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APPENDICES

APPENDIX A

TEST PRODUCTS

Table 33 Test products

Brand name	Manufacturer	Mfg. date
A (Zocor®)	Merck Sharp and Dohm	- (Exp.Jun 00)
B	Locally-made product B	22/05/98
C	Locally-made product C	13/08/96
D	Locally-made product D	07/04/98

Original product = A

APPENDIX B

STANDARD CURVE DETERMINATION

The typical standard curve data for simvastatin in 45% acetonitrile in water, dissolution medium, and total simvastatin hydroxy acid in dog plasma are presented in Table 34 to 36 and Figure 21 to 23 , respectively.

Table 34 Standard curve of simvastatin in 45% acetonitrile in water.

Standard No.	Nominal conc. (mg/ml)	PAR	Inversely estimated conc.[2]	% Analytical recovery[3]
1	1.96	0.393	1.83	93.33
2	3.92	0.862	4.00	102.15
3	5.88	1.286	5.97	101.57
4	7.84	1.687	7.83	99.92
5	9.80	2.121	9.85	100.50
6	11.76	2.513	11.67	99.23
			Mean	99.45
			SD	3.18
			% C.V.[4]	3.20

1. $r^2 = 0.999$, $Y = 0.216X + 0.001$ (Y = PAR, X = nominal conc.)

2. Inversely estimated concentration $= \frac{(PAR - 0.001)}{0.216}$

3. % Analytical recovery $= \frac{\text{Inversely estimated concentration}}{\text{Known nominal concentration}} \times 100$

4. % C.V. $= \frac{SD}{Mean} \times 100$

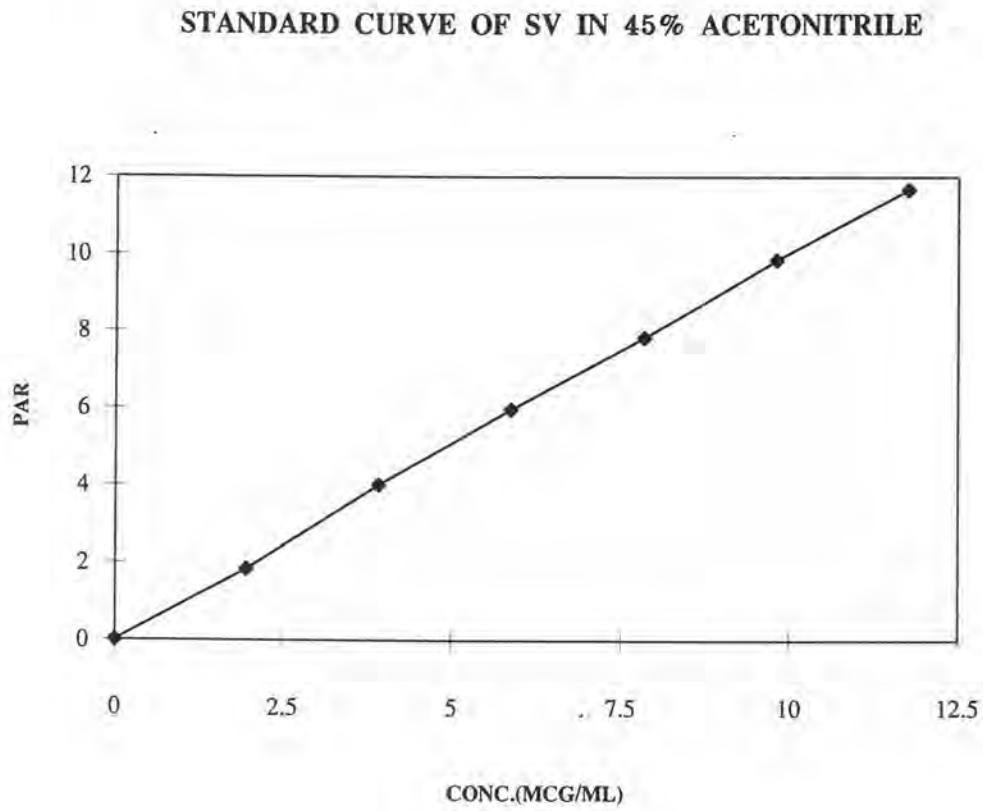

Figure 21 Standard curve of simvastatin in 45% acetonitrile.

