CHAPTER II

EXPERIMENTAL

1. Materials

1.1 Model drug

- Stavudine (Lot No. R3-48/00136, Samchully Pharma Company, Korea)
- Stavudine GPO Working Standard ; 98.19% assay (Control No. WSS 09-33/47, Samchully Pharma Company, Korea)
- Zidovudine GPO Working Standard ; 99.3% assay (Lot No. R3-48/00793, Samchully Pharma Company, Korea)
- Zerit® 40 mg IR. (Lot No. 4L83387, Bristol Mayer Squibb, U.S.A.)

1.2 Additives

- Microcrystallinecellulose (Avicel® PH101, Lot No. R3-47-00193, FMC Ltd., USA)
- Lactose monohydrate (Lot No. R1-47-00487, The Lactose Company of New Zealand, New Zealand)
- Colloidal Ethylcellulose aqueous dispersion (Surelease®, Lot No. 1N 506231, Colorcon, UK)
- Hydroxypropylmethylcellulose (Methocel E15LV, Colorcon, UK)

1.3 Solvents

- Methanol HPLC Grade (Lot No. 0553512, Fisher, England)
- Acetronitrile HPLC Grade (Lot No. 06020080, Labscan, Ireland)

1.4 Dissolution medium

- Hydrochloric acid 37% AR grade (Lot No. A44033, J.T.Baker, USA)
- Sodium hydroxide AR grade (Lot No. B231098243, Merck, Germany)

- Potassium dihydrogen orthophosphate (Lot No. F2H145, Ajax Finechem, Australia)
- Tri-Sodium orthophosphate (Lot No. AF403101, Ajax Finechem, Australia)
- Ammonium Acetate (Lot No. 238 C220716, Merck, Germany)

2. Equipments

- Analytical balance (Model A 200 S, Sartorious, Germany)
- Analytical balance (Model AG204, Metler Toledo, Switzerland)
- Dissolution apparatus (Model VK7000, Vankel, USA)
- Extruder (Model EXKS-1, Fuji Paudal Co., Ltd., Japan)
- Fluidized bed air suspension coater (Model Strea 1, Aeromatic-fielder, UK)
- Friabilator (Model TAR 20, Erweka , Germany)
- Hot air oven (Mammertt, Germany)
- High Performance Liquid Chromatography (Model 10 Avp Shimadzu, Japan)
- Image software (Image Proplus version 4.5 for window)
- Peristatic pump (Model 1B.1003/R, Roto consultant, Germany)
- pH meter (Model 292 Pye Unicam, England)
- Planetary mixer (Model SK5SS, Kitchen Aid, USA)
- Scanning electron microscope (Model S2360N, Hitachi, Ltd., Japan)
- Sieve shaker (Model FT-150 M Filtra, Spain)
- Spheronizer (Model S320, Nitro Fielder, England)
- US standard sieve (Laboratory test sieve ASTM E11, Endecotts. Ltd. U.S.A.)

3. Methods

A. *In vitro* study

1. Formulation of Core Pellets

Amount of ingredients used in experimental formulations are presented in Table 5. The most suitable formulation of stavudine pellets would be chosen for coating.

Table 5 The amount of ingredients and water used in each formulation

Ingredient (%w/w)	P1	P2	P3	P4	P5
Stavudine	40	40	40	40	60
Avicel® PH101	40	30	20	60	40
Lactose	20	30	40	-	-
Optimum water (base on dry basis)*	62	58	55	65	60

*amount of water used in each formulation was optimized to provide the best characteristic pellets.

2. Stavudine pellets Preparation

Stavudine pellets were prepared by extrusion-spheronization method. The ingredients were mixed for 10 minutes in a planetary mixer. Purified water was slowly poured and mixed for 10 minutes until wet mass was obtained. The wet mass was transferred to an extruder and screened through sieve aperture 1.0 mm with speed of 26 rpm/min. Weighed three hundred grams of extrudates and transferred to a spheronizer.

Each formulation was prepared under the various spheronization speeds (400, 600, 860 rpm) and spheronization times (5, 10 and 15 min). The obtained pellets were dried in a hot air oven (Mammertt, Germany) at 55 ^{0}C for 4 hours.

