CHAPTER III

MATERIALS AND METHODS

I. Materials

1. Test products.

Four brands of simvastatin tablets were tested in this study. Each film-coated tablet contains 10 mg of simvastatin, one was the innovator's product which was assigned as the reference standard namely brand A (Zocor®). The letters B, C, and D were given to represent the brand name of each locally manufatured product. Information of the reference standard and the test products was provided in Appendix A.

2. Reagents

2.1 Working standard simvastatin powder (Biolab Co. Ltd, Bangkok, Thailand), potency 98.0%, Lot No. PS12760.

2.2 Internal standard lovastatin powder (Pharmaceutical Fine Chemicals, USA), potency 98.0%, Batch No. 960511.

2.3 Internal standard griseofulvin powder (China).

2.4 Working standard simvastatin hydroxy acid powder (Biolab Co. Ltd, Bangkok, Thailand).

2.5 Methanol HPLC grade (Labscan Ltd, Ireland) Lot No. 3041KPDE.

2.6 Acetonitrile HPLC grade (Labscan Ltd, Ireland) Lot No. 2856KTSS.

2.7 Sodium chloride GR (Farmitalia Carlo Erba, Milano, Italy) Lot No. 479687.

2.8 Sodium hydroxide GR (BDH Laboratory supplies, Bangkok, Thailand) Lot No. 191294DO36.

2.9 Sodium dihydrogen phosphate anhydrous GR (Fluka AG, Buchs, Switzerland) Lot No. 71496.

2.10 Sodium lauryl sulphate (K. H. Co. Ltd, Bangkok, Thailand)

2.11 Phosphoric acid (Farmitalia Carlo Erba, Milano, Italy) Lot No. 4 D050294G.

3. Apparatus

3.1 Analytical balance (Satorius, Scientific Promotion Co. Ltd, Germany)

3.2 Dissolution apparatus (VK650AS, Vankel Industries, Inc., USA)

3.3 Spectrophotometer (Jasco Model 7800, Jasco Corp., Japan)

3.4 pH meter (Beckman 50, Beckman Instrument, Inc., USA)

3.5 Vortex mixer (Vortex Genies-2, Scientific Industries, Inc., USA)

3.6 Centrifuge (Labofuge Model 610, Heraeus-Christ GMBH, Germany)

3.7 High Performance Liquid Chromatograph (Shimadzu LC10AD, Simadzu Corp., Japan) equipped with variable wavelength UV detector (Model LC10A) and data integrating software (LC10)

3.8 Hot air oven (Memmert Model 100, Memmert GMBH, Germany)

3.9 Micropipette (Eppendorf)

3.10 Ultrasonic bath (Transonic digital, Diethelm & Co.Ltd, Germany)

3.11 Speed Vacuum Concentrator (Maxy Dry Plus, Heto, Germany)

3.12 Glassware

II. <u>Methods</u>

A. *In Vitro* Studies

Simvastatin powder was described in the third supplement of the USP 23 for some characteristics but simvastatin tablet is not available in any pharmacopeias. Since the tablet is in a coated-tablet form thus the USP 23 dissolution test for coated tablet was used for the *in vitro* testing of simvastatin tablet. Various dissolution media and related dissolution parameters have been previously investigated to choose the optimal conditions that can explain different dissolution profiles of each brand. In addition, content uniformity test was performed on the products to check on the integrity of the individual tablets before conducting the *in vivo* bioavailability comparison. The tests are described as follows.

1. Assay of the content of active ingredient

The percent labeled amount of simvastatin tablet was determined by HPLC method, which was modified from that of Kaufman and the third supplement of USP 23 as follows (Kaufman, 1990; USP 23, 1995).