Table 35 Standard curve of simvastatin in dissolution medium.

Standard No.	Nominal conc.(mg/ml)	PAR	Inversely estimated conc.[2]	% Analytical recovery[3]
1	2.05	0.995	2.16	105.33
2	4.11	1.760	3.96	96.41
3	6.16	2.750	6.29	102.01
4	8.22	3.474	7.99	97.21
5	10.27	4.504	10.40	101.32
6	12.32	5.326	12.34	100.10
			Mean	100.40
			SD	3.28
			% C.V.[4]	3.27

1. $r^2 = 0.998$, $Y = 0.426X + 0.074$ (Y = PAR, X = nominal conc.)

2. Inversely estimated concentration $= \frac{(PAR - 0.074)}{0.426}$

3. % Analytical recovery $= \frac{\text{Inversely estimated concentration}}{\text{Known nominal concentration}} \times 100$

4. % C.V. $= \frac{SD}{Mean} \times 100$

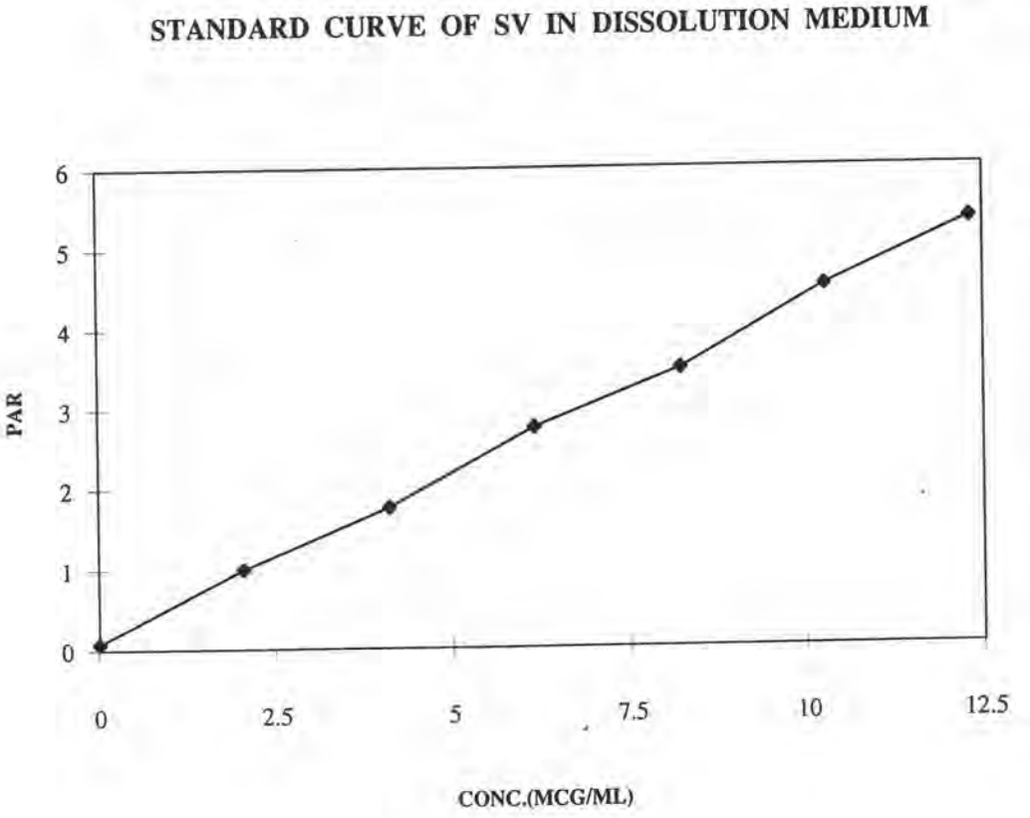

Figure 22 Standard curve of simvastatin in dissolution medium.

Table 36 Standard curve of total simvastatin hydroxy acid in dog plasma.

Standard No.	Nominal conc. (ng/ml)	PAR	Inversely estimated conc.[2]	% Analytical recovery[3]
1	25.77	0.210	27.80	107.88
2	51.55	0.298	55.27	107.22
3	103.10	0.472	109.81	106.51
4	257.74	0.876	236.61	91.80
5	515.48	1.794	524.35	101.72
			Mean	103.02
			SD	6.72
			% C.V.[4]	6.53

1. $r^2 = 0.996$, $Y = 3.19 \times 10^{-3} X + 0.125$ (Y = PAR, X = nominal conc.)