3. Evaluation of Core Pellets

3.1 Appearance of Pellets

Photomicrographs of pellets were taken with a scanning electron microscope. All samples were coated with gold by using ion sputtering coater under vacuum. The shape and surface of pellets were examined in magnification of 25 times.

3.2 Sphericity of Pellets

Image analyzer was used to examine the sphericity of pellets. Three hundreds samples of each formulation were random taken and analyzed by software program; Image Proplus of Image analyzer. The results of aspect ratio of nearly 1.0 indicated the sphericity of pellets.

3.3 Particle Size Distribution

Particle Size Distribution of various formulations was determined by sieve analysis. Fifty grams of pellets were put on the top of the sieve series of opening ranging from 1.40, 1.18, 1.00, 0.84 to 0.71 mm (14, 16, 18, 20 and 25 mesh), respectively. Those sieves were shaken with a mechanical shaker for 10 minutes.

The results, which were averaged from three determinations, were reported as percentage of weight retained on each sieve size. The median pellet size was given corresponding to 50% size on the cumulative percentage undersize axis. Percent yield of desired pellets was calculated from the pellet weight on 16/25 mesh cut.

3.4 Bulk Density and Tapped Density

The bulk density and tapped density were determined from the weight of 40.0 g samples, carefully charged into a 100 ml graduated cylinder. The bulk volume was recorded and the bulk density was calculated. The pellets were tapped from the height of 5 cm until a constant volume was obtained and tapped volume was recorded. Then, tapped volume was divided by weight to attain tapped density. Both densities were averaged from three determinations.

The Carr's compressibility was calculated from the following equation.

$$\% \text{ Carr's compressibility} = \frac{Td - Bd}{Td} \times 100 \qquad (7)$$

Where *Td* and *Bd* are tapped and bulk density, respectively.

3.5 Flow Rate and Angle of Repose

The weight of 40.0 g pellets was filled in a glass funnel with a 6 mm internal stem diameter fixed on a clamp at 4.0 cm height. The time was recorded when the pellets started to flow until finished. Flow rate was calculated in g/min, and angle of repose was calculated from the following equation.

$$Alpha = \tan^{-1}\frac{H}{R} \qquad (8)$$

Where *Alpha* is the angle of repose : *H* and *R* are the height and radius of pellet pile, respectively. The results were average from three determinations.

I95218451

3.6 Percent Friability

Pellet friability determination method was modified from Kavee Chanpaparp, (1989). Ten grams of core pellets retained on 16/25 mesh cut and five spherical metallic balls with a diameter of 0.67 cm and 1.25 gm per ball were filled into a plastic cylindrical container which has diameter of 4.69 cm and height 5.95 cm. The container was firmly closed with the cap and rotated in Erweka TAR20 Friabilator for 5 min. Pellets which were finer than 25 mesh were sieved off. The percent friability, averaged from two determinations, was calculated as percentage of weight loss.

3.7 Content uniformity Determination of Core Pellets

Ten capsules containing core pellets which are equivalent to about 100 mg of stavudine, were weighed. Transferred to a 100 ml volumetric flask. Added approximately 40 ml of water and sonicated the solution for 15 minutes. Adjusted to volume with water and mixed. The stock solution was filtered through a filter paper Wattman No.1 and discarded 5 ml of the first filtrate. Pipetted 5 ml of filtrate into a 50 ml volumetric flask. Adjusted to volume with mobile phase and mixed.

The solutions were analysed by high performance liquid chromatography (HPLC) according to the following chromatographic condition.

Column	: Zobrax Eclipse x DB-C18, 5 micron, 4.6×150 mm
Mobile phase	: Methanol : H_2O (20 : 80)
Flow rate	: 1.0 ml/min
Detector wavelength	: UV 260 nm
Injection volume	: 20 µl
Retention time	: 3.8 minutes

The content of stavudine in pellets was calculated from a standard curve at concentration range 0.01, 0.03, 0.05, 0.1 and 0.12 mg/ml.

3.8 In-vitro Analytical Method Validation

The analytical method validation was performed according to the methods as specified in the United States Pharmacopeia (USP) 29th edition.

