4. RESULTS

4.1 Method validation

4.1.1 Linearity and range

Linear regression analysis of the calibration curves of serum ivermectin concentrations ranging from 0.2-100.0 ng/ml (3 replicates of each concentration) showed good correlation (r = 0.9991) (Figure 7). The linear regression equation of such calibration curves was y = (0.272 ± 0.005) x + (-0.133 ± 0.241), where x is the ivermectin concentration and y is the peak area ratio.

4.1.2 Precision

Intra-day (repeatability) and inter-day (reproducibility) precisions determined from four different serum ivermectin concentrations (10 replicates of each concentration) and expressed as coefficient of variation were less than 5.96% and 7.90%, respectively (Table 2).

4.1.3 Accuracy

The method for determination of ivermectin concentrations ranging from 1.0-40.0 ng/ml (10 replicates of each concentration) was accurate. Percentage of accuracy ranged from 95.33-103.83%. Coefficient of variation values were less than 7.53% for all concentrations (Table 3).

4.1.4 Recovery

Extraction of ivermectin from 0.1, 10.0, and 40.0 ng/ml serum samples (5 replicates of each concentration) was complete. The percentages of recovery were 111.11, 110.28 and 101.85%, respectively (Table 4).

4.1.5 Limit of detection and limit of quantification

The limit of detection and limit of quantification of ivermectin

concentration in serum were 0.17 ng/ml and 0.53 ng/ml, respectively.

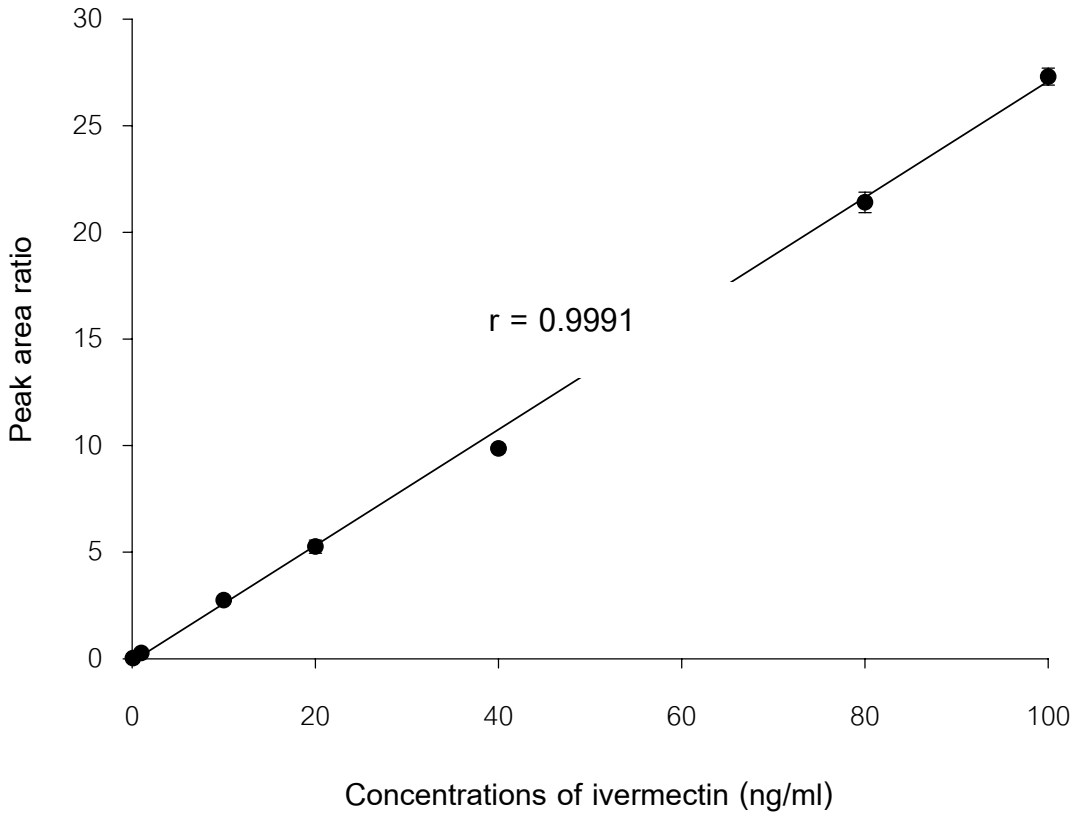

Figure 7 Linearity curve (mean ± S.E.) of different concentrations of ivermectin spiked in serum; correlation coefficient (*r*) = 0.9991

Table 2 Intra-day (repeatability) and inter-day (reproducibility) precisions of the HPLC method for ivermectin determination

Concentration of ivermectin (ng/ml)	Intra-day (repeatability)		Inter-day (reproducibility)	
	Mean peak area ratio ± S.D. (n = 10)	CV (%)	Mean peak area ratio ± S.D. (n = 10)	CV (%)
1	0.319 ± 0.019	5.96	0.779 ± 0.060	7.70
10	3.181 ± 0.159	4.99	3.239 ± 0.256	7.90
20	6.153 ± 0.146	2.37	6.149 ± 0.262	4.26
40	11.798 ± 0.593	5.03	11.385 ± 0.670	5.88

Table 3 Accuracy of the HPLC method for ivermectin determination

Concentration of ivermectin (ng/ml)	Measured concentration (ng/ml) Mean ± S.D. (n = 10)	Accuracy (%)	CV (%)
1	0.957 ± 0.033	95.75	3.43
2.5	2.543 ± 0.161	101.70	6.33
5	5.221 ± 0.393	104.42	7.53
10	10.304 ± 0.542	103.04	5.26
20	20.447 ± 0.497	102.23	2.43
40	39.713 ± 2.026	99.28	5.10

Table 4 Recovery of extraction of ivermectin from serum

Concentration of ivermectin (ng/ml)	Mean peak area ratio ± S.D. (n = 5)		Recovery (%)
	Direct injection	After extraction	
1	0.027 ± 0.002	0.030 ± 0.002	111.11
10	3.045 ± 0.119	3.358 ± 0.239	110.28
40	11.256 ± 0.335	11.464 ± 0.396	101.85

4.2 Chromatography

Since human serum was used for validation method, separation of ivermectin spiked in either human serum, or cat serum was initially compared. Ivermectin (10 ng/ml; 3 replicates) was spiked in human serum and cat serum, and was undergone the same condition of analysis. Ratios of retention time and peak area between ivermectin and abamectin were compared. Results show that those parameters were not statistically different by Mann-Whitney *U*-test with $p<0.05$. In addition, there was apparently no peak interference during separation of ivermectin in either human serum, or cat serum (Figure 8).

