determined by using HPLC.

For every sample, six determinations were performed on hairless mice skin, and four determinations were carried out through artificial silicone membrane. The mean values were used for correlation and comparison purposes.

Drug in the receptor chamber was also determined using HPLC. The steady-state flux (J_{ss}) was estimated from the slope of the linear portion of the cumulative amount of drug absorbed against time profiles. The permeability coefficient (K_p) was also calculated using the following equation;

$$K_p = \frac{J_{ss}}{C} \qquad (1.9)$$

As stated above, after the removal of receptor solution, the receiver compartment was refilled with an identical volume of receptor fluid. This causes of the dilution of receptor solution leading to an under determination of the sample concentration. As a consequence, it was necessary to mathematically correct each successive sample concentrations, which was determined by applying the following equation:

$$C_t = C_{mt} + \left[V_s . \frac{\sum_{t=1}^{t=n-1} C_m}{V_r} \right] \qquad (1.10)$$

where C_t is the actual current concentration of drug in the receptor phase at time t, C_{mt} is the apparent (*i.e.* measured) current concentration of drug in the receptor phase, V_s is the sample volume withdrawn for analysis, V_r is the volume of receptor solution, and ΣC_m is the summed total of the previous measured concentrations.

Finally, the amount of drug penetrating the skin *per* unit area was calculated by dividing the obtained concentrations by the surface area available for diffusion cell and this value was different for each individual cell.

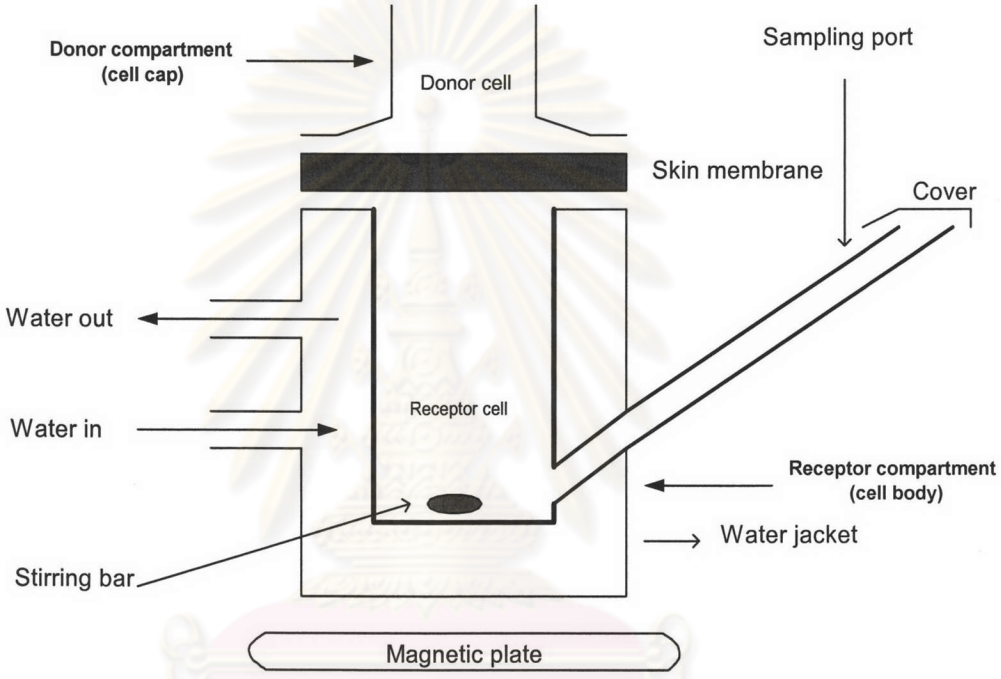

Figure 21. Schematic illustration of a diffusion cell

3.8 *In vitro* skin retention studies

After 24 h of skin permeation studies as described above, the skin were carefully washed with distilled water. The skin stripping technique was then performed in order to remove the stratum corneum (SC) (De Rosa *et al.,* 2003). The skins were stretched and fixed in the Petri dish. The SC was removed by stripping the skin ten times with adhesive tape (3 M Microspore®). Ten tape strips were incubated

in 20 mL of acetronitrile and water (80:20), left overnight, sonicated for 1 min, and then filtered. The amount of drug in the filtrate was determined by HPLC.

3.9 Statistical analysis

The significant difference in permeation rate or permeability coefficiency value of TMZ-HE between two preparations was calculated using Student's t test ($p= 0.05$).