3.8.1 Linearity and Range

Fifty milligrams of standard stavudine was weighed accurately and transferred to 100 ml volumetric flask, then adjusted to volume with methanol. This solution was used as a stock solution. Pipetted 1, 3, 5, 5 and 6 ml of stock solution into 50, 50, 50, 25 and 25 ml volumetric flasks, respectively. Adjusted to volume with methanol and mixed. The final concentration of each solution was 0.01, 0.03, 0.05, 0.1 and 0.12 mg/ml, respectively. Each concentration was prepared for triplicate assay. All samples were assayed by HPLC as described previously.

The peak areas against the concentration of each solution were fitted to a straight line using linear regression analysis.

The coefficient of determination (R^2) was calculated and it should be greater than 0.999

3.8.2 Limit of detection (LOD) & Limit of quantification (LOQ)

Various concentrations of standard stavudine solution were prepared. All samples were assayed by HPLC as previous condition and the signal-to-noise ratio was calculated.

The limit of detection (LOD) is the minimum concentration at which the analyte can be detected. A signal-to-noise ratio between 3 or 2 : 1 is considered acceptable for estimating the detection limit.

The limit of quantification (LOQ) is the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio 10 : 1 is considered acceptable for estimating the detection limit.

3.8.3 Accuracy (Spiked placebo method)

Prepared five standard solutions of stavudine having final concentrations of 0.01, 0.03, 0.05, 0.1 and 0.12 mg/ml. The 250 mg of placebo pellets were added into each volumetric flask, then added methanol and sonicated for 30 minutes. Adjusted to volume with methanol. All samples were analysed according to the previous chromatographic condition.

The percent recovery of stavudine was calculated and it should be within 85-115%

3.8.4 Precision

Precision of the analytical method was evaluated by intra-day and inter-day precision. Six different samples of standard solution of stavudine were prepared having final concentration of 0.05 mg/ml. The content of stavudine was determined by HPLC. Each sample was analysed in duplicate within one day for intra-day precision assessment. Another six samples of the same concentration were analysed in three different days for inter-day precision assessment.

The deviation (S.D.) and percent coefficient of variation (%CV) should not be more than 2%

4. Preparation of Film Coated stavudine pellets

From a preliminary study, a suitable formulation and condition for preparing stavudine pellets were formulation P4 using the spheronization speed 860 rpm and the spheronization times 10 minutes. Therefore, pellets on sieve fraction 16/25 cut of formulation P4 with these spheronization conditions were chosen for coating in next step.

4.1 Film Coating Preparation

Ethylcellulose aqueous dispersion (Surelease®) was diluted to 15 % w/w dispersion, based on the manufacturer's recommendation, by adding distilled water while stirring.

A solution of HPMC E15LV in water was separately prepared by dispersing HPMC powder in pre-heated water (80-90^{0}C), diluted with an additional cold water and kept overnight. HPMC E15LV solution was added to the diluted Surelease® to produce the required HPMC contents and stirred throughout the coating processes.

4.2 Film Coating Formulation

The 100 g of pellets were coated with ethylcellulose aqueous dispersion (Surelease®) containing HPMC E15LV with various ratios to 5, 7.5, 10, 15 and 20% of coating levels. The composition of coating dispersion is shown in Table 6

Table 6 The composition of coating dispersion with the mixture of Surelease® and HPMC E15LV

Formulation	Surelease® (%)*	HPMC E15LV (%)	% Coating level (w/w)
S5	100	-	5
S7.5	100	-	7.5
S10	100	-	10
S10H5	95	5	10
S10H7.5	92.5	7.5	10
S10H10	90	10	10
S15	100	-	15
S15H5	95	5	15
S15H7.5	92.5	7.5	15
S15H10	90	10	15
S20	100	-	20
S20H5	95	5	20
S20H7.5	92.5	7.5	20
S20H10	90	10	20

*Ethylcellulose aqueous dispersion had solid content of 25.1%, 70% of which was ethylcellulose

4.3 Coating Conditions

The 100 g of 16/25 mesh cut stavudine pellets were placed in the bottom spraying of fluidized bed coating machine (Wurster type). In preliminary study, various factors in coating condition such as inlet/outlet air temperature, fan capacity, atomizing air pressure and feed rate of coating solution were studied and adjusted to the most optimal condition. A suitable coating condition for stavudine pellets was presented in Table 7

Table 7 The coating conditions using Wurster type bottom spray method.