Chromatograms of separation of ivermectin spiked in human serum and in cat serum after drug administration are shown in Figure 9. Peaks of ivermectin and internal standard were completely separated from other serum components. The mean retention times of ivermectin and abamectin (10 replicates for each) were 12.5±1.0 min and 7.0±1.0 min, respectively.

Conc. (ng/ml) (n=3)	Retention time ratio		*p*-value*	Peak area ratio		*p*-value*
	Human serum	Cat serum		Human serum	Cat serum	
10	1.73±0.01	1.73±0.01	0.70	3.45±0.22	3.22±0.14	0.40

*Mann-Whitney *U*-test; α=0.05 for each

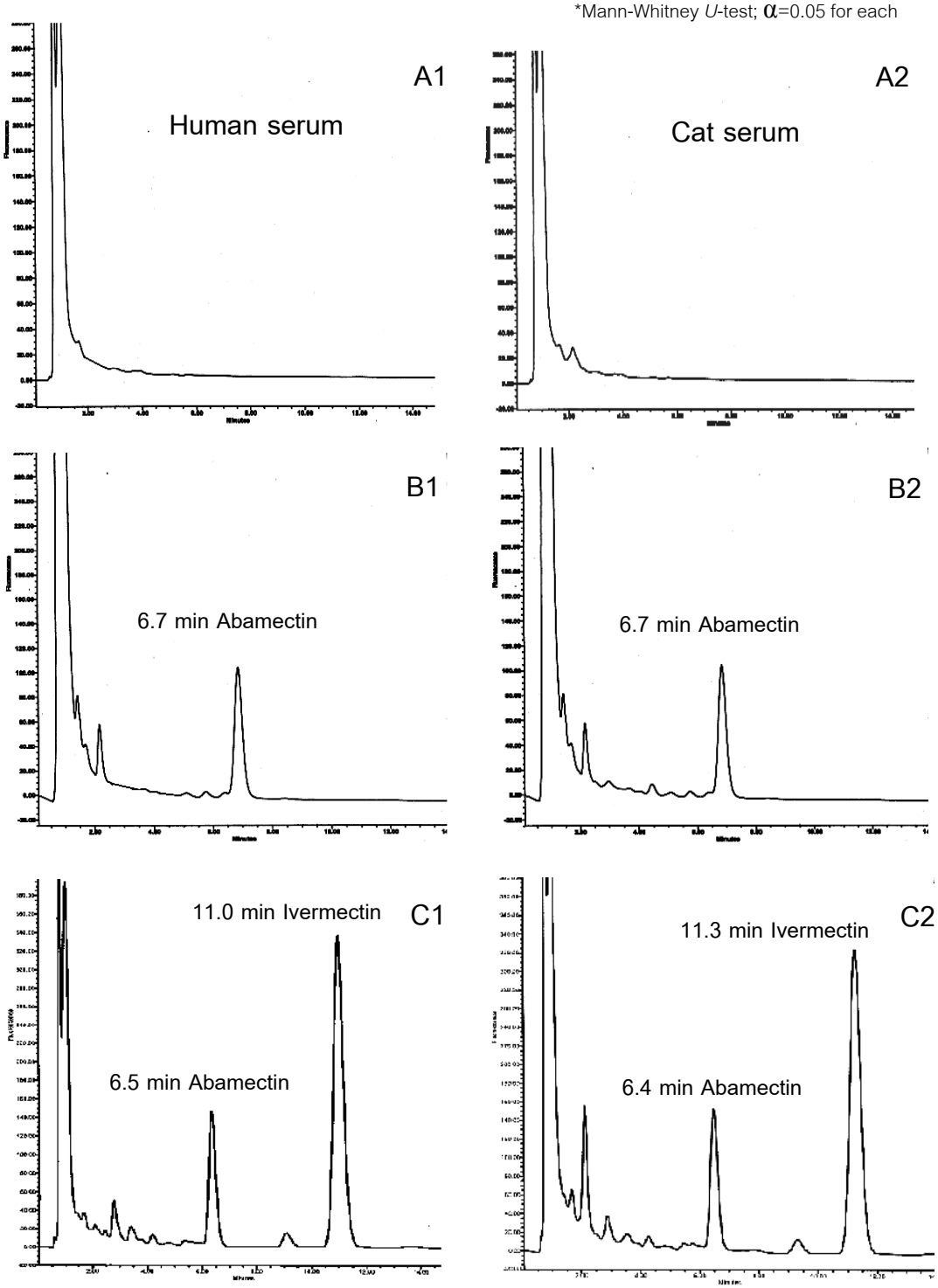

Figure 8 Representative chromatograms for comparison of separation of ivermectin in human serum and cat serum; serum blank (A1, A2), serum spiked with internal standard abamectin (30 ng/ml) (B1, B2), serum spiked with internal standard abamectin (30 ng/ml) and ivermectin (10 ng/ml) (C1, C2)

