CHAPTER III

MATERIALS AND METHODS

Equipment

1. Analytical balance (Satorius basic BA 210S and Satorius MC 1)
2. Elemental analyser (Perkin-Elmer 2400 Series II CHNS/O Analyzer)
3. Fluorescent microscope (Olympus IX 51)
4. Heating bath (Buchi B-490)
5. Heating magnetic stirrer (VELP Scientifica, model ARE)
6. Infrared spectrometer (IR) (Perkin Elmer FT-IR Spectrometer Spectrum 2000)
7. Microplate reader (Anthos htl, model A-5022)
8. Nuclear magnetic resonance spectrometer (Avance DPX-400, 400 MHz)
9. NMR tubes 5 mm 7 inches in length (Wilmard 507-PP, Sigma)
10. pH meter (Consort, model C832T)
11. Reversed-phase high performance liquid chromatography, RP-HPLC (Shimadzu)
12. Rotary evaporator (Buchi R-200)
13. Silica gel 60 F_{254} thin layer chromatography (TLC) aluminium sheets (precoated, Merck)
14. Ultrasonic cleaner (Mettler Electronics, model ME 4.6)
15. UV-VIS light detector, 254 and 366 nm (Spectroline, model CM-10)
16. Vacuum desiccator 220 (Heraeus, Berli Jucker)

Chemicals

1. Acetanilide, Reference material grade (Perkin Elmer)
2. Acetonitrile (HPLC grade) (Fisher Scientific UK and Lab-Scan)
3. Benzoic acid, Reference material grade (Perkin Elmer)
4. Chloroform, AR Grade (Lab-Scan)
5. Chloroform-D (Sigma)
6. Citric acid (Ajax Finechem)
7. Dextrin (Sigma Chemical), the weight-average molecular weight of 6,600 (Mw/Mn = 1.8), determined by gel permeation chromatography
8. N, N′-dicyclohexylcarbodiimide (DCC) (Sigma Chemical)
9. 4-dimethylaminopyridine (DMAP) (Sigma Chemical)
10. Dulbecco's modified Eagle's medium (DMEM) (Invitrogen)
11. Di-sodium hydrogen orthophosphate (Na_2HPO_4) (Ajax Finechem)
12. N, N′-dimethylformamide (DMF), Dry (Sigma-Aldrich Chemical)
13. Dimethylsulfoxide (DMSO), Dry (Sigma-Aldrich Chemical)
14. Fetal bovine serum (Invitrogen)
15. Hoechst 33342 (bisBenzimide H 33342 trihydrochloride) (Sigma)
16. Hydrochloric acid (Lab-Scan)
17. 1-hydroxybenzotriazole (HOBt) (Epochem)
18. L-glutamine (200 mM, 100x, Invitrogen)
19. Methanol (HPLC grade) (Fisher Scientific UK and Lab-Scan)
20. Methyl sulfoxide-D (Aldrich Chemical)
21. Penicillin-Streptomycin (Invitrogen)
22. Phosphate buffered saline (Sigma Chemical)
23. Phosphoric acid (*ortho*-phosphoric acid, Merck)
24. Sephadex LH-20 (Amersham Biosciences)
25. Sodium sulfate, anhydrous (Ajax Finechem)
26. SpectraPor membrane (MWCO: 3,500) (Spectrum Laboratories)
27. Succinic anhydride (Aldrich Chemical)
28. Tetrahydrofuran (THF), Dry (Sigma Chemical)
29. Triethylamine (Carlo Erba Reagent)

30. Trysin (0.25 %, QB perbio)
31. Zidovudine (Samchully Pharmaceuticals, Seoul, South Korea, obtained as a gift from The Government Pharmaceutical Organization, Bangkok, Thailand)

Cells

Immortalized BEAS-2B lung epithelial cells were from ACTT (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C in humidified atmosphere containing 5 % CO_2.

Animals

Male Sprague Dawley rats weighing 250-300 g were obtained from National Laboratory Animal Centre (Nakhon Pathom, Thailand). The rats did not show any signs of abnormality.