Coating conditions	
Inlet air temperature	55 ± 2^0 C
Outlet air temperature	50 ± 2^0 C
Atomising air pressure	1.5 bar
Fan capacity	7
Partition height	2.0 cm
Peristatic pump setting	5-7 rpm/min

Coated pellets were dried in hot air oven (Mammertt, Germany) at 55 ^{0}C for 4 hours.

5. Evaluation of Film Coated stavudine pellets

5.1 Stavudine Content in Film Coated Pellets

5.1.1 Standard Preparation

Stavudine standard solution was prepared by weighing accurately about 25 mg of stavudine WS into a 25 ml volumetric flask, dissolved and adjusted to volume with methanol. Pipetted 1 ml of stock solution into 50 ml volumetric flask and adjusted to volume with methanol. The final concentration of solution was 0.02 mg/ml.

5.1.2 Sample Preparation

Twenty capsules containing coated pellets were weighed and calculated for average weight per capsule. An accurately weighted portion of coated pellets, equivalent to about 100 mg of stavudine was transferred to a 100 ml volumetric flask. Methanol was added to dissolve the film, then sonicated for 15 minutes and adjusted to volume with methanol. The suspension was centrifuged at 3,000 rpm for 10 mins. Pipetted 1 ml of supernatant into a 50 ml volumetric flask then adjusted to volume with methanol.

5.1.3 Assay Procedure

Standard and sample preparation were analysed by HPLC at chromatographic condition as previously described.

The % labeled amount (%L.A.) of stavudine in each capsule was calculated by the formula :

$$\%\ \text{L.A. of Stavudine capsule} = \frac{C_{sam.} \times 100 \times 50 \times Wt._{avg}\ (g)}{Wt._{sam}\ (g) \times 100} \times 100$$

Where ; $C_{sam.}$ = Concentration of sample preparation (mg/ml)

$Wt._{avg}$ = Average weight of pellets per capsule (g)

$Wt._{sam}$ = Weight of pellets (g)

5.2 The release of stavudine from Film Coated Pellets

5.2.1 Calibration Curve of stavudine

Calibration curves of stavudine were done in three mediums; water, phosphate buffer pH 6.8 and 0.1 N HCl. Standard stavudine was accurately weighed about 100 mg and transferred to a 100 ml volumetric flask, then adjusted to volume with water. This solution was used as stock solution. Pipetted 1, 3, 5, 5 and 6 ml of stock solution into 50, 50, 50, 25 and 25 ml volumetric flasks, respectively. Adjusted to volume with medium and mixed.

Calibration curves of stavudine are presented in the Appendix A.

5.2.2 Dissolution study of Film Coated stavudine pellets

An accurate weight portion of coated pellets equivalent to 100 mg stavudine, were hand filled in capsule No.2 and six capsules were placed in vessels. Hydrochloric acid buffer pH 1.2 and phosphate buffer (KH_2PO_4) pH 6.8 were prepared by following the direction in USP 24. Dissolution testing was performed by using the USP apparatus II (paddle) operated at 50 rpm with 900 ml of hydrochloric acid buffer pH 1.2 and phosphate buffer pH 6.8 as the dissolution medium equilibrated at 37 $\pm$ 0.5^{0}C. One milliliter of specimen was withdrawn at predetermined time interval of 0.5, 1, 2, 4, 6, 9, 12, 15, 18, 21 and 24 hours. The average of % drug released was calculated from six determinations.

For pH change system, stavudine pellets were tested in 750 ml of hydrochloric acid buffer pH 1.2 at the first two hours and after that adjust to pH 6.8 by adding 250 ml of 18.5 g $Na_3HPO_4.12\ H_2O$ in water. The operation was continued until completing 24 hours. One milliliter of specimen was withdrawn at the same time interval described above.