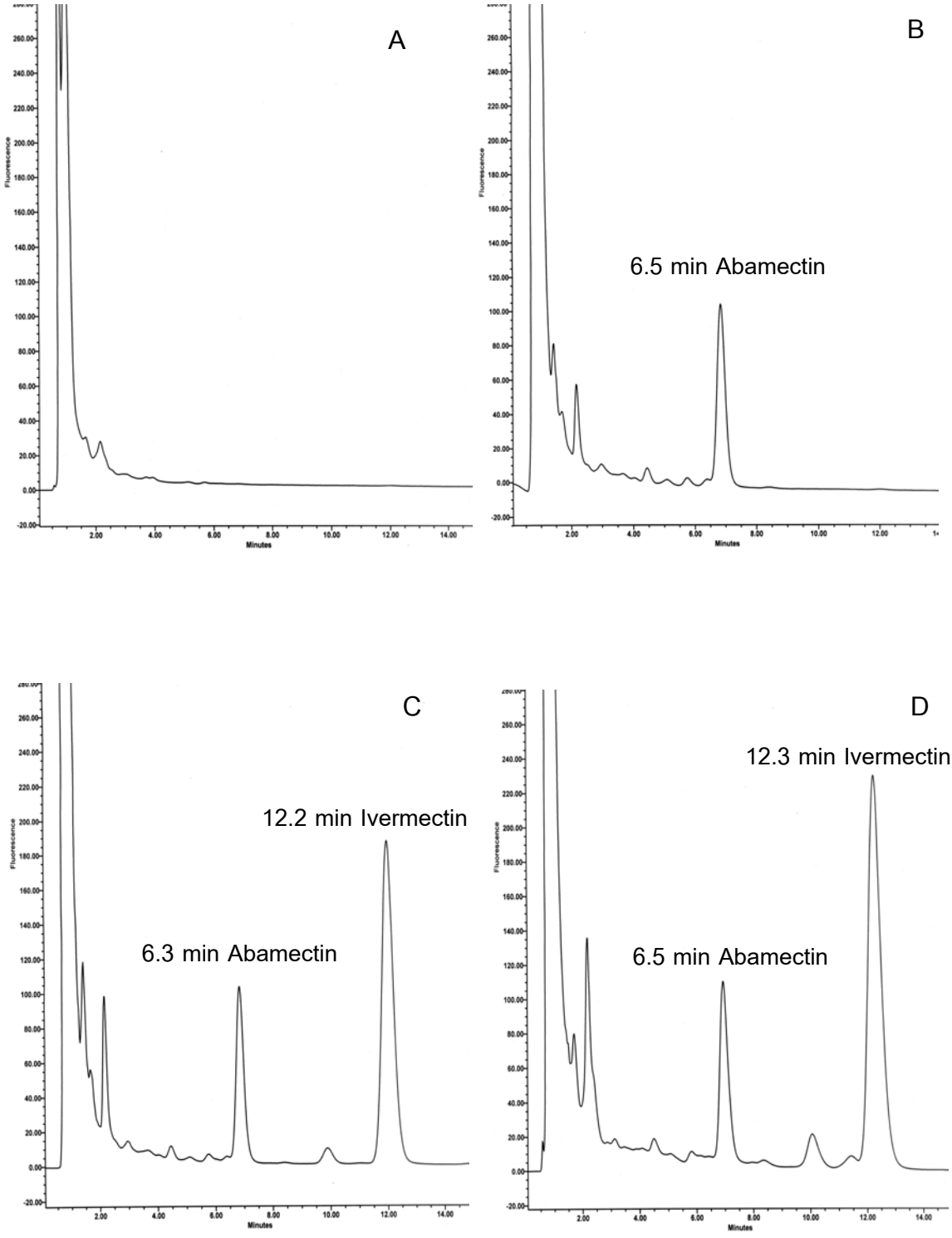

Figure 9 Representative chromatograms of separation of ivermectin; serum blank (A), serum spiked with internal standard abamectin (30 ng/ml) (B), serum spiked with internal standard abamectin (30 ng/ml) and ivermectin (10 ng/ml) in human serum (C), and serum sample collected from a normal cat at 12 hour after ivermectin administration (D)

4.3 Demographic data

All experimental animals were considered to be physically healthy based on essential laboratory tests (blood chemistry and hematology). The parameters were compared with reference values in normal cats (Martin, 1998; Aiello, 1998). The study values of experimental cats selected must be adjacent values or within normal range of normal values (Table 5).

Table 5 Demographic data of normal and infected cats (Normal range modified from Aiello, 1998 and Martin, 1998)

	Normal cats								Infected cat
Code	01	02	03	04	05	06	07	08	09
Sex	M	M	M	M	F	F	F	F	F
Weight (kg)	2.80	3.30	2.70	3.10	2.60	3.15	2.55	2.15	2.90
Blood chemistry (normal range)									
- BUN (15.4-31.2 mg %)	27.00	24.00	23.00	30.00	26.00	21.00	20.00	27.00	19.00
- Cr (1.2-2.1 mg %)	1.40	1.60	1.50	1.80	0.90	1.70	1.00	1.70	1.20
- SGOT (9.2-39.5 U/L)	43.00	35.00	50.00	37.00	53.00	35.00	38.00	55.00	52.00
- SGPT (8.3-52.5 U/L)	49.00	56.00	46.00	56.00	76.00	58.00	41.00	64.00	61.00
- ALP (12.0-65.1 U/L)	98.00	37.00	10.00	49.00	53.00	18.00	18.00	36.00	25.00
Hematology (normal range)									
- Hb (8.0-15.0 g %)	7.30	11.70	11.30	13.30	9.00	10.30	8.70	9.00	N.A.
- Hct (24.0-45.0 %)	22.00	35.00	34.00	40.00	27.00	31.00	26.00	27.00	N.A.
- WBC ($x10^3$cell/mm^3) (5.5-19.5)	14.50	11.40	13.50	19.50	19.50	17.40	11.80	10.00	N.A.
- PMN ($x10^3$cell/mm^3) (3.0-13.0)	9.70	9.80	9.10	14.60	12.10	12.70	6.80	2.80	N.A.
- Lymph ($x10^3$cell/mm^3) (1.2-9.0)	4.80	1.60	4.40	4.50	7.40	4.50	4.90	7.20	N.A.

