CHAPTER III

MATERIALS AND METHODS

Materials

Drug: -

Zidovudine (AZT) : 100.1% assay, AR grade, was from Brantford Chemical, Ontario, Canada)

Chemicals: -All chemicals were AR geades.

Disodium hydrogen phosphate, potassium chloride, sodium bicarbonate, sodium carbonate anhydrous, and sodium phosphate were obtained from Farmitalia Carlo Erba, Milano, Milan, Italy.

Sodium acetate anhydrous, sodium chloride, sodium dihydrogen phosphate, and sodium phosphate (tribasic) were from Merck, Darmstadt, Germany.

Lauric acid was from Uniqema, Emmarich, Germany.

Monobasic potassium phosphate was obtained from Ajax chemical, Auburn, Australia.

Sodium hydroxide was from Eka Nobel, Bohus, Sweden

Reagents: - Almost all reagents were AR grade, unless they were mentioned otherwise.

Bis (d – ethylhexyl) adipate purum, Isopropyl myristate, n-decanol, n-heptanol, n-hexanol, n-nonanol, n-octanol, n,n-dimethylacetamide were obtained from Fluka Chemie, Buch, Switzerland

Isopropyl palmitate and medium chained triglycerides were from Uniqema, Selango Darul Ehsan, Malaysia.

Absolute ethanol was from E. Merck, Darmstadt, Germany.

C_{12-15} alkyl benzoate was from Finsolv TM TN, Fintex, USA.

Glacial acetic acid was from F16828, J. T. Baker, USA.

Hydrochloric acid was from Univar, Auburn, Australia.

Methanol, HPLC grade, was from Lab Scan, Bangkok, Thailand.

N – methyl – 2 – pyrrolidone (Pharmasolve®) was from ISP Pharmaceutical, Texas City, USA.

Oleic acid was from Sigma, Buchs, Switzerland.

Polyethylene glycol 400, commercial grade, was received from Srichand UnitedDispensary co., LTD, Thailand.

Membranes: -

Controlled caliper ethylene vinyl acetate (9% EVA) membrane (3M™ Cotran™ 9702 Membrane), and microporous polyethylene film (No. 9711 Cotran™ Membrane) were from 3M, St. Paul, USA.

Porous polycarbonate membrane (Isopore™ Membrane filter was from Schleicher &Schuell, Dassel, Germany.

Newborn pig skin was generously supplied by, Local farm in Nakornpathom, Thailand.

Equipment: -

Analytical balance (Model A200S, Sartorius GmbH, Germany and Model PB3002 Mettler, Switzerland)

High Performance Liquid Chromatography (HPLC) (Thermo Separation Product Sanjose, USA) consists of Autosampler : AS 3000, Pump: Spectra SYSTEM P1000, UV absorption detector: Spectra SYSTEM P1000 and data station operated by software P1000.

Magnetic stirrer (Model SP 46920 – 26, Cimarec 2, Thermolyne, USA)

Modified Franz diffusion cells (Crown Glass Company, Inc., New Jersey, USA)

pH meter (Model 232, Pye Unicam Ltd., England)

HPLC column (water Spherisorb® ODS 5 μm, 250 x 4.6 mm, MA, USA)

Ultrasound transonic digital sonicator (Model T900, Elma, Germany)

Ultraviolet-visible spectrophotometer (Model Spectronic 3000, Milton Roy, USA)

Vortex Mixer (Vortex Genie – 2, Model G – 560 E, Scientific Industries Inc., Bohermia, New York, USA)

Water bath with shaker (Model TBVS HETOMIX, Heto, Allerod, Denmark)

Methods

There are three experimental steps for developing AZT transdermal drug delivery by membrane controlling system, they were as following:

1. Determination of physicochemical properties of AZT
2. Preformulation study of AZT
3. Evaluation of AZT transdermal delivery system

1. Determination of physicochemical properties of AZT

Physicochemical properties of drug compound such as dissociation constant (pK_a) and partition coefficient are crucial in designing transdermal drug delivery systems (TDS) (Guy, 1988). According to little in formation about the dissociation constant (pK_a) and partition coefficient (log K) of AZT, it was necessary to these parameter values in this study.

1.1 Determination of dissociation constant (pK_a) of AZT

The dissociation constant of AZT was determined by spectrophotometric method (Martin, 1993). The standard solutions of 1×10^{-7} M of AZT were prepared in a variety of buffer solutions as shown in Table 5.

