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Appendix

Table 1 Polarities of Chemically Bonded Phases in Ascending Order

Type	Functional Group
RP-F (Perfluorinated)	$-C_nF_{2n+1}$
RP-n (N-Alkyl)	$-C_nH_{2n+1}$
Cyclohexyl	$-C_6H_{12}$
Phenyl	$-C_6H_6$
Cyano-propyl	$-C_3H_6-CN$
Diol	$-CHOH-CH_2-OH$
Amine	$-NH_2$
Silica	-Si-OH

(From Szepesi G. HPLC in Pharmaceutical Analysis Vol.1 Boston : CRC Press, 1990.)

Table 2 Effect of Mobile Phase Composition on Retention for Erythromycin, Its Related Substances and Degradation Products.

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M phosphate buffer pH 5.0 (v/v) ; flow rate, 1.0 ml/min.

$CH_3CN:CH_3OH:0.05$ M NaH_2PO_4 (v/v)	Capacity factor (k')				
	Erythromycin C	Erythromycin A	Erythromycin B	Anhydroerythromycin A	Erythromycin A enol ether
40 : 0 : 60	0.68	1.09	1.96	2.44	2.61
30 : 15 : 55	0.87	1.42	2.42	2.77	3.35
25 : 23 : 52	1.02	1.58	2.64	3.03	3.60
20 : 31 : 49	1.12	1.70	2.76	3.17	3.73
18 : 34 : 48	1.51	1.77	2.91	3.32	3.99
17 : 36 : 47	1.39	1.73	2.68	3.13	3.82
16 : 37 : 47	1.48	1.88	3.01	3.50	4.25
15 : 38 : 47	1.55	2.05	3.00	3.78	4.92
15 : 40 : 45	1.27	1.70	2.36	3.01	3.63
10 : 46 : 44	1.31	1.93	2.73	3.42	4.24
5 : 55 : 40	1.06	1.67	2.60	2.60	3.31
0 : 62 : 38	0.99	1.63	2.16	2.39	3.19

Capacity factor (k')

$$k' = (t_R - t_o) / t_o$$

where t_R = distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the interested peak.

t_o = distance along the baseline between the point of injection and a perpendicular dropped from the maximum of an unretained peak.

Table 3 Effect of Buffer Salts on Plate Number (N) and Tailing Factor for Erythromycin

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M buffer salts pH 5.0 (15:38:47, v/v); flow rate, 1.0 ml/min.

Buffer Salts	N	Tailing Factor
Ammonium acetate	6,325	1.21
Sodium acetate	4,977	1.22
Sodium dihydrogen phosphate	7,581	1.02

Column efficiency

This can be expressed as the theoretical plate number (N)

$$N = 5.54 \left(t_R / W_{1/2} \right)^2$$

where t_R = distance, in millimetres, along the baseline between the point of injection and a perpendicular dropped from the maximum of the interested peak

$W_{1/2}$ = peak width, in millimetres, at half height

Peak asymmetry (Tailing factor)

It can be determined by dropping a perpendicular from the peak maximum to the baseline and calculating the ratio of the rear (b) to the front (a) baseline segment at 10% of the peak height.

$$A_{sf} = b / a$$

Table 4 Effect of Phosphate Buffer Concentration on Retention for Erythromycin, Its Related Substances and Degradation Products.

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol phosphate buffer pH 5.0 (15:38:47, v/v) ; flow rate, 1.0 ml/min.

Phasphate Concentration (M)	Capacity factor (k')				
	Erythromycin C	Erythromycin A	Erythromycin B	Anhydroery-thromycin A	Erythromycin A enol ether
0.010	2.85	3.70	5.72	7.38	8.98
0.025	1.58	2.46	4.00	4.61	5.68
0.050	1.46	1.99	3.22	3.69	4.44
0.075	1.50	1.86	2.87	3.46	4.15
0.100	1.53	1.79	2.85	3.37	4.06

Capacity factor (k')

$$k' = (t_R - t_o) / t_o$$

where t_R = distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the interested peak.

t_o = distance along the baseline between the point of injection and a perpendicular dropped from the maximum of an unretained peak.