2. Inversely estimated concentration $= \frac{(PAR - 0.125)}{3.19 \times 10^{-3}}$

3. % Analytical recovery $= \frac{\text{Inversely estimated concentration}}{\text{Known nominal concentration}} \times 100$

4. % C.V. $= \frac{SD}{Mean} \times 100$

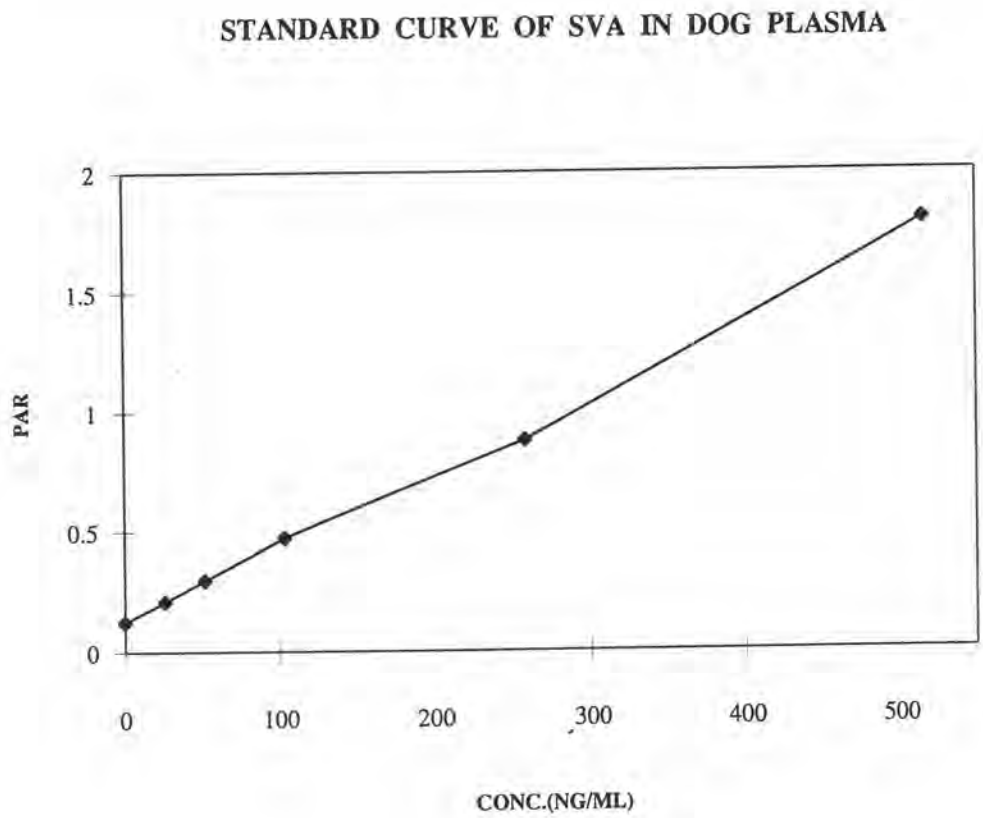

Figure 23 Standard curve of total simvastatin hydroxy acid in dog plasma.

APPENDIX C

REAGENT PREPARATION

0.5% Sodium lauryl sulphate 0.01 M Monobasic sodium phosphate buffer pH 5.5

Dissolved 7.2 g of monobasic sodium phosphate in 6 L of water, mix. Adjust the resulting solution with 1.0 N sodium hydroxide to a pH of 5.5 ± 0.02. Dissolved 30.0 g of sodium lauryl sulphate in above buffer.

0.025 M Monobasic sodium phosphate buffer pH 4.5

Dissolved 3.0 g of monobasic sodium phosphate in 1000 ml of water, mix. Adjust the resulting solution with 0.1 N hydrochloric acid to a pH of 4.5 ± 0.02.

0.5 M Potassium hydroxide solution

Dissolved 28.1 g of potassium hydroxide in 500 ml of water, mix, and diluted with water to 1000 ml.

0.25 M Phosphoric solution

Diluted 85% phosphoric acid 28.8 g with water to 1000 ml.