Note: N.A.; Not available due to clotting of blood sample

M; Male

F; Female

4.4 Study 1: Pharmacokinetics of ivermectin in normal cats

- Concentration-time profile

Serum concentration-time profiles, expressed in arithmetic scale and logarithmic scale, of ivermectin in normal cats receiving a single dose of 200 μg/kg of ivermectin were shown in Figures 10 and 11, respectively. Ivermectin concentrations were detectable in the serum within 1 hour post-dose but declined to undetectable levels after 11 (one cat), 15 (five cats) and 25 (two cats) days post-dose (Figures 10A and 11A). Overall, ivermectin was seen in serum until day 25. The mean serum concentrations of ivermectin were shown in Figures 10B and 11B.

- Pharmacokinetic analyses

a) Compartment model

Results of pharmacokinetic of ivermectin analyzed using 1-compartment model are shown in Table 6. The mean ± S.E. value of the parameters are as follows: T_{max}, 1.13 ± 0.18 day; C_{max}, 16.17 ± 1.43 ng/ml; k_{ab}, 1.72 ± 0.66 day^{-1}; $t_{1/2\ ab}$, 0.27 ± 0.09 day; k_e, 0.20 ± 0.05 day^{-1}; $t_{1/2\ el}$, 2.53 ± 0.79 days; V_d/F, 8.18 ± 1.82 L/kg; Cl/F, 5.68 ± 0.66 L/day; and $AUC_{0\rightarrow\infty}$, 94.23 ± 10.79 ng.day/ml, respectively.

b) Non-compartment model

Results of pharmacokinetic of ivermectin analyzed using non-compartment model are shown in Table 7. The mean ± S.E. value of T_{max}, 1.09 ± 0.23 day; C_{max}, 17.53 ± 1.68 ng/ml; k_{ab}, 1.16 ± 0.13 day^{-1}; $t_{1/2\ ab}$, 0.55 ± 0.06 days; λ_z, 0.24 ± 0.04 day^{-1}; $t_{1/2\ \lambda_z}$, 2.30 ± 0.60 days; V_z/F, 7.23 ± 1.64 L/kg; Cl/F, 5.68 ± 0.60 L/day; $AUC_{0\rightarrow\infty}$, 94.84 ± 12.75 ng.day/ml; $AUMC_{0\rightarrow\infty}$, 466.95 ± 124.66 $ng.day^2/ml$; and $MRT_{0\rightarrow\infty}$, 4.58 ± 0.90 days, respectively.

The mean of C_{max} and k_{ab} values analyzed using 1-compartment model were significantly different compared with non-compartment model (16.17 ±

1.43 v.s. 17.53 ± 1.68 ng/ml and 1.72 ± 0.66 v.s. 1.16 ± 0.13 day^{-1}; $p<0.05$).

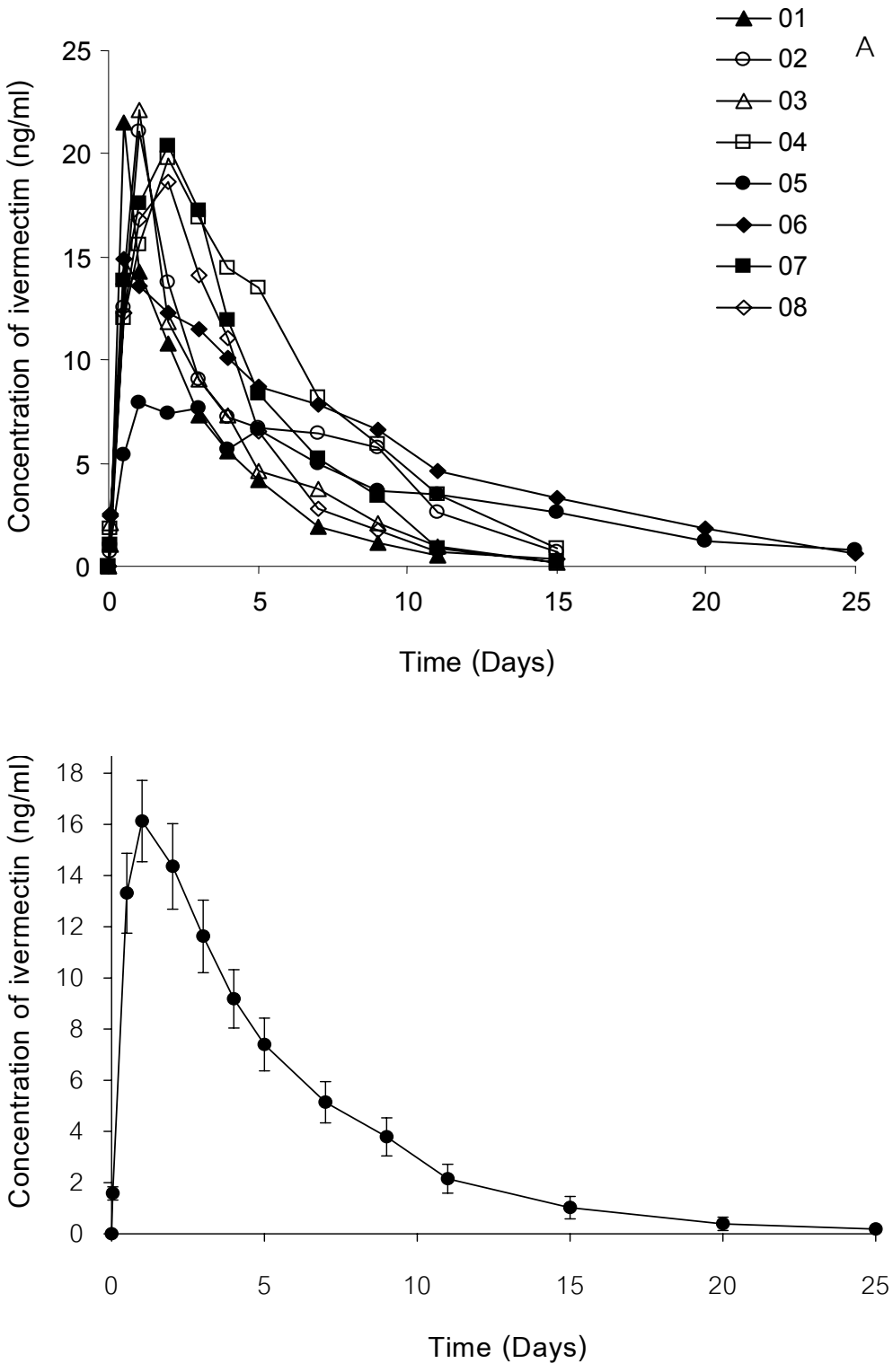

Figure 10 Concentration-time profiles of ivermectin after a single dose of 200 μg/kg of ivermectin in normal cats; individual animal serum concentrations (A) and mean (± S.E.) (n = 8) serum concentrations (B)

