

3. MATERIALS AND METHODS

3.1 Chemicals and Reagents

Reference standard ivermectin (Lot 90K1506) and derivatizing agents; N-methylimidazole (Lot 71K3680) and trifluoroacetic anhydride (Lot 41K3444) were obtained by Sigma-Aldrich (St. Louis, MO, USA). Reference internal standard, abamectin (Lot LB281-125A), was purchased from Supelco, U.S.A. Injectable solution of 1% w/v ivermectin (Ivomec[®], MSD) was obtained from Merial Ltd. (Bangkok, Thailand). Methanol (Lot V40E74) and Acetonitrile (Lot V30806), HPLC grade, were purchased from J.T. Baker Selusorb[®], U.S.A. Water was purified by Milli-Q plus 185 Water Purification System from Millipore Corp. (Milford, MA, U.S.A.). Albendazole (Zeben[®] Suspension) was purchased from Siam Pharmaceutical Co., Ltd. (Bangkok, Thailand).

3.2 Study protocol

3.2.1 Sample size estimation

Some limitation in this experiment was sample size. Normally, the confidence of experimental study depends on the quantity of used animals, but due to the insufficient of experimental animals, we must use an equation to find the sample size. It was calculated as follows.

$$n = \frac{Z_{\alpha}^2 \cdot \sigma^2}{d^2}$$

n = Sample size

Z_{α} = z-parameter $(1 - \frac{\alpha}{2})$, $Z_{\alpha} = 1.96$

σ^2 = Variance (S.D.²), $\sigma = 1$

d = Margin of sampling error, d = 0.7

Z_{α} was proceeded at the confidence interval 95 % ($\alpha = 0.05$) while σ^2

was obtained from the pilot study. n was a determinate value for obtaining the sample size.

3.2.2 Study 1: Pharmacokinetics of ivermectin in normal cats

- Animals

Four male and four female domestic cats (*Felis catus*) aging 1.5 yr and weighing 2.5 kg approximately were included. Before starting the experiment, all of them had general health check-up. Physical examination and rabies vaccination were performed. The animals were dewormed by taking a single oral dose of 40 mg/kg of albendazole (Zeben[®]). Blood samples (2 ml) were drawn from the external jugular vein for blood chemistry tests to assess function of hepatic (SGOT, SGPT, ALP), renal (BUN, Cr), and hematological systems (CBC) and to screen for microfilariae.

During experimental period, the animals were housed in an experimental animal room at the Faculty of Science maintained at constant temperature (25 °C) under a 12 hour/light/day cycle. They were fed a commercial cat food (Me-O[®] S.W.T. Co. Ltd., Samutprakan, Thailand) at the amount of 19 g/kg/day and had free access to water. The experimental protocol was approved by the Ethics Committee for experimental animals, Prince of Songkla University.

- Drug administration

The animals were subcutaneously injected a single dose of 200 µg/kg of ivermectin (Ivomec[®], 1% w/v of ivermectin) at neck region of cats.

- Adverse drug reactions observation

After drug administration, the animals were observed throughout the experiment for common side effects including any abnormal behavior, ataxia, lethargy, weakness, tremors, and recumbency (Lewis *et al.*, 1994).

- Blood sampling

Blood samples (3 ml) were taken from the external jugular vein before and at 1, 12, 24 hours and at 2, 3, 4, 5, 7, 9, 11, 15, 20, 25, 30 days after administration of ivermectin. Samples were left to clot for 1 hour at room temperature. Serum samples were separated after centrifugation at 2000 xg for 15 min and kept frozen at $-70^{\circ}C$ until analysis at 1 month approximately.

3.2.3 Study 2: A preliminary study of pharmacokinetics of ivermectin in an infected cat

- An animal

One *B. malayi*-infected female cat (*Felis catus*) age 1.5 yr, weight 2.5 kg and living in filariasis-endemic area in Narathiwat Province was used. Before starting the experiment, its health was checked in the same aspect as in the normal cats. The animal was housed and cared in an experimental animal room at the Filariasis Project Pigultong Development Center in Narathiwat Province. Caring conditions were also as same as in normal cats.

Being *B. malayi* infected was confirmed using Giemsa staining of thick-blood films. A sample of blood (50 μ l), in duplicate, was collected from the ear vein. Thick-blood samples were immediately made on a clean glass slide and microfilariae were counted under a light microscope after Giemsa staining of blood films (Filariasis Division, 1998).