For Zerit® IR, it was tested using the same procedure as described above but the specimen was withdrawn at predetermined time interval of 0.25, 0.5, 1, 2, 4, 6, 9, 12, 15, 18, 21 and 24 hours.

The amount of drug dissolved at any time interval was measured by HPLC and calculated from a calibration curve. A cumulative amount of drug released as a function of time was determined.

To determine drug release rates, the dissolution data were analysed following the report of Fatemeh et al (2001). Regression analysis was used to obtain the release rate constant (K) and coefficient of determination (r^2). The coefficient of determination for the best fit was used as the main criterion to evaluate the models. The release equation with the highest correlation coefficient was judged to be the most appropriate model for each system.

6. Stability of Zerit® IR and stavudine pellets

Zerit® IR and stavudine pellets were kept in the tight polyethylene bottles at 30 ^{0}C, 65 %RH and at 40 ^{0}C, 75 %RH for 6 months. Both products at two storage conditions were assayed and made dissolution test every two weeks.

Percent assay content of active ingredient should be within 90-110 %L.A. and thymine degradation compound should not be more than 3%w/w over the stability period.

For dissolution test, the dissolution profile at initial time was the reference and the other dissolution profiles at stability periods were the test samples. All profiles were compared with reference using the difference factor (f_1) and similarity factor (f_2).

The two factors were calculated by the following equations :

$$f_1 = \left((\Sigma^{n}_{t=1} \left| R_t - T_t \right|) / \Sigma^{n}_{t=1} R_t \right) \times 100$$

$$f_2 = 50 \times \log \left(\{1 + (1/n) \Sigma^{n}_{t=1} (R_t - T_t)^2 \}^{-0.5} \times 100 \right)$$

in which ;

n = Number of sampling points

R_t = Mean percentage dissolved of reference sample at sampling time t hours

T_t = Mean percentage dissolved of test sample at sampling time t hours

The similarity factor is the principal factor for considering the equivalence of dissolution profiles between test samples over stability period and reference at initial time.

The difference factor is a factor which measures the relative error of dissolution profiles between test samples over stability period and reference at initial time.

Criteria for dissolution profiles comparison, the difference factor and similarity factor should be between 0 to 15 and 50 to 100, respectively.

B. *In vivo* studies

This single-dose, randomized, two-way crossover study was conducted at the Faculty of Pharmaceutical Science, Chulalongkorn University (Bangkok, Thailand). The protocol of this study was approved by the Independent Ethical Committee of the faculty.

1. Test and reference products

Two products were tested in this study. The test product was stavudine pellets 100 mg and the reference product was Zerit® IR 40 mg (d4T IR) which the content of stavudine had been modified to be 50 milligram.

2. Study design

The study was conducted in a randomized two-way crossover design. Twelve white New Zealand rabbits were divided into two groups. Zerit® IR and stavudine pellets were given to each subject according to a single dose, two-treatments, two periods and two sequences with a washout period of two weeks between each administration as shown in Table 8.

Table 8 Randomization Schedule

Sequence	Subject no.	Period	
		1	2
1	1 - 6	A	B
2	7 - 12	B	A

A = Test product (stavudine pellets)

B = Reference product (Zerit® IR)

3. Drug administration, sample collection and handling

All subjects were fasted 10 hours before each drug administration. A single dose of both products given to each subject (Stavudine pellets 100 mg and Zerit® IR 50 mg). with 5 ml of water. Approximately 5 ml of blood samples were withdrawn from ear vein of each subject at the following time; predose, 1, 2, 2.5, 3, 3.25, 3.5, 3.75, 4, 6, 9, 12, 15, 18, 21, 24 and 30 hours after dosing for stavudine pellets and at predose, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 9 and 12 hours after dosing for Zerit® IR.

All blood samples were collected in heparinized tubes and chilled at 0 ^{0}C. Blood samples were centrifuged at 3,000 rpm for 15 minutes. Plasma samples were removed using plastic pipette, divided into two aliquots and placed in glass tubes. They were immediately frozen at -20 ^{0}C until analysis. After a washout period of two weeks, the same manners were repeated to complete the crossover design.