Table 5 Effect of Mobile Phase pH on Retention for Erythromycin, Its Related Substances and Degradation Products.

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M phosphate buffer (15:38:47, v/v) ; flow rate, 1.0 ml/min.

Buffer pH	Capacity factor (k')				
	Erythromycin C	Erythromycin A	Erythromycin B	Anhydroery-thromycin A	Erythromycin A enol ether
3.5	1.09	1.72	2.78	3.18	3.74
4.0	1.54	1.71	2.77	3.18	3.73
5.0	1.52	1.96	3.07	3.60	4.37
6.0	1.55	2.53	3.50	4.53	6.01
6.5	1.61	3.92	6.52	7.50	10.44

Capacity factor (k')

$$k' = (t_R - t_o) / t_o$$

where t_R = distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the interested peak.

t_o = distance along the baseline between the point of injection and a perpendicular dropped from the maximum of an unretained peak.

Table 6 Stability of Erythromycin Solution in Buffer pH 5.0

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M NaH_2PO_4 pH 5.0 (15:38:47, v/v); flow rate, 1.0 ml/min.

Time	Content (mg) calc.as Erythromycin A		
	Erythromycin A	Anhydroerythromycin A	Erythromycin A enol ether
Initial	20.15	0.59	0.57
17 min	20.37	NC	0.93
53 min	20.70	NC	1.52
1 hr 10 min	21.02	1.30	1.64
2 hr 5 min	19.87	0.49	0.53
4 hr 23 min	19.36	0.65	2.07
5 hr 57 min	18.85	NC	2.07
1 day 46 min	18.40	0.65	6.09
7 day 13 min	17.09	2.53	35.95
14 day 3 hr 5 min	16.35	4.01	67.83

NC = not calculated

Table 7 Stability of Erythromycin Solution in Buffer pH 3.0

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M NaH_2PO_4 pH 5.0 (15:38:47, v/v); flow rate, 1.0 ml/min.

Time	Content (mg) calc.as Erythromycin A		
	Erythromycin A	Anhydroerythromycin A	Erythromycin A enol ether
Initial	20.62	0.22	6.25
18 min	15.66	1.62	48.86
36 min	11.24	3.06	74.01
55 min	6.54	3.21	92.08
2 hr 6 min	2.31	12.12	115.80
4 hr 15 min	ND	12.83	107.49
6 hr 8 min	2.79	3.36	91.39
1 day 9 hr 33 min	1.40	NC	20.87
7 day 4 hr 47 min	ND	NC	16.32
14 day 7 hr 27 min	ND	NC	14.04

NC = not calculated

ND = not detected

Table 8 Stability of Erythromycin Solution in Buffer pH 4.0

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M NaH_2PO_4 pH 5.0 (15:38:47, v/v); flow rate, 1.0 ml/min.

Time	Content (mg) calc.as Erythromycin A		
	Erythromycin A	Anhydroerythromycin A	Erythromycin A enol ether
Initial	20.19	0.59	1.34
20 min	20.08	0.37	1.96
39 min	19.80	0.16	2.18
1 hr 14 min	19.29	NC	2.61
1 hr 52 min	19.91	0.94	4.49
4 hr 23 min	18.84	NC	9.26
6 hr 31 min	19.14	NC	12.26
1 day	15.34	2.34	40.37
7 day 2 hr 51 min	5.64	5.26	129.44
14 day 5 hr 31 min	NC	6.05	127.17

NC = not calculated

Table 9 Stability of Erythromycin Solution in Buffer pH 6.0

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M NaH_2PO_4 pH 5.0 (15:38:47, v/v); flow rate, 1.0 ml/min.