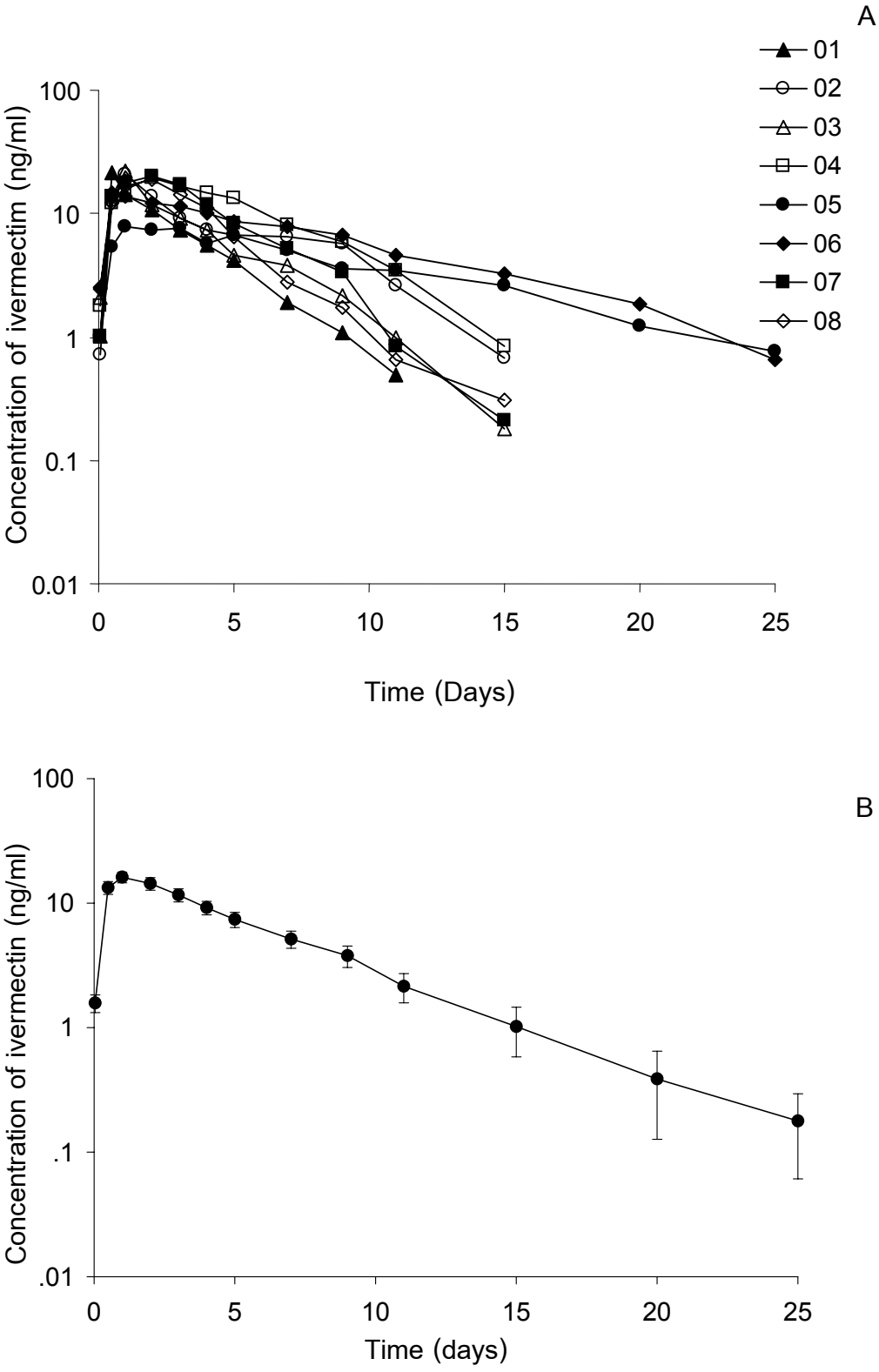

Figure 11 Logarithmic concentration-time profiles of ivermectin after a single dose of 200 μg/kg of ivermectin in normal cats; individual animal serum concentrations (A) and mean (± S.E.) (n = 8) serum concentrations (B)

Table 6 Pharmacokinetic parameters derived from one-compartment model analysis of ivermectin in eight normal cats receiving a single dose of 200 μg/kg of ivermectin by subcutaneous injection

Subject No. (Weight; kg)	T_{max} (day)	C_{max} (ng/ml)	k_{ab} (day^{-1})	$t_{1/2\ ab}$ (day)	k_e (day^{-1})	$t_{1/2\ el}$ (day)	V_d/F (L/kg)	Cl/F (L/day)	$AUC_{0\rightarrow\infty}$ (ng.day/ml)
01 (2.80)	0.55	18.60	5.05	0.14	0.40	1.73	8.65	9.67	57.88
02 (3.30)	1.00	16.61	2.69	0.26	0.22	3.09	9.61	7.10	92.90
03 (2.70)	0.93	18.28	2.37	0.29	0.37	1.87	7.76	7.75	69.71
04 (3.10)	1.89	19.17	1.09	0.63	0.20	3.41	7.10	4.47	138.71
05 (2.60)	1.57	7.88	2.04	0.34	0.09	7.23	21.84	5.44	95.61
06 (3.15)	0.70	14.26	5.81	0.12	0.11	6.53	13.01	4.35	144.77
07 (2.55)	1.59	20.38	1.05	0.66	0.34	2.05	5.72	4.94	103.23
08 (2.15)	1.54	18.83	0.89	0.78	0.45	1.53	5.28	5.14	83.66
Arithmetic mean	1.22	16.75	2.62	0.40	0.27	3.43	9.77	6.11	98.31
Harmonic mean	-	-	1.72*	0.27	0.20	2.53	8.18	5.68	-
Geometric mean	1.13	16.17*	-	-	-	-	-	-	94.23
S.E.	0.18	1.43	0.66	0.09	0.05	0.79	1.82	0.66	10.79