- Microfilarial periodicity

Before administration of ivermectin, blood samples (50 μ l) were collected from the ear vein of an infected cat at 4-hour intervals for 24 hours to investigate microfilarial periodicity (Sasa & Tanaka, 1972; Sasa & Tanaka, 1974; Aikat & Das, 1977) of *B. malayi*.

- Drug administration

An infected cat was also administered a single dose of 200 μ g/kg of

ivermectin by subcutaneous injection at neck region of cats.

- Adverse drug reactions observation

The infected cat was observed for common side effect after drug administration, as same as in normal cats.

- Blood sampling

Blood samples (3 ml) were taken from the external jugular vein before and at 1, 12, 18, 24, 30, 36, 42, 48, 60, 72, 84 hours and at 4, 5, 7, 9, 11, 15, 20, 25, 30 days after administration of ivermectin. Samples were left to clot for 1 hour at room temperature. Serum samples were separated after centrifugation at 2000 xg for 15 min and kept frozen at -70 °C until analysis at 1 month approximately.

- Microfilarial density after ivermectin administration

Microfilarial density after ivermectin administration was determined. Samples of blood (50 µl) drawn from the ear vein were collected in the same day that the blood was drawn for determination of ivermectin concentration. The collecting time was at 24.00 hour which was nearly the peak hour of existing of the microfilariae in blood circulation. Microfilarial density was determined using Giemsa staining as same as microfilarial periodicity.

3.3 Analytical procedure

3.3.1 Instrumentation

The HPLC system consisted of a Waters 2695 Separations Module and a Waters 2475 Multi λ Fluorescence detector (Waters, MA, U.S.A.). Data were collected and processed using the Empower™ Software System. The analytes were concentrated to dryness by using speed vacuum (UNIVAPO 100 ECH, U.S.A.) and extracted with LC 18 cartridge, Sep-Pak® Cartridges, (Waters, MA, U.S.A.).

3.3.2 Chromatographic conditions

Concentrations of ivermectin in serum were determined using method modified from Lifschitz *et al.* (1999a). The HPLC column was Nova-pak[®] C18 (Waters, 4 µm, 3.9x150 mm). It was connected to the guard-pak precolumn module inserted with C18 packing materials. The mobile phase consisted of acetonitrile-methanol-water (60:35:5 v/v/v) and was pumped at a flow rate of 1.5 ml/min. The mobile phase was freshly prepared each day and was filtered through 0.22 µm filtered paper and degassed before using. Detection was made at an excitation wavelength of 365 nm and an emission wavelength of 475 nm. All analysis was performed at room temperature.

3.3.3 Working standard solutions

Stock solutions (1 mg/ml) of reference standard ivermectin and reference internal standard abamectin were prepared in acetonitrile. Working standard solutions were prepared by diluting the stock solutions with acetonitrile to a concentration range of 0.1-100 ng/ml for the reference standard ivermectin and 300 ng/ml for the reference internal standard abamectin. Both solutions were stored at 4-8 °C until analysis.

3.3.4 Calibration curves

Calibration curves were prepared by adding working standard ivermectin solution and 100 µl of internal standard abamectin (300 ng/ml) to blank human serum so that final concentrations of ivermectin were 1, 5, 10, and 25 ng/ml.

3.3.5 Sample extraction

The extraction of ivermectin from serum spiked with ivermectin and animal serum samples were extracted following the technique described by De Montigny *et al.* (1990) and Lifschitz *et al.* (1999a). A serum sample (1 ml) was combined with 100 µl of abamectin (300 ng/ml) and then mixed with 2 ml

of acetonitrile:water (80:20 v/v) to precipitate protein. After 30 sec mixing by vortex, the solvent-sample mixture was centrifuged at 2000 xg for 15 min. The supernatant was passed through a Sep-Pak[®] Classic C18 cartridge previously conditioned by passing 2 ml of methanol and 2 ml of deionized water. The cartridge was flushed with 2 ml of water:methanol (3:1 v/v) to elute non-analyte. The analytes were eluted with 1 ml of methanol and concentrated to dryness under speed vacuum at 45-50 °C.