4. Determination of stavudine in plasma

4.1 Sample Preparation

An aliquot (1 ml) of plasma sample was transferred to a glass test tube. 100 μL of internal standard (Zidovudine 25 μg/ml) and 1 ml of acetonitrile were added for protein precipitation. The mixture was shaken for 10 seconds in a vortex mixer and centrifuged at 3,000 rpm for 15 minutes. Supernatant was then separated and 20 μL aliquot of the solution was injected into the HPLC.

4.2 Chromatographic system

Column	: Apollo C18, 5 micron, 4.6×250 mm
Mobile phase	: 0.025 M Ammonium acetate (pH 3.8) : MeOH (70 : 30)
Flow rate	: 1 ml/min
Detector	: UV 265 nm
Injection volume	: 20 µL
Temperature	: Ambient (25 °C)
Retention time	: Stavudine ≈ 4.8 min,
	Zidovudine ≈ 10.4 min

4.3 Standard preparation

Stock standard solution of stavudine (5 mg/ml) was prepared in water. This solution was diluted with water to give working solutions of 0.5, 1, 2.5, 5, 10, 15 and 20 µg/ml, respectively.

Zidovudine internal standard solution (25 µg/ml) was prepared by weighting accurately about 50 mg of zidovudine WS into 100 ml volumetric flask. Added about 30 ml of water and sonicated for 10 minutes. Adjusted to volume with water and mixed. Pipetted 5 ml of stock solution into 100 ml volumetric flask and adjusted to volume with water.

4.4 Calibration Curve of stavudine in plasma

An aliquot (100 µL) of each working standard solutions of stavudine and zidovudine were spiked to blank plasma to produce a set of calibration standard of stavudine at 50, 100, 250, 500, 1000, 1500 and 2000 ng/ml, respectively. All these standard solutions were analysed by HPLC following the same procedure as described earlier. The peak area ratios of stavudine to internal standard were plotted against the known concentration of stavudine and the calibration curves were fitted to a straight line by linear regression analysis.

5. **In-vivo Analytical Method Validation**

For performance characteristics of suitable and reliable method for the intended analytical application, method validations are performed partially in accordance with the specification given in the Guidance for industry : Bioanalytical Method Validation of Center for Drug Evaluation and Research (CDER) and Center for Veterinary Medicine (CVM), U.S. Department of Health and Human Services, Food and Drug Administration, 2001. Detail of validation were described as following :

5.1 Selectivity

Samples prepared from blank plasma of six subjects were analysed using the same procedure as described earlier. In all cases chromatograms were visually examined for potential interfering peaks. Stavudine coadministered with zidovudine were tested to ensure that there is no interference of plasma protein and/or endogenous substances to the peaks of stavudine and internal standard.

5.2 Lower limit of quantification (LLOQ)

Five determinations of the lowest concentration (50 ng/ml) of standard stavudine in plasma were analyzed. The LLOQ were established by examination of the accuracy and precision data. Analyte peak of these concentrations should be identifiable, discrete, and reproducible with a precision not exceeding 20% and %recovery of 80-120%, and it would serve as the first point of a standard calibration curve.

5.3 Linearity and standard calibration curve

Stock standard solution of stavudine and internal standard were prepared in water. The calibration curve were prepared by adding working of stavudine in blank plasma to provide the standard solutions of 50, 100, 250, 500, 1000, 1500 and 2000 ng/ml. All of standard solutions were analyzed by HPLC. Peak area ratios of stavudine to internal standard versus the corresponding concentrations of stavudine were fitted to a straight line using linear regression analysis.

The coefficient of determination (R^2) was calculated and it should be $\geq$ 0.99. The percent coefficient of variation for precision of the LLOQ should not be more than 20% and percent coefficient of variation for precision of other concentrations should not be more than 15%.

5.4 Accuracy

The accuracy of the method was determined by assessing the agreement between the estimated and nominal concentrations of three concentrations of quality control samples (QC samples)

Three concentrations (100, 750 and 1,750 ng/ml) of QC samples were prepared for low (QCL), medium (QCM) and high (QCH) concentrations of stavudine in plasma, respectively. Five replicate sets of these three concentrations of QC samples were analyzed for the drug content.