Time	Content (mg) calc.as Erythromycin A		
	Erythromycin A	Anhydroerythromycin A	Erythromycin A enol ether
Initial	19.50	0.28	1.35
17 min	19.80	0.53	0.29
35 min	19.91	ND	ND
51 min	20.09	0.62	1.50
1 hr 51 min	20.83	0.35	0.87
3 hr 54 min	20.87	NC	1.32
5 hr 51 min	18.67	NC	1.55
1 day 1 hr	19.29	0.38	3.63
7 day	18.64	2.67	24.27
14 day 1 hr 35 min	16.77	3.92	44.23

NC = not calculated

ND = not detected

Table 10 Stability of Erythromycin Solution in Buffer pH 6.5

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M NaH_2PO_4 pH 5.0 (15:38:47, v/v); flow rate, 1.0 ml/min.

Time	Content (mg) calc.as Erythromycin A		
	Erythromycin A	Anhydroery-thromycin A	Erythromycin A enol ether
Initial	19.77	0.13	1.09
17 min	19.66	0.37	0.82
35 min	19.93	0.49	0.47
1 hr 8 min	19.87	NC	0.82
2 hr 5 min	19.54	NC	0.49
4 hr 1 min	19.21	0.78	1.52
6 hr 11 min	19.87	NC	1.59
1 day 1 hr 8 min	19.28	0.72	2.01
7 day 3 hr 58 min	21.41	1.53	9.63
14 day 6 hr 34 min	23.10	4.89	20.41

NC = not calculated

Table 11 Selection of Internal Standard

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M NaH_2PO_4 pH 5.0 (15:38:47, v/v); flow rate, 1.0 ml/min.

Drug Tested	Relative Retention Time (RRT)
Erythromycin A enol ether	1.00
Cefotaxime sodium	0.21
Phenylpropanolamine HCl	0.24
Metronidazole	0.25
Trimethoprim	0.26
Caffeine	0.27
Piroxicam	0.35
Chlorpropamide	0.37
Pyrimethamine	0.40
Propranolol HCl	0.42
Propyl paraben	0.48
Mebendazole	0.54
Naproxen	0.56
Oxyphencyclimine	0.66
Triamcinolone	0.67
Diclofenac sodium	0.76

Table 11 (Continued)

Drug Tested	Relative Retention Time (RRT)
Ibuprofen	0.82
Diazepam	0.89
Indomethacin	0.98
Mefenamic acid	1.07
Glibenclamide	1.46
Niclosamide	1.66
Cinnarizine	1.97
Betamethasone 17-valerate	2.15

Table 12 Samples of Raw Material and Enteric-Coated Tablets Used

Sample	Manufacturer	Batch No.	Mfg.date	Exp.date
I Raw Material				
1.) RM 1	Abbott	46-544-CR	15.10.90	01.11.95
2.) RM 2	Abbott	57-124-CR	17.09.91	01.10.96
3.) RM 3	Lupin Chemicals	059/91	05.09.91	04.09.96
II Enteric-Coated Tablets				
1.) FP 1	Abbott	54240 TF	17.04.90	01.05.93
2.) FP 2	Abbott	58560 TF	27.08.90	01.09.93
3.) FP 3	Abbott	69344 TF	04.07.91	01.08.94
4.) FP 4	Abbott	73369 TF	26.11.91	01.12.94
5.) FP 5	Abbott	79397 TF	02.06.92	01.07.95

Table 13 Relationship between concentration and peak height ratio of erythromycin A

Concentration (mg/ml)	Peak Height Ratio ± SD
0.48	0.7646 ± 0.0082
0.64	0.9850 ± 0.0014
0.80	1.2088 ± 0.010
0.96	1.4491 ± 0.018
1.12	1.6614 ± 0.0011
1.28	1.8759 ± 0.017
Slope	1.3975
Y-intercept	0.0943
Correlation coefficient	0.9999

Table 14 Intra-Day Precision Data of Erythromycin Raw Material

Sample, RM1

Trial	Content of erythromycin A (μg/mg)
1	904.20
2	912.10
3	908.20
4	903.92
5	909.08
6	908.65
Mean	907.69
% RSD	0.34