APPENDIX D

ANIMALS

Table 37 Animal demographic data.

Dog No.	Weight (kg)	SGPT Pre -treatment[a]	SGPT Post-treatment[a]	δ^b (Post-pre)	Diseases
1	12.2	31	152	+121	-
2	15.0	31	17	-14	-
3	13.5	40	64	+20	Microfilaria⊕ve
4	11.6	14	23	+9	-
5	14.0	57	26	-31	Microfilaria⊕ve
6	16.4	49	29	-20	Microfilaria⊕ve
7	17.0	45	75	+30	-
8	12.0	28	40	+12	-
9	13.0	29	41	+12	Microfilaria⊕ve
10	11.8	7	19	+12	-
11	11.3	7	30	+23	-
12	13.0	65	135	+70	Microfilaria⊕ve
Mean	13.4	33.58	54.25	-20.33	-
SD	1.88	18.0	45.34	40.99	-

+ = increase from the initial value

- = decrease from the initial value

[a] The normal value is 30-40 i.u. (Benjamin, 1966).

δ^b The difference of pre- and post-treatment SGPT value was no statistically significance. The paired t-test results are provided in Appendix E.

APPENDIX E

STATISTICS

1. Mean (X)

$$X = \frac{\sum X}{N}$$

2. Standard deviation (S.D.)

$$SD = \frac{\sum (X - X)^2}{\sqrt{N - 1}}$$

3. Standard error of mean (SEM)

$$SEM = \frac{SD}{\sqrt{N}}$$

4. Bartlett's test

Test of eqaulity of more than two variances

$$\chi^2 = (\Sigma N_i - 1)\ln S^2 - \Sigma[(N_i - 1)\ln S_i^2]$$

where S^2 = Pooled variance

S_i^2 = The variance of the ith sample

The value of $\chi_{0.05}^2$ is refer to a χ^2 distribution with (N-1) d.f. If χ^2 calculated less than $\chi_{0.05}^2$, the variance do not differ. A significant value of χ^2 means that the variances are not equal.

5. Dunnett's Test

Sometimes experiments are designed to compare several treatments against a control not among each other. Dunnett's devised a multiple comparison procedure for treatments versus a control. The critical difference for 2-sided test for any of the comparisons versus control, D, is defined as:

$$D' = t' \sqrt{S^2(1/N_1+1/N_2)}$$

where t' is obtained from Table t value for Dunnett's comparison

S^2 = Error variance from ANOVA (within mean square for one-way ANOVA

N_1, N_2 = number of sample

The difference between the test mean and control mean is considered significant if its absolute value is greater than D'.

6. Randomized block analysis of variance

Testing the difference among treatments

Block	Treatment 1	2	3..... j	Total (block)	Mean(block)
1	X_{11}	X_{12}	X_{13} ..... X_{1j}	R_1	X_1
2	X_{21}	X_{22}	X_{23}.....X_{2j}	R_2	X_2
.	.	.	. ...	.	.
i	X_{i1}	X_{i2}	X_{i3} .....X_{ij}	R_i	X_i
Total (treat)	C_1	C_2	C_3 .....C_j	T	
Mean (treat)	X_1	X_2	X_3 ..... X_j		X

where T = Total of all observations

X = Overall mean

j = Number of treatments

i = Number of blocks

$\mu_1, \mu_2, \mu_3, \ldots\ldots, \mu_i$ = Population mean of block

$\mu_1, \mu_2, \mu_3, \ldots\ldots, \mu_j$ = Population mean of treatment

For block The null hypothesis :Ho = $\mu_1 = \mu_2 = \mu_3 = \ldots\ldots \mu_i$

The alternative hypothesis :Ha = $\mu_1 \neq \mu_2 \neq \mu_3 \neq \ldots\ldots \mu_i$

For treatment The null hypothesis :Ho = $\mu_1 = \mu_2 = \mu_3 = \ldots\ldots \mu_j$

The alternative hypothesis :Ha = $\mu_1 \neq \mu_2 \neq \mu_3 \neq \ldots\ldots \mu_j$