3.3.6 Sample derivatization

The residue was dissolved with 100 μ l of N-methylimidazole:acetonitrile (1:1 v/v). Derivatization was initiated by adding 150 μ l of trifluoroacetic anhydride (1:2 v/v). After mixing by vortex, the mixture was centrifuged at 1000 xg for 5 min. After completion of the reaction (<30 sec) an aliquot (100 μ l) of the supernatant was injected directly into the HPLC system.

3.4 Method validation

Prior to ivermectin analysis, validation characteristics, namely linearity, recovery, intra-day and inter-day precisions, accuracy, limit of detection and limit of quantification were performed in accordance with ICH guideline recommendations (Swartz & Krull, 1997). Drug-free human serum was used throughout the validation.

3.4.1 Linearity and range

Linearity was determined by preparing standard serum samples with different concentrations of ivermectin (0.2, 1.0, 10, 20, 40, 60, 80, 100 ng/ml; 3 replicates of each concentration). The calibration curve was constructed by plotting peak area ratio (Y) of ivermectin to the internal standard versus ivermectin concentrations (X). The regression parameters of slope, intercept and correlation coefficient were determined by least-squares method.

3.4.2 Precision

Both intra-day (repeatability) and inter-day (reproducibility) precisions were determined by adding different concentrations of standard ivermectin 1, 10, 20, 40 ng/ml (10 replicates of each concentrations) into serum and analyze within the same day and 10 consecutive days, respectively. Precision was expressed as coefficient of variation was calculated as follows.

$$\text{Coefficient of variation (\% C.V.)} = \frac{\text{Standard deviation (S.D.)}}{\text{Mean value}} \times 100$$

An acceptable precision was less than 10%.

3.4.3 Accuracy

Accuracy or the closeness of the agreement between the theoretical value and the value found was determined by adding the standard ivermectin into serum at concentrations of 1, 2.5, 5, 10, 20, 40 ng/ml (10 replicates of each concentration). The percent accuracy was calculated as follows.

$$\text{Accuracy (\%)} = \frac{\text{Concentration of ivermectin found in sample}}{\text{Nominal concentration of ivermectin - spiked samples}} \times 100$$

3.4.4 Recovery

Recovery of extraction of ivermectin from serum was determined at low (0.1 ng/ml), medium (10 ng/ml) and high (40 ng/ml) concentrations (5 replicates of each concentration) by comparing the responses after direct injection of standard ivermectin in derivatizing agents with those after extraction. Percent recovery was calculated as follows.

$$\text{Recovery (\%)} = \frac{\text{Response after extraction of ivermectin from serum samples}}{\text{Response after direct injection of ivermectin in derivatization agent}} \times 100$$

3.4.5 Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection and Limit of quantification were determined by using 5 calibration curves of standard serum samples of various ivermectin

concentrations (0.1, 1, 10, 20, 40 ng/ml). LOD and LOQ were calculated as follows.

$$\text{LOD} = \frac{3.3 (\text{S.D. of Y-intercepts})}{\text{Mean value of slope}} \quad ; \quad \text{LOQ} = \frac{10 (\text{S.D. of Y-intercepts})}{\text{Mean value of slope}}$$

3.5 Pharmacokinetic analyses

The serum concentrations of ivermectin in each individual animal and the mean concentrations were plotted against time. Regarding to compartment model analysis, Akaike's Information Criterion (AIC) (Yamaoka *et al.*, 1978) was used for selecting of appropriate model. Pharmacokinetic parameters were determined based on one-compartment and non-compartment models (Gibaldi & Perrier, 1982) with aid of the software WinNonlin™ version 4.1 (Pharsight Corporation, U.S.A.). All pharmacokinetic parameters obtained from one-compartment and non-compartment model analyses in normal cats were compared by the Wilcoxon Signed-Rank test with $p < 0.05$.