The content of 20 tablets of each brand were introduced into a 200-ml volumetric flask and disintegrated in a small volume of water. 150 ml of 45% acetonitrile in water was added to the flask which was sonicated for 10 minutes. The mixture was allowed to cool at room temperature and diluted to volume with the same solution. After centrifugation at 5000 rpm for 5 minutes, 50 µl of the supernatant and 40 µl of the internal standard solution (1 mg/ml griseofulvin in 45% acetonitrile) were transferred to a 10-ml volumetric flask and diluted with the same solution above. An aliquot (20 µl) of this sample was

injected to HPLC. The amount of simvastatin (in mg) contained in tablets was calculated according to the standard curve (Appendix B).

Standard curve

The standard was obtained from the preparation of a series of standard solution with known amount of simvastatin in 45% acetonitrile or dissolution medium at concentration of 2, 4, 6, 8, 10, and 12 µg/ml, respectively, and with griseofulvin at 4 µg/ml. This solution set was analyzed chromatographically according to the condition in Sect. 4. The ratios of the peak area of standard simvastatin to that of the internal standard were plotted against the standard simvastatin concentrations in 45 % acetonitrile or dissolution medium and the resulting curve was fitted to a strainght line using linear regression (Appendix B).

2. Content uniformity

Ten tablets of simvastatin tablets from each brand were sampled and individually assayed according to method described below.

Each tablet was introduced into a 10-ml volumetric flask and disintegrated in a small volume of water. 7 ml of 45% acetronitrile in water was added and the flask was sonicated for 10 minutes before it was allowed to cool at room temperature and diluted to volume with the same solution. After centrifugation at 5000 rpm for 5 minutes, 50 µl of the supernatant and 40 µl of the internal standard solution were transferred to another 10-ml volumetric flask, diluted with the same solution above and an aliquot (20 µl) of this sample was injected HPLC. Griseofulvin internal standard was dissolved in 45% acetronitrile in water to obtain concentration of about 1 mg/ml. The amount of

simvastatin (mg) in each tablet was calculated according to the standard curve (Appendix B). Preparation of simvastatin standard solutions was similar to that of the assay.

3. Dissolution tests

The dissolution tests of each brand were operated according to the method described in USP 23 for coated-tablet. The USP dissolution apparatus type II (paddle) was used to compare the dissolution profiles. 0.5% sodium lauryl sulphate (SLS) in 0.01 M monobasic sodium phosphate buffer (pH 5.5 ± 0.02) were used as the dissolution medium. This concentration of SLS was previously found to be an the optimal solubility of simvastatin and this pH was chosen because no degradation of simvasatin was found in 24 hours (Serrajuddin, Ranadive and Mahoney, 1991). The procedure was described as follows:

Nine hundred ml of dissolution medium was placed into each of the six vessels and equilibrated at 37 ± 0.5°C. . A tablet was introduced into each vessels which covered with a plastic cover. The apparatus was immediately operated and the stirring speed was maintained at 50 rpm for 2 hours. This speed was in agreement with the requirements set by The Food and Drug Administration of Thailand for non-official dissolution method. Eleven ml of the dissolution medium was withdrawn from each vessel at 5, 10, 15, 20, 30, 45, 60, and 120 minutes and the vessel was replaced with an equal volume of the fresh dissolution medium at 37 °C. Each withdrawn sample was then added to a 10-ml volumetric flask which contained 40 µl of the internal standard solution (1 mg/ml of griseofulvin solution). The flask was adjusted to volume with the dissolution medium and mixed. An aliquot (20 µl) of this sample was

then assayed chromatographically using the same HPLC conditions. The amount of the drug dissolved at various time intervals was quantified using the standard curve (Appendix B). Preparation of simvastatin standard solutions was similar to that of the assay. Corrections were also made during the calculation of the amount dissolved to account for the dilution effect due to replacement of the dissolution medium.

4. Chromatographic conditions

The HPLC system consisted of two pumps, an autoinjector, and an UV detector (Shimadzu, Japan). The HPLC columns used were a μ-Bondapak C18 stainless steel column (30 cm x 3.9 mm I.D., 10-μm packing) and a μ-Bondapak C18 guard column, both manufatured by Waters Associates Pty-Ltd., USA. The column was maintained at room temperature and the UV detector was set at 238 nm. For the *in vitro* and the *in vivo* plasma analyses, the mobile phases were 54% and 44% V/V isocratic mixtures of acetonitrile in 0.025 M monobasic sodium phosphate pH 4.5, respectively, and a flow-rate of 1.5 ml/min was used in both analyses.