Randomized block analysis of variance

Sources of variation	Degree of freedom	Sum of square	Mean square	F
Block	i -1	SS_{block}	MS_{block}	MS_{bl}/MS_{err}
Treatment	j -1	$SS_{treatment}$	$MS_{treatment}$	MS_{tr}/MS_{err}
Error	(i-1)(j-1)	SS_{error}	MS_{error}	
Total	ij -1	SS_{total}		

Computation of sum of square

$C.T. = (\sum_{ij} X_{ij})^2/N$

$SS_{total} = \sum X_{ij}^2 - C.T.$

$SS_{block} = \sum R_i^2/j - C.T.$

$Ss_{treatment} = \sum C_j^2/i - C.T.$

$SS_{error} = SS_{total} - SS_{block} - SS_{treatment}$

$MS_{block} = SS_{block} / d.f._{block}$

$MS_{treatment} = SS_{treatment} / d.f._{treatment}$

$MS_{error} = SS_{error} / d.f._{error}$

$F_{block} = MS_{block} / MS_{error}$

$F_{treatment} = MS_{treatment} / MS_{error}$

F_{block} and $F_{treatment}$ have (i-1), {(i-1)(j-1)} and (j-1),{(i-1)(j-1)}degree of freedom, respectively.

If calculated F value is less than tabulated $F_{0.05}$, the null hypothesis is accepted and the alternative hypothesis is rejected. If F value is greater than $F_{0.05}$, the alternative hypothesis stand which shows that there are significant differences among the mean ($p<0.05$).

7. Two one-sided t-tests and 90 % confidence interval procedure for bioequivalence comparison

Schuirman (1987) proposed a procedure which consist of two pairs of testing hypotheses. The tests are performed each at the 5% level with the null hypothesis.

$$\text{Ho}: \frac{T}{R} < 0.8 \qquad \text{and} \qquad \text{Ho}: \frac{T}{R} > 1.20$$

where T, R represent the test product and reference product, respectively.(R = brand A, T = brand B, C, and D in this study)

If both tests are rejected, the products are considered to have a ratio of the pharmacokinetic parameters between 0.8 and 1.20 and are taken to be bioequivalent. The out come of the test is equivalent to forming a 90 % symmetric confidence interval about the mean difference, and accepting equivalent if the limits of the intervals lie between 0.8 and 1.20. If either test (or both) is not rejected, the products are not considered to be equivalent.

Since the two one-sided t-tests are equivalent to the construction of 90 % confidence interval. The latter approach can be used interchangeably for bioequivalence evaluation.

In the example from table 22, a 90% confidence interval for the difference of the t_{max} for brand B versus brand A is

$$= \bar{\Delta} \pm t\sqrt{EMS\,(1/N_1+1/N_2)}$$
$$= (2.25-1.875) \pm 1.69\sqrt{0.428(1/12+1/12)}$$
$$= 0.375 \pm 0.451$$
$$= -0.076 \text{ to } 0.826$$

where $\bar{\Delta}$ = the average difference of the t_{max} (T-R)

t = the t value with 33 d.f. = 1.69, $\alpha = 0.10$

EMS = Error mean square from ANOVA table

N_1, N_2 = Total number of treatment T and R

The confidence interval can be expressed as an approximate percentage relative bioavailibility by dividing the lower and the upper limits for the t_{max} difference by the average t_{max} for brand A, the reference product and then add 1 to each of the resulting values.

Average t_{max} for the reference product = 1.875

approximate 90 % confidence interval for $\frac{T}{R}$

= (1.875-0.076)/1.875 to (1.875+0.826) /1.875

= 0.9595 to 1.4405

= 95.95 to 144.05 %

For the example of the t_{max} ratio, the test product would not pass the USP equivalency test because the upper limit exceeds 1.20.

If the variance among the treatments are not homogenous due to the Bartlett's test result. Log-transformed data were used in the analysis of variance instead of normal data and 90 % confidence interval for the ratios of C_{max}, t_{max}, and AUC can be determined according to the following procedure. Since the difference of the logarithms is equivalent to logarithmic of the ratio [i.e., log T-log R = log (T/R)], thus the anti-log of the average difference of the logarithms is an estimate of the ratio of that parameter.