*$p<0.05$ significantly different compared with non-compartment model analysis (Wilcoxon Signed-Rank test)

Table 7 Pharmacokinetic parameters derived from non-compartment model analysis of ivermectin in eight normal cats receiving a single dose of 200 μg/kg of ivermectin by subcutaneous injection

Subject No. (Weight; kg)	T_{max} (day)	C_{max} (ng/ml	k_{ab} (day^{-1})	$t_{1/2\,ab}$ (day)	λ_z (day^{-1})	$t_{1/2\,\lambda z}$ (day)	V_z/F (L/kg)	Cl/F (L/day)	$AUC_{0\rightarrow\infty}$ (ng.day/ml)	$AUMC_{0\rightarrow\infty}$ ($ng.day^2/ml$)	$MRT_{0\rightarrow\infty}$ (day)
01 (2.80)	0.50	21.52	1.73	0.40	0.35	1.99	9.71	9.46	59.17	184.74	3.12
02 (3.30)	1.00	21.08	1.15	0.60	0.29	2.33	6.86	6.72	98.23	498.67	5.08
03 (2.70)	1.00	22.15	1.36	0.51	0.41	1.67	6.46	7.22	74.74	272.44	3.64
04 (3.10)	2.00	19.75	0.80	0.87	0.33	2.11	4.61	4.69	132.23	672.71	5.09
05 (2.60)	1.00	7.92	0.98	0.71	0.11	6.15	18.94	5.55	93.73	943.64	10.07
06 (3.15)	0.50	14.90	1.67	0.41	0.14	4.99	10.36	4.53	139.08	1187.42	8.54
07 (2.55)	2.00	20.40	0.87	0.80	0.42	1.65	4.64	4.96	102.78	395.54	3.85
08 (2.15)	2.00	18.65	1.48	0.47	0.28	2.45	8.28	5.03	85.43	308.25	3.61
Arithmetic mean	1.25	18.30	1.26	0.60	0.29	2.92	8.73	6.02	98.17	557.93	5.36
Harmonic mean	-	-	1.16*	0.55	0.24	2.30	7.23	5.68	-	-	4.58
Geometric mean	1.09	17.53*	-	-	-	-	-	-	94.84	468.14	-
S.E.	0.23	1.68	0.13	0.06	0.04	0.60	1.64	0.60	12.75	124.66	0.90

* $p<0.05$ significantly different compared with one-compartment model analysis (Wilcoxon Signed-Rank test)

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

- Concentration-time profile

Serum concentration-time profiles, expressed in arithmetic scale and logarithmic scale, of ivermectin in an infected cat receiving a single dose of 200 µg/kg of ivermectin were shown in Figures 12A and 12B. Ivermectin concentration was detectable in the serum within 1 hour post-dose and fell to undetectable levels after 25 days post-dose.

- Pharmacokinetic analyses

a) Compartment model

Results of pharmacokinetic of ivermectin analyzed using 1-compartment model are shown in Table 8. The measured value of the parameters are as follows: T_{max}, 1.57 day; C_{max}, 14.64 ng/ml; k_{ab}, 1.74 day^{-1}; $t_{1/2\ ab}$, 0.40 day; k_e, 0.14 day^{-1}; $t_{1/2\ el}$, 4.88 days; V_d/F, 10.94 L/kg; Cl/F, 4.50 L/day; and $AUC_{0\rightarrow\infty}$, 128.72 ng.day/ml, respectively.

b) Non-compartment model

Pharmacokinetics of ivermectin analyzed using non-compartment model resulted that, the measured value of T_{max}, 1.50 day; C_{max}, 17.14 ng/ml; k_{ab}, 0.85 day^{-1}; $t_{1/2\ ab}$, 0.81 day, λ_z, 0.16 day^{-1}; $t_{1/2\ \lambda z}$, 4.28 days; V_z/F, 9.36 L/kg; Cl/F, 4.39 L/day; $AUC_{0\rightarrow\infty}$, 132.11 ng.day/ml; $AUMC_{0\rightarrow\infty}$, 1013.55 $ng.day^2/ml$; and $MRT_{0\rightarrow\infty}$ 7.67 days, respectively (Table 9).

- Microfilarial density

The microfilariae appeared in the peripheral blood at all times during the 24-hour period. The results show that the microfilariae were nocturnally subperiodic *Brugia malayi*. The parasite counts peaked at 02.00 hour (Figure 13). After a subcutaneous injection of ivermectin, the maximum concentration was 17.14 ng/ml at 1.50 day; with approximately 50% decrease of

microfilariae. The decrease was maximum (65% of microfilariae) at day 4. After that, the ivermectin concentration fell to undetectable level after 25 days post-dose. The reduction of microfilariae was maintained further until day 25 (Figure 14).

4.6 Adverse drug reactions

Eight normal cats and an infected cat were enrolled and completed in this study. No side effect was observed after drug administration.