5. *In vitro* statistical evaluation

Physical characteristics of all four brands of simvastatin tablets were examined and a one-way analysis of variance was performed to compare the dissolution rate constants of the innovator's and those of the three locally manufactured products at 5% significance level. When significant treatment effects were observed from ANOVA, differences between treatment versus a control were examined by means of the multiple comparison Dunnett's test each at 5% significance level.

B. *In vivo* studies

1. Animals

Twelve male mongrel dogs with the weight ranging from 10 to 18 kg were chosen in this study. At first coming, they were bathed, got rid of parasites and worms, and given rebies vaccine. They had their own cages and were fed the same food once a day in the morning. Their cages and wards were cleaned everyday. Their blood samples were withdrawn at first and after completion of this study check for any abnormal values of blood chemistry, especially their hepatic function, and any diseases. They were taking no other medications for at least one week prior to and throughout this study. The results of their initial health examination and blood chemistry are provided in Appendix D.

2. Dosage and administration

Twenty tablets of 10-mg simvastatin were given orally with few ml of water in a single dose. All dogs recieved each dose in the morning after an overnight fast. No food (except water) was permitted until 2 hours after dosing. Food was approximately 200 g of Boss® in each meal.

3. Experimental design

The study was conducted in a crossover fashion using a repeated latin square design. Each dog received the drug according to the sequence shown in Table 4 with at least one-week washout period between each treatment.

Table 4 Schedule of Repeated Latin Square Design for *in vivo* Bioequivalence study.

Dog #	Order of Treatment			
	1 st period	2 nd period	3 rd period	4 th period
1	A	B	C	D
2	B	D	A	C
3	C	A	D	B
4	D	C	B	A
5	A	B	C	D
6	B	D	A	C
7	C	A	D	B
8	D	C	B	A
9	A	B	C	D
10	B	D	A	C
11	C	A	D	B
12	D	C	B	A

4. Sample collection

Five ml of each blood sample was collected from a cephalic vein of the dog's forelimb or a saphenous vein of its hindlimb using a disposible syringe and immediately transferred to heparinized tubes containing 25 µl of 5000 i.u./ ml of heparin solution. The vein was kept patent throughout the sampling period by small flushing dose (1 ml) of 5 i.u./ml heparin solution. Blood samples were collected immediately before drug administration and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 8 hr post dosing. The blood was immediately centrifuged at 2500 rpm for 10 minutes and the plasma was separated and kept at -20°C until subsequent analysis.