From Table 25, 90% confidence interval for the difference log-transformed value for AUC for brand B versus brand A is

$$= (2.629\text{-}2.624) \pm 1.69\sqrt{1.54\text{x}10^{-2}(1/12+1/12)}$$

$$= 0.005 \pm 0.086$$

$$= \text{-}0.081 \text{ to } 0.090$$

The anti-log of these limits are 0.83 to 1.23 that pass the USP equivalency test since the resultant interval contains within 80-125% bioequivalence range for the log-transformed data.

8. Correlation coefficient test (Bolton, 1990)

The correlation coefficient is a quantitative measure of the relationship of correlation between two variables X and Y.

$$r = \frac{N\Sigma XY - \Sigma X\Sigma Y}{\sqrt{[N\Sigma X^2-(\Sigma X)^2][N\Sigma Y^2-(\Sigma Y)^2]}}$$

where r = Correlation coefficient

N = The number of X and Y pairs.

Test of zero correlation

Let ρ (rho) = The true correlation coefficient, estimated by r

The null hypothesis : $Ho = \rho = 0$

The alternative hypothesis : $Ha = \rho \neq 0$

$$t_{N-2} = \frac{|r\sqrt{N-2}|}{\sqrt{1-r^2}}$$

The value of $t_{0.05}$ is referred to a t distribution with (N-2) d.f. If t calculated is greater than $t_{0.05}$, the null hypothesis is rejected. If t is not significant, the null hypothesis stands.

9. Paired student's t-test (dependent sample)

In the paired-sample experiment, the treatments are applied to experimental units which are closely related.

The null hypothesis Ho : $\Delta = 0$

The alternative hypothesis Ha : $\Delta \neq 0$

Δ = hypothetical difference of the means of the paired samples

the t-test is: $$t = \frac{|\bar{\delta} - \Delta|}{S/\sqrt{N}}$$

where δ = means difference of the paired-samples

S = standard deviation of δ

N = pairs of samples

The value of $t_{0.05}$ is referred to a t-distribution with (N-1) d.f. If calculated t is greater than $t_{0.05}$, the null hypothesis is rejected. If not significant. The null hypothesis stands.

For the differences of pre- and post-treatment SGPT value from Table 37, a two-sided test ,

$$t = \frac{|20.33 - 0|}{40.99 / \sqrt{12}} = 1.718$$

Tabulated $t_{0.05}$ (11) is 1.80. The calculated t is less than $t_{0.05}$ thus the null hypothesis stands.

The percentage differences of TC from the initial values of each brand are also determined in the same procedure as described above.

10. Friedman test

The Friedman test is a nonparametric test applied to data which is, at least, ranked and which in the form of two-way ANOVA design (randomized block). This test, which may be applied to ranked or individual / ratios type data, is used when more than 2 treatment gruops are included in the experiment. In Friedman test, the treatment are ranked with in each block (e.g., animal or person), disregarding differences between blocks. The procedure will be illustrated using data from Table 27. The parenthetical values in Table 27 are the ranks of the t_{max} for each dog over four treatments.

If the sample sizes are sufficiently large, a chi-square distribution can be used to approximate the test of significance. The chi-square test is

$$\chi^2_{c-1} = \frac{12 (\Sigma R_i^2)}{r\, c\, (c+1)} - 3r\,(c+1)$$

where χ^2_{c-1} = the χ^2 statistic with c-1 degree of freedom

r = number of rows (blocks)

c = number of columns (treatments)

R_i = sum of ranks in ith group (column)

The significance is considered if calculated equal or more than tablated . In our data, the chi-square statistic has 3 degree of freedom:

$$\chi^2_3 = \frac{12\,(31^2+37^2+19.5^2+30.5^2)}{(12)\,(4)\,(4+1)} - 3(12)\,(4+1)$$

$$= 2.02$$

Tabulated $\chi^2_{0.05(3)}$ is 7.81, hence, there were no statistically significant difference in t_{max} value over the four treatments at the 5% significant level.

APPENDIX F

CALCULATION FOR DISSOLUTION RATE CONSTANT (K_d)

The dissolution rate constants (K_d) are calculated according to sigma-minus method as shown in equation

$$\ln (X_\infty - X_t) = - K_d t + \ln X_\infty$$

where X_∞ = The amount of drug dissolved at infinity (t_∞)

X_t = The amount of drug dissolved at time t

The above equation describes the relationship for the amount of un dissolved simvastatin ($X_\infty - X_t$) versus time. A linear curve is obtained by graphing the natural logarithm of the amount of undissolved simvastatin ($X_\infty - X_t$) versus time. The dissolution rate constants (K_d) is obtained from the slope of this curve. Using the dissolution data in dissolution medium of brand B (tablet 1) in Table 7, determine the rate of dissolution as follow:

1. Construct the following table (Table 38) and
2. Determine the amount of undissolved simvastatin ($X_\infty - X_t$) and then plot ($X_\infty - X_t$) on the log scale versus time (semi-log plot).