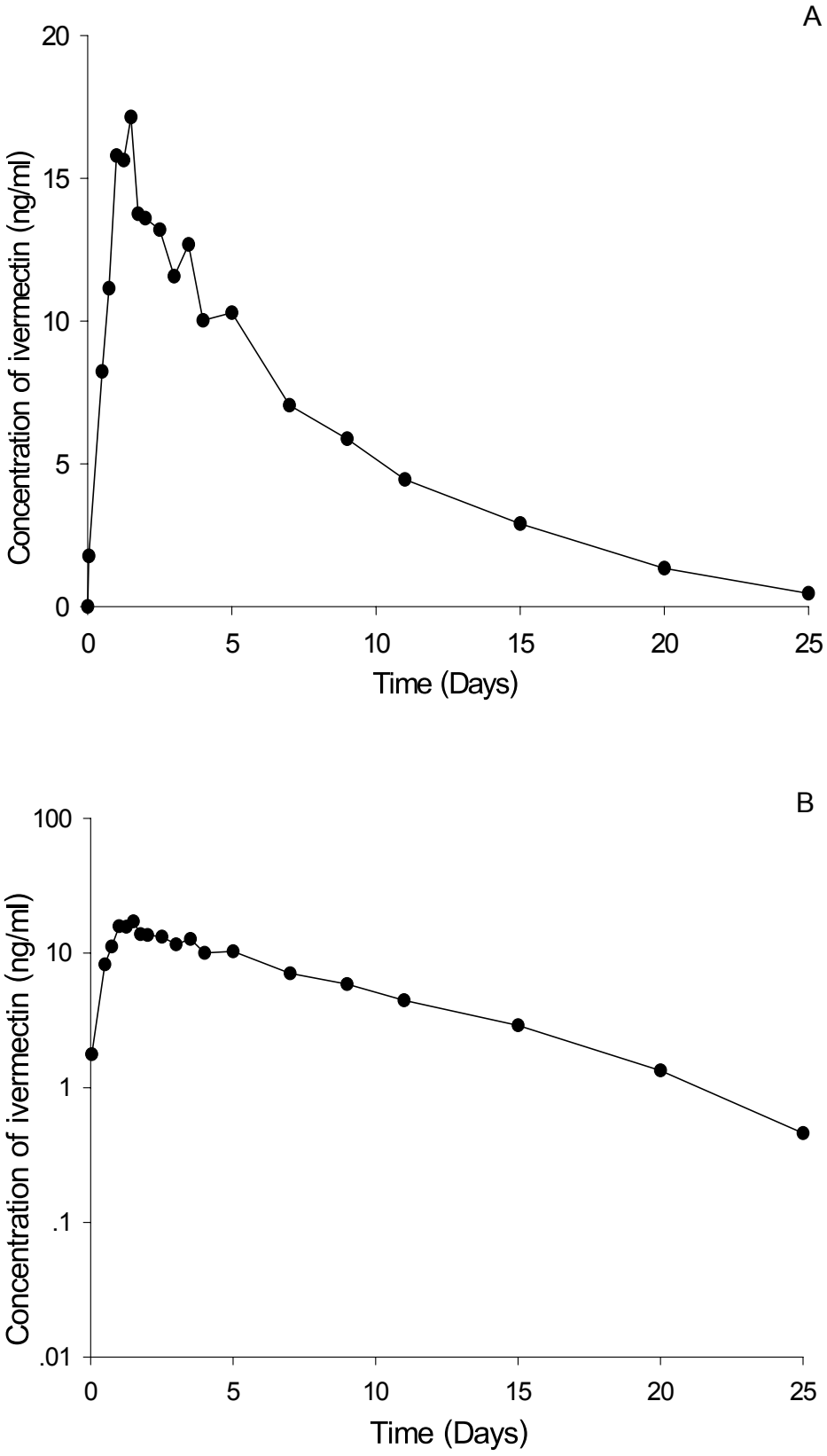

Figure 12 Concentration-time profiles of ivermectin after a single dose of 200 μg/kg of ivermectin in a *Brugia malayi*-naturally infected cat; an arithmetic plot (A) and a logarithmic plot (B)

Table 8 Pharmacokinetic parameters derived from one-compartment model analysis of ivermectin in an infected cat receiving a single dose of 200 μg/kg of ivermectin by subcutaneous injection

Parameter	Measured value
T_{max} (day)	1.57
C_{max} (ng/ml)	14.64
k_{ab} (day^{-1})	1.74
$t_{1/2\ ab}$ (day)	0.40
k_e (day^{-1})	0.14
$t_{1/2\ el}$ (day)	4.88
V_d/F (L/kg)	10.94
Cl/F (L/day)	4.50
$AUC_{0\rightarrow\infty}$ (ng.day/ml)	128.72

Table 9 Pharmacokinetic parameters derived from non-compartment model analysis of ivermectin in an infected cat receiving a single dose of 200 μg/kg of ivermectin by subcutaneous injection

Parameter	Measured value
T_{max} (day)	1.50
C_{max} (ng/ml)	17.14
k_{ab} (day^{-1})	0.85
$t_{1/2\ ab}$ (day)	0.81
λ_z (day^{-1})	0.16
$t_{1/2\ \lambda z}$ (day)	4.28
V_z/F (L/kg)	9.36
Cl/F (L/day)	4.39
$AUC_{0\rightarrow\infty}$ (ng.day/ml)	132.11
$AUMC_{0\rightarrow\infty}$ ($ng.day^2$/ml)	1013.55
$MRT_{0\rightarrow\infty}$ (day)	7.67

Hour	06:00	10:00	14:00	18:00	22:00	02:00	06:00
No. of mf /50 μl	144	119	164	333	398	635	144
No. of mf (%)	22.60	18.70	25.80	52.40	62.60	100.00	22.60