Table 15 Inter-Day Precision Data of Erythromycin Raw Material

Sample, RM1

Day	Mean content of erythromycin A (μg/mg $\pm$ S.D., n=3)
1	907.69 $\pm$ 3.13
2	898.61 $\pm$ 5.54
3	907.29 $\pm$ 2.83
4	905.21 $\pm$ 3.68
5	904.93 $\pm$ 2.91
6	908.29 $\pm$ 3.16
Mean	905.34
% RSD	0.39

Table 16 Intra-Day Precision Data of Erythromycin Tablets Sample, FP4

Trial	content of erythromycin A (mg/tab)	% Labeled amount
1	228.76	91.51
2	232.84	93.14
3	229.78	91.91
4	230.95	92.38
5	225.05	90.02
6	227.10	90.84
Mean	229.08	91.63
% RSD	1.21	1.21

Table 17 Inter-Day Precision Data of Erythromycin Tablets Sample, FP4

Day	Mean content of erythromycin A (mg/tab ± S.D.,n=3)	% Labeled amount
1	225.96 ± 2.05	90.38
2	227.34 ± 2.33	90.94
3	228.24 ± 4.74	91.30
4	234.85 ± 1.77	93.94
5	229.62 ± 1.38	91.85
6	229.08 ± 2.77	91.63
Mean	229.18	91.67
% RSD	1.34	1.34

Table 18 Precision of Recovery of Erythromycin from Tablets Powder Spiked with Reference Standard Sample, FP5

Trial	Erythromycin added, mg	Erythromycin found, mg	% Recovery
1	15.04	14.43	95.96
	15.04	14.84	98.69
	15.04	15.05	100.08
	15.04	14.34	95.36
	15.04	14.92	99.22
Mean			97.86
% RSD			2.13
2	15.10	15.60	103.31
	15.10	16.34	108.21
	15.10	14.62	96.82
	15.10	15.39	101.92
	15.10	15.91	105.36
Mean			103.12
% RSD			4.12
3	15.09	14.99	99.34
	15.09	15.14	100.33
	15.09	15.37	101.86
	15.09	14.44	95.69
	15.09	14.11	93.51
Mean			98.15
% RSD			3.51

Table 19 Linearity of Recovery of Erythromycin from Tablets Powder Spiked with Reference Standard Sample, FP5

Trial	Erythromycin added, mg	Erythromycin found, mg	% Recovery
1	4.05	4.14	102.22
	8.09	8.12	100.37
	12.14	11.69	96.29
	16.18	15.77	97.47
	20.23	19.91	98.42
Mean			98.95
% RSD			2.38
Slope		0.9688	
y-intercept		0.1661	
Correlation coefficient		0.9997	
2	4.58	4.64	101.31
	9.16	9.13	99.67
	13.74	13.79	100.36
	18.32	18.55	101.26
	22.90	21.85	95.41
Mean			99.60
% RSD			2.45
Slope		0.9572	
y-intercept		0.4400	
Correlation coefficient		0.9983	

Table 19 (Continued)

Trial	Erythromycin added, mg	Erythromycin found, mg	% Recovery
3	4.70	4.98	105.96
	8.34	8.60	103.12
	12.88	12.89	100.08
	18.17	18.29	100.66
	21.71	20.78	95.72
Mean			101.11
% RSD			3.76
Slope		0.9432	
y-intercept		0.6960	
Correlation coefficient		0.9989	

Table 20 Peak Height of Erythromycin Peak with and without Erythromycin B, Anhydroerythromycin A and Erythromycin A Enol Ether.