Table 5 Buffer solutions for pK_a determination of AZT

Buffer solutions	pH range
Glacial acetic acid / sodium acetate	5-6.5
Dihydrogen sodium phosphate / disodium hydrogen phosphate	7 - 8
Sodium bicarbonate / sodium carbonate	8.5 - 10.5
Disodium hydrogen phosphate / sodium phosphate (tribasic)	11 - 11.5

The pH values of AZT buffer solutions were adjusted to be 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, and 11.5, meanwhile, the ionic strength (μ) was fixed at 0.1 M by utilizing sodium chloride.

A 60 mcl of AZT standard solution ($1x10^{-7}$ M) was mixed with a 3 ml of various buffer solutions to the required pH values. The absorbance of AZT buffer solution was triplicately determined at 267 nm through Milton Roy Spectronic 3000.

The absorbance of AZT was plotted against pH values, and the absorbance of the unprotonated (A_o) and protonated (A_α) species were determined from this plot. The pK_a was then estimated from the pH corresponding to absorbance midpoint. For more accurate determination, these pK_a values were transformed to K_a and then substituted into eqn.8 for calculating the absorbances of AZT

$$A = (A_0[K_a] + A_\alpha[H^+]) / ([H^+] + K_a) \quad \text{.....(8)}$$

where A is the calculated absorbance at any given pH value, A_o is the absorbance of the unprotonated species, A_α is the absorbance of the protonated species, and $[H^+]$ is the concentration of hydrogen ion.

The similarity factor f_2, (Shah, 1998) was used for comparing the absorbance-pH profiles obtained from the experiment by using the following eqn.9.

$$f_2 = 50\log\{[1+\frac{1}{n}\sum(A-A')^2]^{-0.5}.100\} \text{.....(9)}$$

where f_2 is the similarity factor. A is the calculated absorbance, and A´ is the experimental absorbance at any given pH value. It was noticed that the higher the f_2 value, the more similarity becomes.

1.2 AZT partition coefficient (K_{app}) determination

The partition coefficient (K_{app}) value of AZT is crucial factor for designing suitable co-solvent system for AZT to permeate through the skin. Therefore, the partition of AZT between different organic solvents as an organic phase and phosphate buffer pH 7.4 as an aqueous phase was determined at 30 ± 1^0C in water bath. Nine organic solvents with different in polarities values were employed, they were n-hexanol, n-heptanol, n-octanol, n-nonanol, n-decanol, dichloromethane, chloroform, carbon tetrachloride, and hexane. The phosphate buffer pH 7.4 was saturated by organic solvent and organic solvents were also saturated by 0.01 M

phosphate buffer pH 7.4 overnight before using. An equal volume of AZT at 20 mcg/ml in 0.01M phosphate buffer pH 7.4 was added into a glass stoppered flask. The flask was then shaken at 50 rpm for 24 hours in a shaking water bath with temperature controlled at 30±1°C. At the due time, the separated aqueous phase was spectrophotometrically analyzed for AZT. The concentration of AZT in the organic phase can be calculated from the difference of AZT between the initial time and at the equilibrium. Therefore, the K_{app}, and log K_{app} was then calculated from eqn.10 (James, 1995)

$$\log K_{app} = \log \frac{C_o}{C_w} \quad \text{.....(10)}$$

where C_0 is the concentration of AZT in the organic phase; C_w is the concentration of AZT in the aqueous phase (James, 1995).

2. Preformulation study of AZT

In order to design an AZT transdermal delivery system various preformulation techniques were conducted. The effect of various the vehicles on solubility of AZT was determined. Then the of appropriate solvents as the vehicle for AZT were selected and permeation characteristics of the drug through the skin were elucidated. In addition, various types of enhancers were employed into the formulations and the *in vitro* permeation studies were therefore investigated.

2.1 Solubility characteristic of AZT in variety of solvent systems

The purpose of this experiment was to study the solubility character of AZT in various solvents with a wide range of dielectric constants as tabulated in Table 6.

The non-equilibrium method (synthetic method) was utilized for approximating solubility value of AZT. This method is simple and less time consuming (James, 1986). Ten milligrams of AZT was accurately weighed into a 10-ml test tube. The graduated volume of solvent was gradually added by using a micropipette, and then vortex mixed until AZT was completely dissolved. The experiments were performed under a controlled temperature of 30±1° C using a water bath. The solubilities of AZT in various solvents were triplicately determined. The approximate solubility of AZT was calculated according to eqn.11

$$S = Q/V \qquad \text{.....(11)}$$

where S is the solubility of AZT (mg/ml), Q is the amount of AZT added (mg), V is the total volume of solvent used (ml).