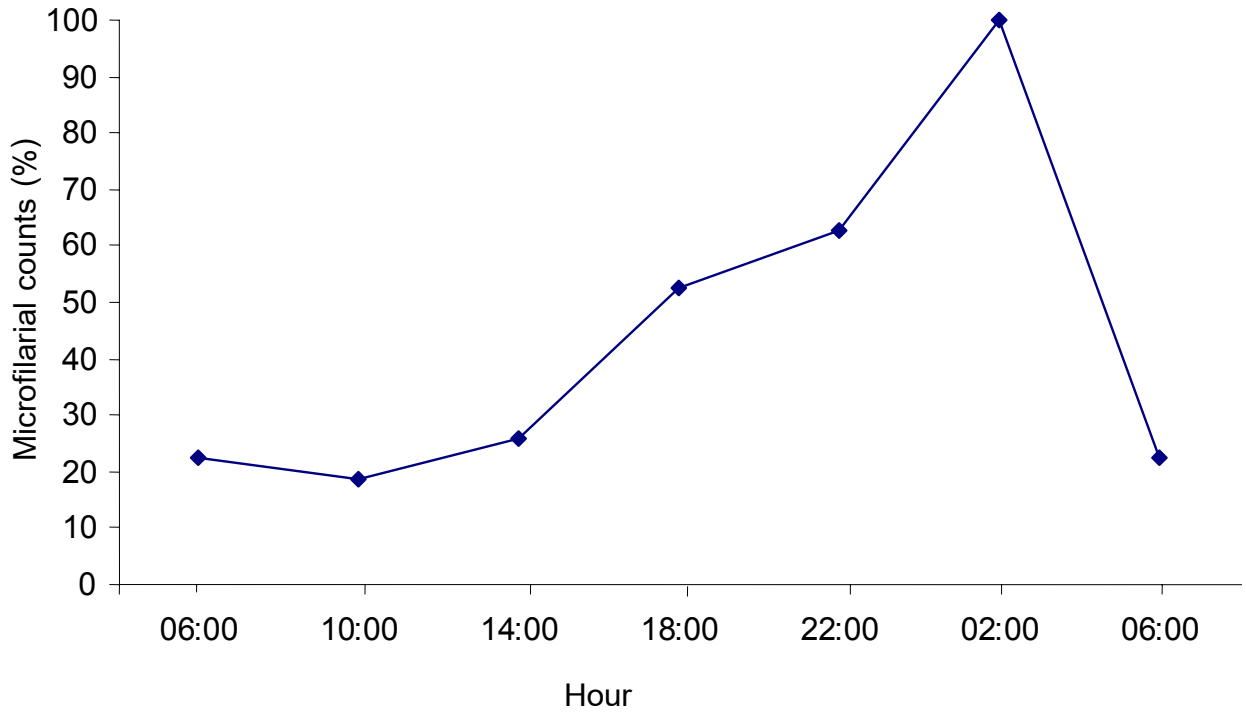

Figure 13 Periodicity of *Brugia malayi* microfilariae of the infected cat

Days	0	1	2	3	4	5	7	9	11	15	20	25
No. of mf /50 μl	560	348	250	281	196	224	181	228	175	184	219	195
No. of mf (%)	100.00	62.14	44.64	50.18	35.00	40.00	32.32	40.71	31.25	32.86	39.11	34.82
Concentrations (ng/ml)	0.00	15.79	13.60	11.56	10.02	10.29	7.05	5.86	4.45	2.90	1.39	0.46

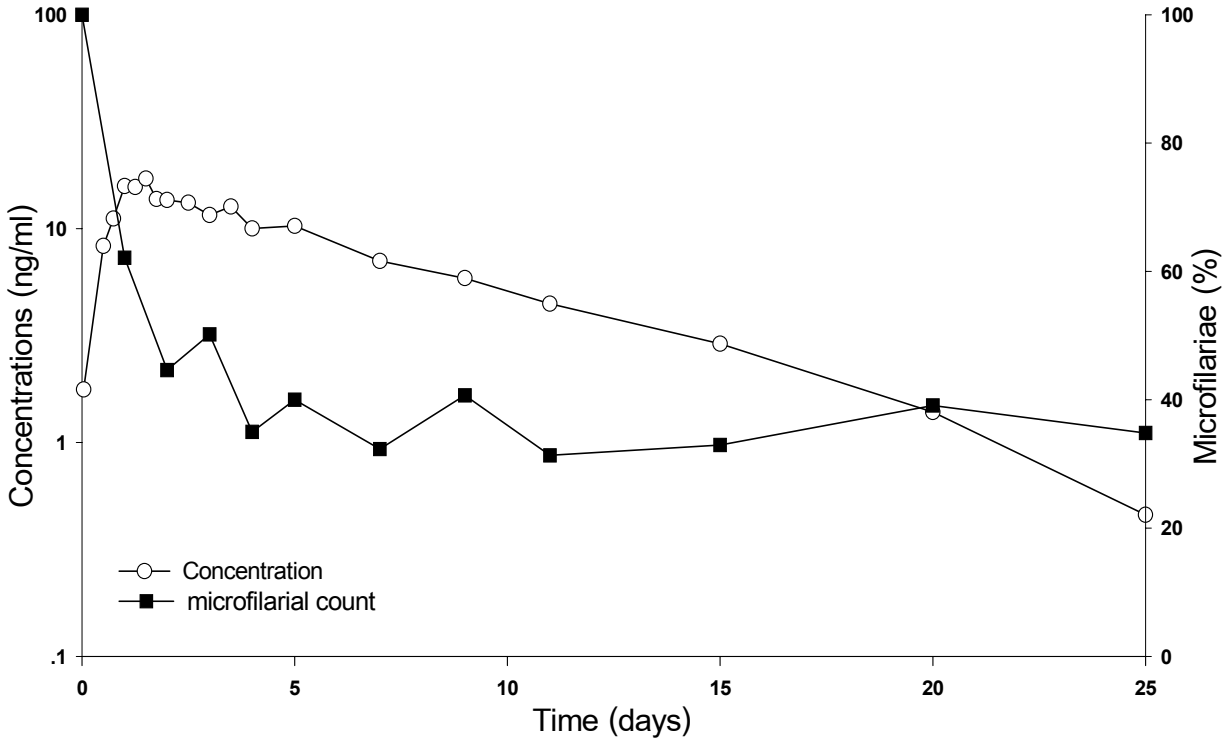

Figure 14 Number of blood microfilariae and serum concentrations in a *Brugia malayi*-infected cat receiving a single dose of 200 μg/kg of ivermectin by subcutaneous injection