Methods

1. Synthesis and characterization of succinylated zidovudine

1.1 Synthesis of succinylated zidovudine

Zidovudine was characterized by FT-IR and ^{1}H-NMR before synthesis. Succinylated zidovudine was synthesized by modified Giammona's process (Giammona et al., 1998). The synthetic scheme was shown in figure 1.

Zidovudine (1.98 mmol) was dissolved in 25 ml of dry THF, in a 50 mL round bottomed flask. Succinic anhydride (3.16 mmol) and triethylamine (2.57 mmol) were added into a solution of zidovudine. The reaction mixture was heated at 69 °C. The progress of reaction was followed by TLC with the TLC condition described below. The reaction mixture was stirred for 6 h. After 6 h, succinic anhydride (0.99 mmol) and triethylamine (1.38 mmol) were added again and the reaction was left at 69 °C for 6 h. After reaction, the solvent was evaporated and the residual was dissolved in distilled water (50 mL) and then acidified to pH 2 by 1M HCl. The solution was transferred to a separatory funnel and extracted with ethyl acetate several times. The ethyl acetate phase were collected and dried over anhydrous sodium sulfate overnight. The organic phase was filtered and evaporated. The residual was purified by flash column packed with sephadex LH-20 and eluted with mixed solvent (chloroform:methanol, 9:1, v/v). The fraction containing succinylated zidovudine were evaporated and dried under vacuum. The product was characterized by FT-IR, ^{1}H-NMR and elemental analysis.

Thin layer chromatography (TLC) for succinylated zidovudine

Technique:	one way, ascending
Stationary phase:	Silica gel 60 F_{254} on aluminium plate
Plate size:	6.5 cm
Developing solvent system:	chloroform: methanol = 9:1
Solvent front:	5 cm
Detection:	UV (254 nm)

1.2 Characterization of succinylated zidovudine

1.2.1 Infrared spectroscopy (IR)

Two mg of succinylated zidovudine was blended with dry potassium bromide and pressed to form a thin transparent pellet under vacuum and high pressure. The percentage of IR transmittance was measured by infrared spectrometer.

1.2.2 Nuclear magnetic resonance spectroscopy (NMR)

Five mg of succinylated zidovudine was dissolved in $CDCl_3$. The ^{1}H-NMR of succinylated zidovudine was recorded with NMR spectrometer.

1.2.3 Elemental analysis

One - three mg of succinylated zidovudine was weighted accurately within $\pm$ 0.02 mg. The reference materials, acetanilide and benzoic acid were used to calibrate the instrument. Benzoic acid was used for nitrogen zero matrix blank. The sample was analyzed by elemental analyzer.

2. Synthesis and characterization of dextrin-zidovudine conjugate

2.1 Synthesis of dextrin-zidovudine conjugate

Dextrin was characterized by FT-IR and ^{1}H-NMR before synthesis. The synthetic scheme for dextrin-zidovudine conjugate was shown in figure 2.

Dextrin (0.98 mmol) was dissolved in 17 mL of dry DMF and 3 mL of dry DMSO at 50 °C with agitation overnight. To start reaction, the solution of succinylated zidovudine (0.39 mmol) and DCC (1.56 mmol) in 10 mL of dry DMF was stirred for 15 min under nitrogen. The solution of HOBt (1.17 mmol) in 4 ml of dry DMF was added and the reaction was left for 30 min. The overnight-prepared dextrin solution and DMAP (0.98 mmol) were added into the reaction mixture. The reaction was left at room temperature with stirring under nitrogen for 48 h. Then, the precipitate of dicyclohexylurea was filtered out and filtrate was collected. The solvent was removed under vacuum and the residual was precipitated in vigorously stirring diethyl ether. The precipitate was collected and dissolved in distilled water (50 mL). The impurity was successively extracted out with ethyl acetate 4 times (40 mL) and chloroform 4 times (40 mL). The aqueous phase was then dialyzed against distilled water. The dextrin-zidovudine conjugate solution was dried by using freeze-dry technique. The white residual was characterized by FT-IR and ^{1}H-NMR. The mole % of linked zidovudine was estimated by UV and ^{1}H-NMR.