Table 6 Solubility study of AZT in various solvent systems

Solvents	Dielectric constant (ε)	Polarity classification according to ε
Mineral oil	2.1[a]	Non - polar
Silicone	2.2[b]	
Isopropyl mysirtate (IPM)	3.3[c]	
PEG 400	12.4[d]	Semi - polar
Isopropyl alcohol (IPA)	18.3[e]	
Ethanol	24.3[a]	
Propylene glycol	32.0[a]	
n,n-dimethylacetamide	37.8[d]	
DMSO	46.68[b]	
Water	80.4[a]	Polar

[a] From the pharmaceutical codex principles and practice of pharmaceutics 12[ed] 1994

[b] From ASI instrument inc., [c] From Harada,2000

[d] From the U.S. Nation Bureau of Standard Circular 514, [e] From Mathin,1993 p.87

2.2 Determination of appropriate combination of vehicles for AZT

From Section 1.2, the log partition coefficient (log K) value of AZT between octanol and phosphate buffer pH 7.4 was determined be 0.02 that implied the difficulty in partitioning or diffusing of AZT through stratum corneum. From Section 2.1 AZT could be more solubilized in many semi-polar solvents. An attempt to increase the partition of AZT into stratum corneum and also to enhance diffusion through the skin in order to increase the maximum flux was then conducted by determining the potential binary solvent systems.

IPM, PEG 400, IPA, ethanol and water was chosen for combination study. Two of these solvents were combined in the volume ratio 50/50 and the dielectric constant of all these binary vehicles were calculated according to eqn.12

$$\varepsilon_{CAL} = (\%A \times \varepsilon_A) + (\%B \times \varepsilon_B) \qquad \text{.....(12)}$$

The ε_{cal} is the calculated dielectric constant, %A is the volume of solvent A; ε_A is the dielectric constant of solvent A; %B is the volume of solvent B, ε_B is the dielectric constant of solvent B.

AZT was formulated in these binary vehicles at the concentration 70% of saturated AZT in the volume ratio of 50:50 as shown in Table 7.

Table 7 Preformulation of AZT in various binary vehicles

Composition	Formulation (binary vehicle, V/V)			
	F1	F2	F3	F4
Ethanol	50			50
Water	50	50	50	
IPA		50		
PEG 400			50	
IPM				50
AZT	√	√	√	√

√ = 70% of saturated AZT in each binary vehicle

The solubility of AZT in these binary vehicles were determined using the equilibrium method (analytical method) (James, 1986). An excess amount of AZT was added into a screw-capped test tube containing 5 ml of a solvent or a binary vehicle. The test tube was continuously rotated using the top to bottom rotator (Higuchi and Connors, 1965) for 24 h at 33±1°C that was the ambient temperature for *in vitro* permeation studies at the donor compartment of diffusion cell. The saturated AZT solution was then centrifuged at 5000 rpm (relative centrifugal force =

5245 g) for 10 min. A 500 mcl of supernatant was pipetted to a 10-ml volumetric flask and adjusted to volume with absolute ethanol. This solution was diluted with HPLC mobile phase (methanol: water; 60: 40) containing internal standard (methyl paraben) to obtain the final AZT concentration of 2-10 mcg/ml. The solution was then injected into HPLC for AZT determination in triplicate.

High-performance liquid chromatographic technique for AZT analysis

The HPLC system for AZT analysis was processed through an Spherisorb® ODS column (5 mcm, 250 x 4.6 mm i.d, Water) with methanol:water (60 : 40) as the mobile phase at the flow rate of 1.0 ml/min and detected at 267 nm .

Analytical method validation for AZT

The analytical method used for AZT determination was validated under USP criteria. The analytical parameters were system suitability, specificity, accuracy, precision, linearity, and limit of quatitation. The standard solutions of AZT in the concentration range of 2-10 mcg/ml were used for this validation. The validation results are given in APPENDIX A.

***In vitro* Permeation study**

The *in vitro* permeation study was a tool for determining the most suitable combination of vehicles for AZT, which could provide the highest flux and permeability coefficient of AZT.

Preparation of skin

The newborn pigs (local pig, 1.1-1.4 kg.) were obtained from the province of Nakornpathom, Thailand. Abdominal skin of a newborn pig was carefully excised and inspected for any defect; e.g. scratches, bruises, or bites. Subcutaneous fat and other extraneous tissues adhering to the dermis were completely removed and trimmed, if necessary, using forceps and scissors. The skin was cleaned with the phosphate buffer saline pH 7.4 (PBS). The skin specimen was cut to the size of 4x4 cm^2 wrapped in aluminium foil and stored at -20^{0}C. The frozen skin was thawed to room temperature prior to use.