Adding erythromycin B, anhydroerythromycin A, and erythromycin A enol ether	Peak Height (± S.D., n=3)
-	1604 ± 32.58
+	1619 ± 46.76

Table 21 Detection Limit of Erythromycin

At concentration of 9.26 ppm

	Signal/Noise Ratio (from peak height response)
1.	2.9091
2.	2.7273
3.	2.7500
4.	2.6667
5.	2.5833
6.	2.5833
7.	2.9091
8.	2.6667
9.	2.5833
10.	2.9167
Mean	2.7296
% RSD	5.06

Table 22 Quantitative Analysis of Raw Material

Sample	Water content[a] (%)	HPLC (n=6)		Microbiological assay (n=2)	
		Mean content of erythromycin A (μg/mg)[b] $\pm$ S.D.	%RSD	Mean content of erythromycin (μg/mg)[b] $\pm$ S.D.	%RSD
1. RM1	4.62	911.07 $\pm$ 10.73	1.18	893.11 $\pm$ 68.10	7.63
2. RM2	5.30	909.71 $\pm$ 7.40	0.81	896.65 $\pm$ 42.53	4.74
3. RM3	5.15	840.60 $\pm$ 9.72	1.16	834.49 $\pm$ 68.19	8.17

[a] Water content by using Karl Fischer titration method

[b] μg/mg calc. on anhydrous basis $\pm$ standard deviation

Table 23 Quantitative Analysis of Enteric-Coated Tablets

Sample	HPLC (n=6)		Microbiological assay (n=2)	
	Mean content of erythromycin A (mg/tab) ± S.D.	%RSD	Mean content of erythromycin (mg/tab) ± S.D.	%RSD
1. FP1	247.95 ± 4.92	1.98	244.72 ± 15.26	6.24
2. FP2	238.68 ± 4.54	1.90	238.65 ± 14.89	6.24
3. FP3	230.94 ± 4.39	1.90	235.07 ± 10.08	4.29
4. FP4	229.36 ± 2.83	1.23	234.04 ± 14.57	6.23
5. FP5	227.86 ± 2.21	0.97	229.86 ± 6.98	3.04

Table 24 Statistical Test of HPLC and Microbiological Assay

t test at 95% confidential limit

Sample	t - value		
	t (critical)	ν	t (calculated)
(A) Raw material			
1. RM1	± 3.1414	3.10	0.5231
2. RM2	± 3.1333	3.12	0.6080
3. RM3	± 3.1495	3.08	0.1780
(B) Enteric-coated tablets			
1.FP1	± 3.0115	3.42	0.4094
2.FP2	± 3.0277	3.38	0.003910
3.FP3	± 2.306	8	-0.9035
4.FP4	± 3.1211	3.15	-0.6345
5.FP5	± 3.0155	3.41	-0.5548

Comparison of Two Variance: The F-Test

$H_0 : \sigma_1^2 = \sigma_2^2$

$$F = \frac{s_1^2}{s_2^2}$$

$$\nu_1 = n_1 - 1 \quad , \quad \nu_2 = n_2 - 1$$

The observed value of F is compared with $F_{\alpha}(\nu_1, \nu_2)$ which is obtained from F-Tables.

Table 24 (Continued)

Comparison of Means: The t-Test

$H_0 : \mu_1 = \mu_2$

n_1 and $n_2 < 30$

$\sigma_1^2 = \sigma_2^2$ or $n_1 = n_2$ (though $\sigma_1^2 \neq \sigma_2^2$)	$\sigma_1^2 \neq \sigma_2^2$ and $n_1 \neq n_2$
$t = \dfrac{(\bar{x}_1 - \bar{x}_2)-(\mu_1 - \mu_2)}{\sqrt{s_p^2 \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$	$t = \dfrac{(\bar{x}_1 - \bar{x}_2)-(\mu_1 - \mu_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$
$\nu = n_1 + n_2 - 2$	$\nu = \dfrac{(s_1^2/n_1 + s_2^2/n_2)^2}{\frac{(s_1^2/n_1)^2}{n_1 - 1} + \frac{(s_2^2/n_2)^2}{n_2 - 1}}$
$s_p^2 = \dfrac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$	

The value obtained for t is then compared with a tabulated value of t that corresponds to the chosen α, the level of significance.