Thin layer chromatography (TLC) for the dextrin-zidovudine conjugate

Technique, stationary phase, plate size, developing solvent system, solvent front and UV detection used were the same as for succinylated zidovudine.

2.2 Characterization of dextrin-zidovudine conjugate

2.2.1 Infrared spectroscopy (IR)

Two mg of the dextrin-zidovudine conjugate was blended with dry potassium bromide and pressed to form a thin transparent pellet under vacuum and high pressure. The percentage of IR transmittance was measured by infrared spectrometer.

2.2.2 Nuclear magnetic resonance spectroscopy (NMR)

Approximately fifteen mg of the dextrin-zidovudine conjugate was dissolved in D_2O. The ^{1}H-NMR of the dextrin-zidovudine conjugate was recorded with NMR spectrometer.

3. HPLC analysis

3.1 Analytical method development

The reversed-phase high performance liquid chromatography (RP-HPLC) was used to determine the amount of zidovudine, succinylated zidovudine. The HPLC condition was developed in this study and is shown as following:

Liquid Chromatography :	LC-10AD VP (Shimadzu)
System Controller:	SCL-10Avp (Shimadzu)
UV-VIS Detector:	SPD-10A VP (Shimadzu)
Auto Injector:	Sil-10Advp (Shimadzu)
Degasser:	DGU-14A (Shimadzu)
HPLC column:	C18 column, ODS-3 (250 mm x 4.6 mm i.d., particle size 5 μm, from Inersil) connected to C-18 guard column.
Injection volume:	10 μl
Detector wavelength:	266 nm
Temperature:	ambient temperature

The mobile phase system for quantification of zidovudine and succinylated zidovudine consisted of 2 components: A and B. Mobile phase A was 0.01 % (v/v) of *ortho*-phosphoric acid in water and mobile phase B was the mixture of acetonitrile: methanol (1:1, v/v).

The gradient elution program was followed, at 0 to 14 min, mobile phase A was fixed at 93 % with linearly increase in flow rate from 1.5 to 2 mL/min. At 14 min, mobile phase B was increased immediately from 7 % to 22 % and linearly increase to 40 % at 28 min. After 28 min, mobile phase A returned to 93 % with a flow rate of 1.5 mL/min for 7 min to re-equilibrate column. The gradient elution program is shown in table 5.

Table 5 The gradient elution program of HPLC.

Minute	Mobile phase A (%)	Mobile phase B (%)	Flow (mL/min)
0 – 13.99	93	7	1.5
14	78	22	2
28	60	40	2
28.01 - 38	93	7	1.5

Stavudine (165.3 μg/mL) was used as an internal standard. The calibration curves of peak area ratio of drug/stavudine against drug concentrations were constructed and used to quantify the amount of unknown samples.

3.2 Preparation of standard curve

3.2.1 Preparation of internal standard solution (Stock I)

Stavudine internal standard of 49.6 mg was dissolved in water. The volume was adjusted to 10 mL (conc = 4.96 mg/mL).

3.2.2 Preparation of internal standard solution (Stock II)

Stavudine internal standard stock I of 100 μl was adjusted to 3 mL by water (conc = 165.3 μg/mL).

3.2.3 Preparation of zidovudine working standard stock solution

Zidovudine working standard of 39.39 mg was dissolved in water. The volume was adjusted to 10 mL (conc = 3.94 mg/mL).

3.2.4 Preparation of succinylated zidovudine working standard stock solution

Succinylated zidovudine working standard of 10.05 mg was dissolved in water. The volume was adjusted to 5 mL (conc = 2.01 mg/mL).

3.2.5 Preparation of trichloroacetic acid

Trichloroacetic acid of 5 g was dissolved in water. The volume was adjusted to 25 mL (conc = 20 % w/v).