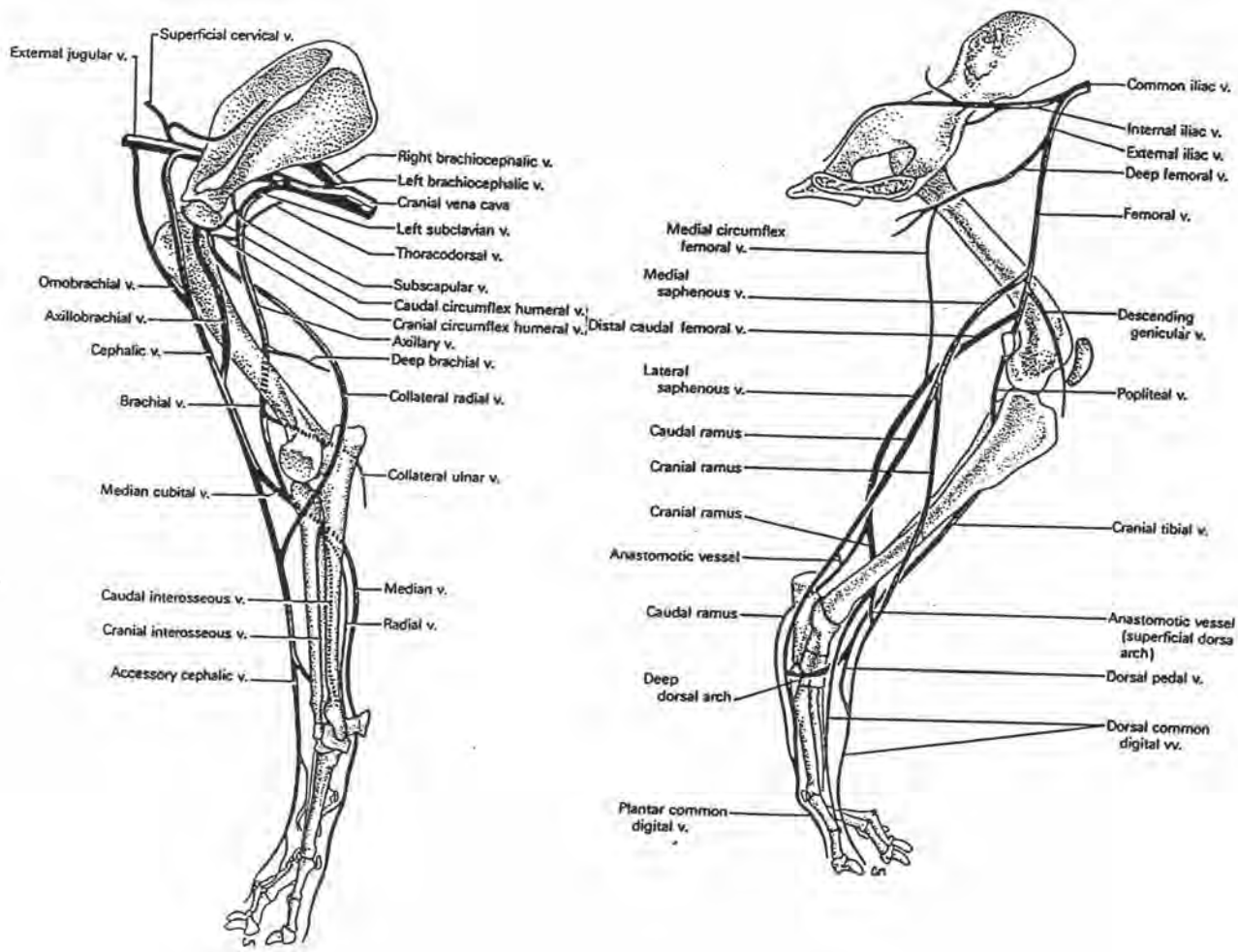

Figure 5 The major veins of the forelimb and the hindlimb of the dogs.

5. Determination of simvastatin in plasma

Concentrations of simvastatin in plasma were determined by conversion of the prodrug to simvastatin hydroxy acid, the hydrolysed form, to avoid interference from partial hydrolysis of simvastatin to simvastatin hydroxy acid during clean-up and analytical processing. Simvastatin hydroxy acid was determined using HPLC method modified from that described by Stubbs et al. (1990). The procedure was described as follows.

Pooled blank dog plasma (1 ml) was separately spiked with 10 µl of various concentrations of simvastatin working solution (2.5, 5, 10, 25, 50 µg/ml, in 45% acetonitrile in water which contained lovastatin hydroxy acid internal standard at 28 µg/ml) to obtain standard concentrations ranging from 25 to 500 ng/ml. The plasma samples were mixed with 10 µl of 45% acetonitrile in water. The standards and samples were treated by the same procedure. Plasma protein was precipitated from the standards and samples by mixing with 2 ml of acetonitrile. The mixtures were centrifuged at 4000 rpm for 5 minutes, then, the supernatant was separated and evaporated to dryness by vacuum concentrator (Maxi Dry Plus, 30°C). The residue was reconstituted in 250 µl of 80% acetonitrile in water, and hydrolysed with 10 µl of 0.5 N potassium hydroxide solution for 20 min, and neutralized with 10 µl of 0.25 M phosphoric acid, centrifuged at 4000 rpm for 5 minutes and an aliquot of the supernatant (25µl) was assayed by HPLC. The peak area ratios of total simvastatin hydroxy acid to the internal standard (lovastatin hydroxy acid) were plotted against the standard simvastatin concentrations in plasma and the resulting curve was fitted to straight line using linear regression (Appendix B).