3.5.1 One-compartmental analysis

The maximum concentration (C_{max}) was the concentration at T_{max} . The time to maximum concentration (T_{max}) was calculated as follows.

$$T_{\text{max}} = \frac{\ln(k_{\text{ab}}/k_e)}{(k_{\text{ab}} - k_e)}$$

The area under the concentration time-curve from the time of dosing extrapolated to infinity ($\text{AUC}_{0 \rightarrow \infty}$) was calculated by using the linear trapezoidal rule. The absorption rate constant (k_{ab}) was estimated from best-fitted curve of observed data. The elimination rate constant (k_e) was derived from the slope of the linear regression of time-log concentration and calculated as follows.

$$k_e = \frac{-\text{slope}}{2.303}$$

The absorption half-life ($t_{1/2\text{ab}}$) and the terminal half-life ($t_{1/2\text{el}}$) were

calculated as follows.

$$t_{1/2ab} = \frac{0.693}{k_{ab}} \quad ; \quad t_{1/2el} = \frac{0.693}{k_e}$$

The total body clearance (Cl/F) was calculated as follows.

$$Cl/F = \frac{\text{Dose}}{AUC_{0 \rightarrow \infty}}$$

The volume of distribution (V_d/F) was calculated as follows.

$$V_d/F = \frac{\text{Dose}}{k_e \cdot AUC_{0 \rightarrow \infty}}$$

3.5.2 Non-compartmental analysis

The maximum concentration (C_{max}) and the time to maximum concentration (T_{max}) derived were read from the curve of concentration versus time. The area under the concentration time-curve (AUC) and the area under the first moment curve (AUMC) were calculated by using the linear trapezoidal rule. AUC_{last} is the area under the curve from the time of dosing to the last measurable concentration. The area under the curve from the time of dosing extrapolated to infinity ($AUC_{0 \rightarrow \infty}$) was calculated as follows.

$$AUC_{0 \rightarrow \infty} = AUC_{last} + \frac{C_{last}}{\lambda_z}$$

C_{last} is the last concentration corresponding to T_{last} , which is time of last measurable concentration. The first order elimination rate constant (λ_z) associated with the terminal (log-linear) portion of the curve was estimated via linear regression of time-log concentration calculated as follow.

$$\lambda_z = \frac{-\text{slope}}{2.303}$$

The absorption rate constant (k_{ab}) was obtained by using the method of residuals (Gibaldi & Perrier, 1982) as follows.

- 1) Plot curves of log drug concentration versus time
- 2) Back extrapolate the log linear portion of the decline phase. Let C'_1 , C'_2 and C'_3 denotes the serum concentration along this extrapolated line.
- 3) Subtract the observed serum concentration (C_1 , C_2 and C_3) from the

corresponding extrapolate value at each time point.

4) Plot the residuals ($C'_1 - C_1$, $C'_2 - C_2$, and $C'_3 - C_3$) against time on the same semi-logarithmic graph.

The absorption rate constant was obtained from the slope of the straight line fitted to residuals calculated as follows.

$$k_{ab} = \frac{-\text{slope}}{2.303}$$

The absorption half-life ($t_{1/2ab}$) and the terminal half-life ($t_{1/2\lambda_z}$) were calculated as follows.

$$t_{1/2ab} = \frac{0.693}{k_{ab}} \quad ; \quad t_{1/2\lambda_z} = \frac{0.693}{\lambda_z}$$

The total body clearance (Cl/F) was calculated as follows.

$$Cl/F = \frac{\text{Dose}}{AUC_{0 \rightarrow \infty}}$$

The volume of distribution (V_d/F) based on the terminal phase, which was calculated as follows.

$$V_d/F = \frac{\text{Dose}}{\lambda_z \cdot AUC_{0 \rightarrow \infty}}$$

$AUMC_{last}$ is the area under the first moment curve from the time of dosing to the last measurable concentration. The area under the first moment curve extrapolated to infinity ($AUMC_{0 \rightarrow \infty}$) was calculated as follows.

$$AUMC_{0 \rightarrow \infty} = AUMC_{last} + \frac{T_{last} \cdot C_{last}}{\lambda_z} + \frac{C_{last}}{\lambda_z^2}$$

The mean residence time extrapolated to infinity ($MRT_{0 \rightarrow \infty}$) was calculated as follows.

$$MRT_{0 \rightarrow \infty} = \frac{AUMC_{0 \rightarrow \infty}}{AUC_{0 \rightarrow \infty}}$$

All pharmacokinetic parameters were expressed as mean \pm S.E. T_{max} , C_{max} , $AUC_{0 \rightarrow \infty}$ and $AUMC_{0 \rightarrow \infty}$ parameters were presented as geometric mean. $t_{1/2ab}$, $t_{1/2el}$, $t_{1/2\lambda_z}$, k_{ab} , k_e , λ_z , V_d/F , Cl/F , and $MRT_{0 \rightarrow \infty}$ were presented as harmonic mean.