The dissolution rate constants (K_d) obtained from this data is 0.129. As for other brands, the dissolution rate constants are also determined in the same procedure as discussed above.

Table 38 Exemplified data for dissolution rate constants (K_d) determined according to sigma-minus method.

Time (min)	X_t(μg %)	$X_\infty - X_t$(μg %)
5	9.68	101.98
10	45.72	92.30
15	81.62	56.27
20	93.27	20.36
30	101.98	8.72

APPENDIX G

Table 39 The difference in total cholesterol (TC) level at 2 hr after 200 mg single oral dose of four brands of simvastatin tablets (raw data n=2).

Dog no.		Brand A			Brand B			Brand C			Brand D		
		TC 0 hr	TC 2 hr	δ (ΔTC)	TC 0 hr	TC 2 hr	δ (ΔTC)	TC 0 hr	TC 2 hr	δ (ΔTC)	TC 0 hr	TC 2 hr	δ (ΔTC)
1	nmol/L	108	106.5	-1.5	128	136.5	+8.5	117	115.5	-1.5	138.5	143.5	-5
	%	100	98.6	-1.4	100	106.6	+6.6	100	98.7	-1.3	100	103.6	+3.6
2	nmol/L	147.5	146	-0.5	142.5	141	-1.5	146.5	146	-0.5	139.5	129	-10.5
	%	100	99.3	-0.7	100	98.9	-1.1	100	99.7	-0.3	100	92.5	-7.5
3	nmol/L	193	191.5	-1.5	203.5	218.5	+15.5	127	89	-38	174	177.5	+3.5
	%	100	99.2	-0.8	100	107.4	+7.4	100	70.1	-29.9	100	102	+2.0
4	nmol/L	262	262.5	+0.5	323.5	287.5	-36.0	229	250.5	+21.5	260	244.5	-15.5
	%	100	100.2	+0.2	100	88.9	-11.1	100	109.4	-9.4	100	94	-6.0
5	nmol/L	216.5	198.5	-18	284	268	-16.0	252	232.5	-19.5	202	196	-6.0
	%	100	91.7	-8.3	100	94.4	-5.6	100	92.3	-7.7	100	97	-3.0
6	nmol/L	228.5	212	-16.5	353	361	-8.0	199.5	238.5	+39	221	224	-3.0
	%	100	92.8	-7.2	100	102.3	+2.3	100	119.5	+19.5	100	101.4	+1.4
7	nmol/L	138.5	141.5	+3.0	88.6	81.5	-7.1	139	133.5	-5.5	142	148	+6.0
	%	100	102.2	+2.2	100	92	-8.0	100	96	-4	100	104.2	+4.2
8	nmol/L	176.5	177.5	+1.0	196.5	168	-28.5	163.5	164	+0.5	164	157.5	-6.5
	%	100	100.6	+0.6	100	85.5	-14.5	100	100.3	+0.3	100	96	-4.0
9	nmol/L	169	172	+3.0	179.5	172	-7.5	160	159	-1	161	161	0
	%	100	101.8	+1.8	100	95.8	-4.2	100	99.4	-0.6	100	100	0
10	nmol/L	156.5	144	-12.5	234	241	+7.0	131	168	+37	172	177	+5
	%	100	92.0	-8.0	100	103	+3.0	100	128.2	+28.2	100	102.9	+2.9
11	nmol/L	203.5	192.5	-11.0	217	186	-31.0	325	280.5	-44.5	228	205	-23
	%	100	94.6	-5.4	100	85.7	-14.3	100	86.3	-13.7	100	89.9	-10.1
12	nmol/L	209	220.5	+11.5	247.5	247.5	0	202	138.5	-63.5	206.5	198	-8.5
	%	100	105.5	+5.5	100	100	0	100	68.6	-31.4	100	95.9	-4.1

VITA

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