Accuracy of the analytical method was estimated by the percent recovery of each concentration level using an equation.

$$\% \text{ Recovery} = \frac{\text{Estimated concentration}}{\text{Know concentration}} \times 100 \qquad (9)$$

The mean value of the percent recovery of each concentration level should be within $\pm$15% of nominal concentration or ranged between 85-115%.

5.5 Precision

The precision of the method was determined by assessing the agreement between the replicate measurements of three concentrations of plasma stavudine QC samples (low, medium and high concentrations).

Within-run Precision

Five replicate sets of three concentrations of plasma stavudine QC samples (100, 750 and 1,750 ng/ml) were prepared and analyzed at the same day.

The percent coefficient of variation of each concentration level was computed and it should not exceed 15%.

Between-run Precision

Five replicate sets of three concentrations of plasma stavudine QC samples (100, 750 and 1,750 ng/ml) were prepared and analyzed at five consecutive day (one set/day).

The percent coefficient of variation of each concentration level was computed and it should not exceed 15%.

5.6 Stability of spiked plasma and extracts

5.6.1 Short-term Room Temperature Stability

Three aliquots of the low and high concentrations (100 and 1,750 ng/ml) of plasma stavudine samples were stored at –20 ^{0}C in freezer for 24 hours and thawed at room temperature and kept at this temperature. At 4, 8 and 12 hours of storage, samples were analysed and compared to that at time zero.

Percent deviation was calculated and it should be within $\pm$15%

5.6.2 Long-term Stability

Three aliquots of the low and high concentrations (100 and 1,750 ng/ml) of plasma stavudine samples were stored at –20 ^{0}C in freezer for 8 weeks. Samples were taken to be analysed at the end of 2, 4, 6 and 8 weeks and compared to that at time zero.

Percent deviation was calculated and it should be within $\pm$15%

5.6.3 Freeze-thaw Stability

Three aliquots of the low and high concentrations (100 and 1,750 ng/ml) of plasma stavudine samples were at –20 ^{0}C in freezer for 24 hours and thawed unassisted at room temperature. This procedure was one freeze-thaw cycle. When completely thawed, the samples were refrozen for 24 hours under the same conditions. The freeze-thaw cycle was repeated two more times, then analyzed on the third cycle.

Percent deviation between the concentration at time zero and that after three free-thaw cycle was calculated and it should be within ±15%.

5.6.4 Post-preparative Stability

Three aliquots of the low and high concentrations (100 and 1,750 ng/ml) of plasma stavudine samples were analyzed after extracting and placing in the autosampler at 4, 8, 12 and 16 hours and they were compared with those at time zero.

Percent deviation was calculated and it should be within ±15%.

6. Pharmacokinetic analysis

Individual plasma stavudine concentration-time profiles from each treatment was analysed using non-compartmental method for relevant pharmacokinetic parameters (C_{max}, t_{max}, AUC_{0-t} and $AUC_{0-\infty}$).

The peak plasma stavudine concentration (C_{max}) and the time to peak plasma stavudine concentration (t_{max}) were observed from the plots of the plasma drug concentration-time.

The area under the plasma stavudine concentration-time curve from time zero to the last point of collection, AUC_{0-t} was calculated using trapezoidal rule from time zero to the last measurable plasma drug concentration.

The area under the plasma stavudine concentration-time curve from time zero to infinite time, $AUC_{0-\infty}$ was calculated by adding the as AUC_{0-t} with $\hat{c}/k$ term ; where $\hat{c}$ is the last measurable drug concentration and k is the terminal elimination rate

constant. At least three points during the terminal Ln-linear phase were used to obtain an accurate estimate of k from linear regression.

The half-life ($t_{1/2}$) was calculated using an equation : $t_{1/2} = 0.693/k$

The relative bioavailability was calculated using an equation :

$$\%\text{Relative bioavailability} = \frac{AUC_{0-\infty\,pellet}}{AUC_{0-\infty\,Zerit\,IR}} \times \frac{Dose_{Zerit\,IR}}{Dose_{pellet}} \times 100$$

The corresponding pharmacokinetic parameters of stavudine obtained from oral administration of 100 mg stavudine pellets and 50 mg Zerit® IR in the rabbits were compared.