Procedure of the *in vitro* permeation study

The i*n vitro* permeation of AZT across newborn pig skin was conducted using modified diffusion cells. The excised skin was mounted between the donor and the receptor compartments. The diffusion cells possess an available diffusion area of 1.81±0.01 cm^2 that can carry the receptor volume approximately varied between 11.5-12.1 ml. The donor compartment that faced the stratum corneum surface contained 1.5-ml of AZT saturated solution in binary vehicle. The receptor compartment, which faced the dermis side, was filled with 12 ml of PBS pH 7.4 with the temperature controlled at 37 ± 1°C.

The donor compartment was covered with polyester film laminate occlusive (Scotchpak ^{TM}, 3M, St. Paul, MN, USA.) and aluminium foil to prevent the evaporation of donor solution. The temperature of donor solutions was 33 ± 1°C. A 2-ml sample solution was withdrawn from the receptor at appropriate time intervals

up to 12h period. The sink condition of the receptor was maintained by adding PBS. The internal standard was added into the withrawal solution from the receptor compartment and analyzed for AZT using HPLC.

Calculation of permeation parameter

The amount of permeated AZT was calculated by multiplying AZT concentration with the receptor volume. For each skin specimen, drug permeated per unit area was plotted against time. The steady-state flux (J_{ss}) and lag time (L) were calculated from the slope and x-intercept of the linear portion of the plot that was fitted through the regression analysis.

The permeability coefficient (K_p, cm/h) of AZT was calculated from eqn.13

$$K_p = \frac{J_{ss}}{C_d} \quad \text{.....(13)}$$

where C_d is the saturated solubility of AZT in the binary vehicle (mg/ml).

For prediction of AZT permeation through human skin, the steady state flux (J_{ss}) can be related to steady state AZT plasma concentration as shown below:

$$J_{ss} = \frac{C_{ss} Cl_t BW}{A} \quad \text{.....(14)}$$

If the maximum surface of transdermal patch are supposed to be 100 cm^2 (A), the standard human body weight (BW) is 60 Kg, the therapeutic AZT level (C_{ss}) equals to 0.2672 mcg/ml, and AZT total clearance in human (Cl_t) is 1.30 L/Kg/h.

Then flux (J_{ss}) can be calculated to be 208 mcg/cm^2/h. This is the expected flux that being targeted for the proposed AZT transdermal delivery system in this study.

2.3 Preformulation of AZT in suitable binary vehicles

To preformulate AZT with suitable binary vehicles, two steps were preformed. The first step was to select the appropriate volume ratio of combination vehicles from 2.2. The second step was to use the selected volume ratio from the first step for determining the most suitable one on AZT permeation.

Saturated solutions of AZT were used in all formulations in order to obtain maximum driving force (Theeuwes, 1976). The prepared mixtures were visualized to ensure that no crystallization of AZT occurred. The preformulations for the first step are shown in Table 8. In the second step, ethanol in several hydrophobic vehicles at 20/80-volume ratio were used since the low ethanol concentration could induce physical perturbation of lipid bilayer of stratum corneum.

From the results obtained from Section 2.2 indicated that hydrophilic and hydrophobic cosolvents could synergistically enhance AZT penetration. The importance of the vehicle for enhancing hydrophilic drug permeation had been documented, and most researchers had paid attention to hydrophilic vehicles whereas the uses of hydrophobic vehicles are limited. The hydrophobic solvents that have ever been studied were tricaprylin, triacetin, squalene, liquid paraffin, isopropyl myristate, silicone fluid etc. (Goto *et al.,* 1993, Lee *et al.*, 1993, Jin *et al.*, 1996; Kim and Chien, 1996). In this study, four hydrophobic vehicles, with polarity indexes are close to IPM (24.3 mN/m), were chosen in order to mix with ethanol at 20/80, and

the permeation parameter will be compared with IPM. They were medium – polar and non-oleaginous emollients usually in various topical pharmaceutical formulations and cosmetics with difference in polarity index. The C_{12-15} alkyl benzoate (FIN), medium chained triglycerides (MCT), isopropyl palmitate (IPP), and dioctyl adipate (ADI), the sequence of polarity index of these vehicles are 18.8, 20.2, 25.2 and 27.0 mN/m, respectively. The preformulations for this study are shown in Table 9.

The solubility of AZT at saturated concentration in each binary vehicle was determined as stated in Section 2.2. The preformulations were freshly prepared by accurately weighed AZT, being calculated from AZT solubility, into a 10-ml volumetric flask. The binary vehicle was added and sonicated until AZT completely dissolved and the volume was adjusted with the binary vehicle.