6. Assay validation (Shah et. al., 1992)

Within-run precision was determined by analyzing three sets of standard curves in the same day. The percent coefficient of variation (%CV) of the peak area ratios of simvastatin hydroxy acid to the internal standard for each concentration was determined.

Between-run precision was determined by comparing the peak area ratios of the daily prepared standard curves for three different days. The percent coefficient of variation (%CV) of each concentration was determined.

Physical recovery was determined by analyzing six sets of standard curves of simvastatin hydroxy acid prepared in dog plasma and in aqueous solution that were treated with the same clean-up procedure. Comparison of the PAR was made at each same concentration of the standards between the plasma and the aqueous solution to obtain percentage recovery at that particular concentration.

Limit of quantitation (LOQ) was determined by analyzing ten samples at limit of the lowest possible concentration that still gave percent coefficient of variation (%CV) less than 20%.

Acceptance criteria for the run :

The acceptance criteria are not greater than 15% CV for both between and within-run precision and not more than 15% deviation from the nominal value for analytical recovery. However, for the LOQ a higher value of 20% is acceptable for both precision and accuracy. For physical recovery, the percentage should not less than 80%.

7. Determination of total cholesterol level at pre- and 2 hours post-administration

Total cholesterol was determined using an enzymatic colorimetric test (Cholesterol liquicolor®, Human, Germany) by automatic analyzer (Imply®, Olympus, Japan). The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinineimine is formed from hydrogen peroxide and 4-aminophenazene in the presence of phenol and peroxidase, and its absorbance was measured at 500 nm. The reaction principle was described as follows:

cholesterol ester + H_2O —CHE → cholesterol + fatty acid

cholesterol + O_2 —CHO → cholestene-3-one + H_2O_2

$2H_2O_2$ + 4-aminophenazene + phenol —POD → quinineimine + $4H_2O$

CHE = Cholesterol esterase, CHO = Cholesterol oxidase, POD = Peroxidase

8. Pharmacokinetic analysis

Standard pharmacokinetic parameters were directly calculated from the plasma-time data, i.e. C_{max}, t_{max}, AUC and elimination rate constant. The C_{max} was the highest observed concentration and t_{max} was the time at which C_{max} occurred. The AUC or area under the plasma drug concentration time curve was calculated by linear trapezoidal rule up to the last quantifiable time point (AUC_{0-8}). The terminal elimination rate constant (K_{el}) was determined by linear regression analysis of the log-linear terminal phase of the plasma

concentration-time profile and the elimination half-life ($t_{1/2}$) was calculated as natural log of 2 divided by the terminal elimination rate constant.

9. Statistical evaluation of bioequivalence

The difference in C_{max}, t_{max}, and AUC_{0-8} between innovator's product and the locally-formulated brands were analyzed by randomized block analysis of variance (ANOVA). Differences between treatments versus control (brand A) were also examined by means of post-ANOVA Dunnett's test. The test brands are considered to be similar to innovator's product when their C_{max}, t_{max} and AUC show no statistically significant differences (α=0.05) from the innovator's product. In addition the 90% confidence intervals were also constructed for the C_{max}, t_{max} and AUC value according to the method of Schuirmann (1987).

The differences in total cholesterol level at 2 hours pose dosing and at time 0 before drug administration (ΔTC) in each product were determined by paired t-test. When significant differences were observed from paired t-test , differences between treatments were further analyzed by randomized block ANOVA.