3.2.6 Preparation of calibration curve of zidovudine

Zidovudine standard stock solutions (3.94 mg/mL) were diluted to various concentrations. Standard working solutions (720 µl) were mixed with internal standard solution stock II (60 µl) to obtain final zidovudine concentrations of 0.12-37.17 µg/mL. The solution mixture (10 µl) was injected into HPLC. Peak area ratio of zidovudine/internal standard and concentrations of zidovudine were used for construction of the calibration curve.

3.2.7 Preparation of calibration curve of succinylated zidovudine

Succinylated zidovudine standard stock solutions (2.01 mg/mL) were diluted to various concentrations. Standard working solutions (720 µl) were mixed with internal standard solution stock II (60 µl) to obtain final succinylated zidovudine concentrations of 0.11-549.23 µg/ml. The solution mixture (10 µl) was injected into HPLC. Peak area ratio of succinylated zidovudine/internal standard and concentrations of succinylated zidovudine were used for construction of the calibration curve.

3.3 Analytical method validation of zidovudine and succinylated zidovudine

3.3.1 Accuracy

Accuracy in term of recovery was determined by calculating the ratio of observed concentration and actual concentration. Observed concentration was obtained from linear regression equation of a calibration curve.

The preparations were processed in triplicate at three different concentrations. The actual concentrations, the observed concentrations, and % recovery of zidovudine was determined.

$$\% \text{ Recovery} = \frac{\text{Observed concentration}}{\text{Actual concentration}} \times 100$$

3.3.2 Precision

3.3.2.1 Intra-day precision

Six replicates of three different concentrations of aqueous solutions of zidovudine and succinylated zidovudine were prepared and analysed by HPLC. The percentage of coefficient of variation (% CV) was calculated.

$$\% \text{ CV} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

3.3.2.2 Inter-day precision

The same range of concentrations of zidovudine and succinylated zidovudine in the intra-day precision was used and analysed in other

three different days. The percentage of coefficient of variation (% CV) was calculated.

3.3.3 Linearity

The linearity graphs of zidovudine and succinylated zidovudine were constructed from the actual concentrations and the observed concentrations of zidovudine and succinylated zidovudine. The graph was fitted with a straight line using the least square linear regression analysis.

3.3.4 Limit of quantitation (LOQ)

The minimum level that zidovudine and succinylated zidovudine can be determined with % CV of precision of less than 20 %.

3.3.5 Limit of detection (LOD)

The concentration of zidovudine and succinylated zidovudine was reduced until the signal-to-noise ratios from HPLC chromatogram was 3:1.

3.3.6 Specificity

The buffer pH 5.5 and pH 7.4 with and without zidovudine and succinylated zidovudine were analyzed by HPLC to ensure that the peaks of buffers are not overlapped with peaks of analytes.

4. HPLC analysis in plasma

For analysis in plasma samples, the calibration curves of zidovudine and succinylated zidovudine were prepared in plasma matrices. Six hundred and fifty µl of human plasma in a microcentrifuge tube was spiked with 100 µl of various zidovudine standard solutions and 75 µl of stavudine internal standard solution. The mixture was mixed and 150 µl of 20% (w/v) of trichloroacetic acid was then added. The tubes were vertexed for 30 s and then centrifuged at 12000 rpm for 7 min to obtain clear supernatant. The final concentrations of zidovudine were in the range of 0.02-40.40 µg/mL. Ten µl of supernatants were injected into HPLC.

For succinylated zidovudine, the calibration curve was prepared in the concentration range of 0.03-103.08 µg/mL.

4.1 Accuracy

The preparations of zidovudine and succinylated zidovudine in plasma were processed in triplicate at three different concentrations. The actual concentrations, the observed concentrations, and % recovery of zidovudine were determined.

4.2 Precision

4.2.1 Intra-day precision

Six replicates of three different concentrations of zidovudine and succinylated zidovudine in plasma were prepared and analysed by HPLC. The percentage of coefficient of variation (% CV) was calculated.

4.2.2 Inter-day precision

The same range of concentrations of zidovudine and succinylated zidovudine in plasma in the intra-day precision was used and analysed in other three different days. The percentage of coefficient of variation (% CV) was calculated.