In vitro permeation of AZT was determined according to the method mentioned in Section 2.2. In addition, the appropriate time intervals for sampling AZT from receiving solution were adjusted to achieve the steady-state flux.

Table 8 Preformulated AZT for volume ratio selection ethanol and IPM

Composition	Formulation (binary vehicle, V/V)		
	F5	F6	F7
Ethanol	20	30	40
IPM	80	70	60
AZT	√	√	√

√ = saturated AZT in each binary vehicle

Table 9 Preformulated AZT with ethanol and other hydrophobic vehicles

Composition	Formulation (binary vehicle, v/v)			
	F8	F9	F10	F11
Ethanol	20	20	20	20
FIN	80			
MCT		80		
IPP			80	
ADI				80
AZT	√	√	√	√

√ = saturated AZT in each binary vehicle, FIN = C_{12-15} alkyl benzoate; MCT = medium chained triglycerides; IPP = isopropyl palmitate; and ADI = dioctyl adipate

Statistical analysis

In order to select the suitable binary vehicles, the statistical analysis was performed. The one way analysis of variance was operated under SPSS version 9.0.

2.4 Study the addition of enhancers into preformulated AZT

The preformulated AZT with selected binary vehicles at fixed volume ratio from Section 2.3 were used. Three compounds with different in polarities, they are pyrrolidone (*N*-methyl-2-pyrrolidone), oleic acid, and lauric acid were chosen to represent a hydrophilic enhancer, lipophilic enhancers with unsaturated fatty acid and saturated fatty acid character, respectively. These three enhancers with three different concentrations were individually added into preformulated AZT to obtain the different AZT formulations as displayed in Table 10. The permeation of AZT in these formulations were then determined across newborn pig skin *in vitro*. Any enhancer solution that can give the highest permeation rate of AZT through pig skin would be included in preparing AZT transdermal delivery system.

Table 10 Preformulated AZT with three different enhancers

Vehicle	Preformulations											
	F12	F13	F14	F15	F16	F17	F18	F19	F20	F21	F22	F23
ETHANOL	20	20	20	20	20	20	20	20	20	30	30	30
IPM	80	80	80	80	80	80	80	80	80	70	70	70
NMP (%V/V)												
1	*									*		
5		*									*	
10			*									*
OLEIC ACID (%V/V)												
1				*								
5					*							
10						*						
LAURIC ACID(%V/V)												
1							*					
5								*				
10									*			
AZT	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔

✔ = Saturated AZT in each binary vehicle

3. Evaluation of AZT transdermal delivery system

The effect of controlling synthetic membrane on the release and permeation of AZT from the prepared transdermal delivery system was investigated. The candidate formulations, which had provided the highest flux of AZT formulations, were selected from section 2.4. Two types of controlling synthetic membranes were used in the study. They were microporous membranes (polyethylene (PE) membrane, 3M, average pore size of 0.2 mcm) and non-porous membrane (9% ethyl vinyl acetate (EVA) nonporous membrane, 3M).

3.1 Effect of controlling membrane on the release of AZT

Both PE microporous membrane and 9% EVA nonporous membrane were utilized instead of the former pig skin in the modified diffusion cell. Either membrane was clamped between the donor and the receptor compartment of diffusion cell. The receptor compartment contained PBS pH 7.4 which was maintained at 37±1°C by a circulating water bath. The donor compartment was exposed to ambient temperature. A 2.0-ml from the receptor compartment was withdrawn at different time intervals for further AZT analysis via HPLC. The sink condition of receptor compartment was maintained using freshly prepared PBS. The concentration of AZT was determined from the receptor and was used for calculation of permeation parameter as in Section 2.2.

3.2 Effect of controlling membrane on the permeation of AZT in *in vitro*

In vitro permeation study of AZT was performed using a modified diffusion cell (as already described in Section 2.2). The controlling synthetic membranes were equilibrated over night before use by soaking in the formulation without AZT. They were then adhered on the newborn pig skin and clamped between the donor and the receptor compartments section of diffusion cell. The procedure for the in *vitro* permeation was then followed as already described in 3.1. The previous investigation it was found that polyethylene microporous membrane retarded AZT flux so polycacbonate microporous membrane (S&S average pore size of 0.4 mcm) was used to observed the effect of microporous membrane on AZT permeation together in order to confirm effect of microporous membrane on AZT permeation.

Statistically analysis was performed on SPSS 9.0. The parametric tests, independent T-test was used.