4.3 Linearity

The linearity graphs of zidovudine and succinylated zidovudine in plasma were constructed from the actual concentrations and the observed concentrations of zidovudine and succinylated zidovudine. The graph was fitted with a straight line using the least square linear regression analysis.

4.4 Limit of quantitation (LOQ)

The minimum level that zidovudine and succinylated zidovudine can be determined with % CV of precision of less than 20 %.

4.5 Limit of detection (LOD)

The concentration of zidovudine and succinylated zidovudine was reduced until the signal-to-noise ratios from HPLC chromatogram was 3:1.

4.6 Specificity

Specificity of analytical method was determined by analyzing plasma, plasma spiked with zidovudine and plasma spiked with succinylated zidovudine to ensure that the peak of plasma is not overlapped with peaks of analytes.

5. *In vitro* drug release

5.1 Drug release study in buffer solution at pH 5.5

The releases of zidovudine and succinylated zidovudine from the dextrin-zidovudine conjugate were investigated in citrate-phosphate buffer solution at pH 5.5. One mL of aqueous solution of the dextrin-zidovudine conjugate (7.23 μmol) was added into 5 mL of preheated buffer solutions and were maintained at 37 ± 0.1 °C with continuously stirring at 420 rpm. Samples (300 μl) were taken at various time intervals (0, 0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24 and 48 h) and 25 μl of stavudine internal standard (165.3 μg/mL) was added. The amount of zidovudine and succinylated zidovudine released were determined by HPLC analysis.

5.2 Drug release study in buffer solution at pH 7.4

The releases of zidovudine and succinylated zidovudine from the dextrin-zidovudine conjugate were investigated in phosphate buffered saline at pH 7.4. One mL of aqueous solution of the dextrin-zidovudine conjugate (7.23 μmol) was added into 5 mL of preheated buffer solutions and were maintained at 37 ± 0.1 °C with continuously stirring at 420 rpm. Samples (300 μl) were taken at various time intervals (0, 0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24 and 48 h) and 25 μl of stavudine internal standard was added. The amount of zidovudine and succinylated zidovudine released were determined by HPLC analysis.

5.3 Drug release study in human plasma

One ml of aqueous solution of the dextrin-zidovudine conjugate (7.23 μmol) was added into 5 mL of preheated plasma and were maintained at 37 ± 0.1 °C with continuously stirring at 420 rpm. Samples (300 μl) were taken at different time intervals (0, 0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24 and 48 h). Thirty μl of stavudine internal standard was added and mixed into the collected sample. Then, 60 μl of 20%

(w/v) of trichloroacetic acid was added to precipitate plasma proteins. The mixture was vertexed for 30 s and centrifuged at 12,000 rpm for 7 min. The supernatant was analyzed by HPLC to determine the amounts of zidovudine and succinylated zidovudine released from the dextrin-zidovudine conjugate.

6. Hemolysis study

Human red blood cells (RBC) were separated and washed three times with cold PBS. The red blood cells were diluted in cold PBS to obtain 2 % (w/v) of RBC suspension.

Various concentrations of polymers (1, 2, 3, 4, 5 mg/mL), zidovudine (concentrations equivalent to zidovudine loading in the dextrin-zidovudine conjugate; 0.19, 0.38, 0.57, 0.76 and 0.95 mg/mL) and the mixture of dextrin and zidovudine (dextrin concentrations of 0.81, 1.62, 2.43, 3.24, 4.05 mg/mL containing zidovudine concentrations of 0.19, 0.38, 0.57, 0.76 and 0.95 mg/mL, respectively) were prepared in PBS. The solutions of samples (100 µl) were incubated with RBC suspension (100 µl) at 37 °C for 4 h. The incubated samples were then centrifuged at 1,000 g for 10 min to remove the unlysed RBC. The supernatant (100 µl) was transferred to 96-well plate. The absorbance was measured at 550 nm using microplate reader. The percentage of hemolysis was calculated relative to 100 % hemolysis obtained from incubation with 1 % of Triton X-100.

7. Cytotoxicity study

Immortalized lung epithelial BEAS-2B cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in humidified atmosphere of 5 % CO_2.

Various concentrations of polymers (1, 50, 100, 500 μg/mL), zidovudine (concentrations equivalent to zidovudine loading in the dextrin-zidovudine conjugate; 0.19, 9.46, 18.92 and 94.60 μg/mL) and the mixture of dextrin and zidovudine (dextrin concentrations of 0.81, 40.54, 81.08, 405.40 μg/mL containing zidovudine concentrations of 0.19, 9.46, 18.92 and 94.60 μg/mL, respectively) were prepared in serum free culture medium.

The cells were seeded in 96-well plates at a density of 1 x 10^4 cells/well. After 24 h, the culture medium was removed. The cells were washed with 150 μl of PBS. The solutions (100 μl) of various concentrations of samples in serum free culture medium were added. The cells were incubated for 48 h at 37 °C. After incubation, the Hoechst 33342 staining assay was performed with fluorescent microscope.

8. *In vivo* study

In vivo study of zidovudine released from the dextrin-zidovudine conjugate was investigated in male Sprague Dawley rats weighing approximately 280 g. The clear freshly prepared solution of free zidovudine (8.46 mg/kg body weight) or the dextrin-zidovudine conjugate (containing zidovudine equivalent to dose of 8.46 mg/kg body weight) in sterile normal saline was administered as a single intravenous bolus via tail veins. Blood samples (150 μl) were taken from tail veins at different time intervals and collected in microcentrifuge tubes. The plasma was separated by centrifugation at 12,000 rpm for 10 min. The plasma samples were processed as described in section 4 and analyzed by HPLC. The pharmacokinetic parameters;

elimination rate constant (k_e), half-life ($t_{1/2}$), area under the plasma concentration-time curve ($AUC_{0->\infty}$), area under the first moment of the plasma concentration-time curve ($AUMC_{0->\infty}$), mean residence time (MRT), clearance (CL), apparent volume of distribution (Vd) were calculated individually according to following equations (Abu-Izza et al., 1997, Callender et al., 1999, Shargel et al., 2005);

for $t_{1/2}$;

$$t_{1/2} = \frac{0.693}{k_e} \quad \text{Eq. 1}$$

for $AUC_{0->\infty}$;

$$AUC_{0->\infty} = AUC_{0->last} + AUC_{last->\infty} \quad \text{Eq. 2}$$

$AUC_{0->last}$ was calculated using trapezoidal rule according to following equation;

$$AUC_{0->last} = \sum \left[\frac{(C_n + C_{n-1})}{2} \times (t_n - t_{n-1}) \right] \quad \text{Eq. 3}$$

$$AUC_{last->\infty} = \frac{C_{last}}{k_e} \quad \text{Eq. 4}$$

Where C_{last} is the plasma drug concentration at the last measured point.

for $AUMC_{0->\infty}$;

$$AUMC_{0->\infty} = AUMC_{0->last} + AUMC_{last->\infty} \quad \text{Eq. 5}$$

$AUMC_{0->last}$ was calculated using trapezoidal rule according to following equation;

$$AUMC_{0->last} = \sum \left[\frac{(C_n t_n + C_{n-1} t_{n-1})}{2} \times (t_n - t_{n-1}) \right] \quad \text{Eq. 6}$$

$$AUMC_{last->\infty} = \frac{C_{last} \times t_{last}}{k_e} + \frac{C_{last}}{k_e^2} \quad \text{Eq. 7}$$

for MRT;

$$MRT = \frac{AUMC_{0->\infty}}{AUC_{0->\infty}} \quad \text{Eq. 8}$$

for Vd;

$$Vd = \frac{\text{Dose x } AUMC_{0->\infty}}{(AUC_{0->\infty})^2} \quad \text{Eq. 9}$$

for CL;

$$CL = \frac{\text{Dose}}{AUC_{0->\infty}} \quad \text{Eq.10}$$

Statistical significances of differences in these studies were assessed using Student's *t* test and analysis of variance (ANOVA) with post-hoc Turkey's comparison. The *P*-value of < 0.05 was considered statistically significant.