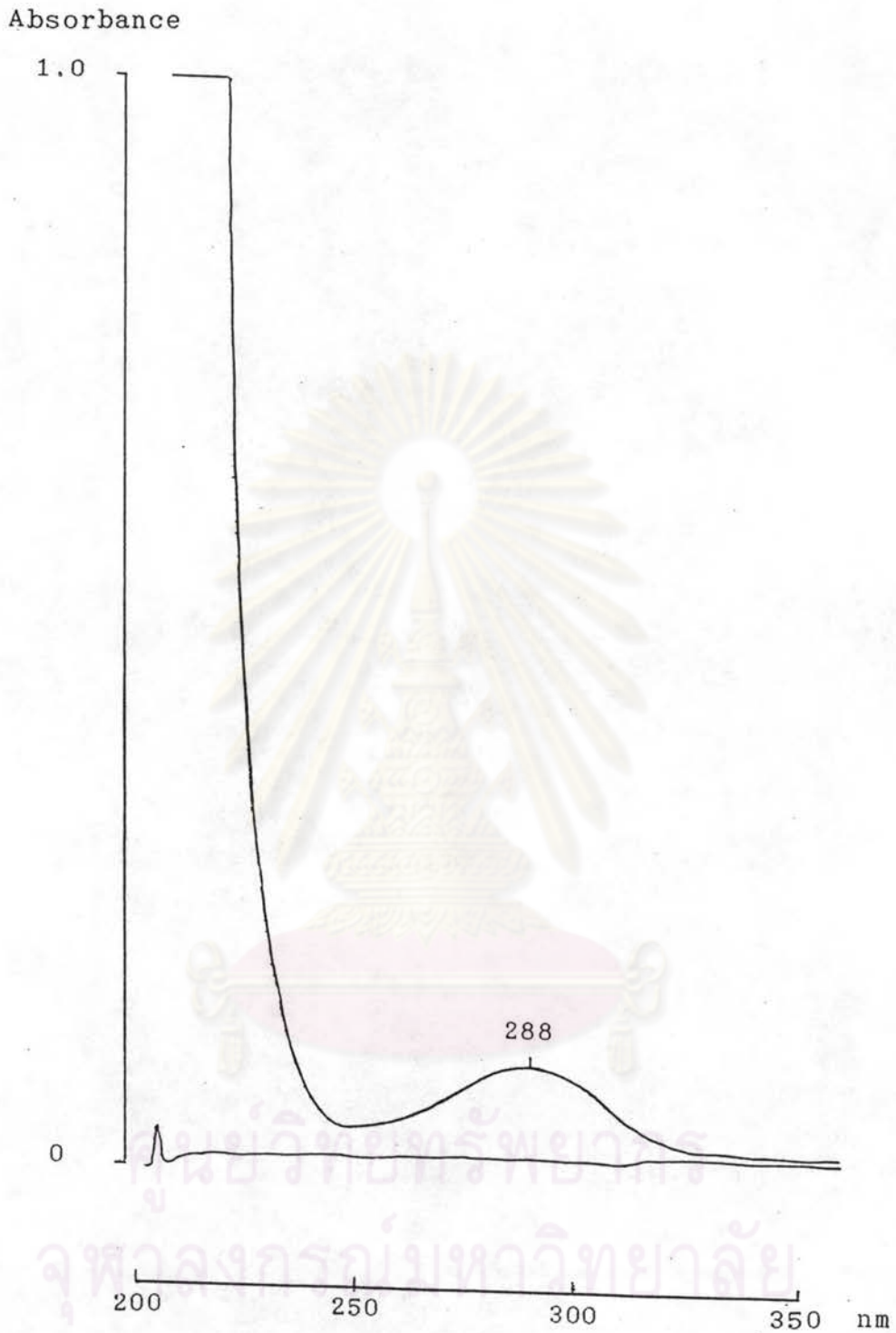

Figure 3. UV absorption spectrum of erythromycin A in mobile phase (1.0 mg/ml)

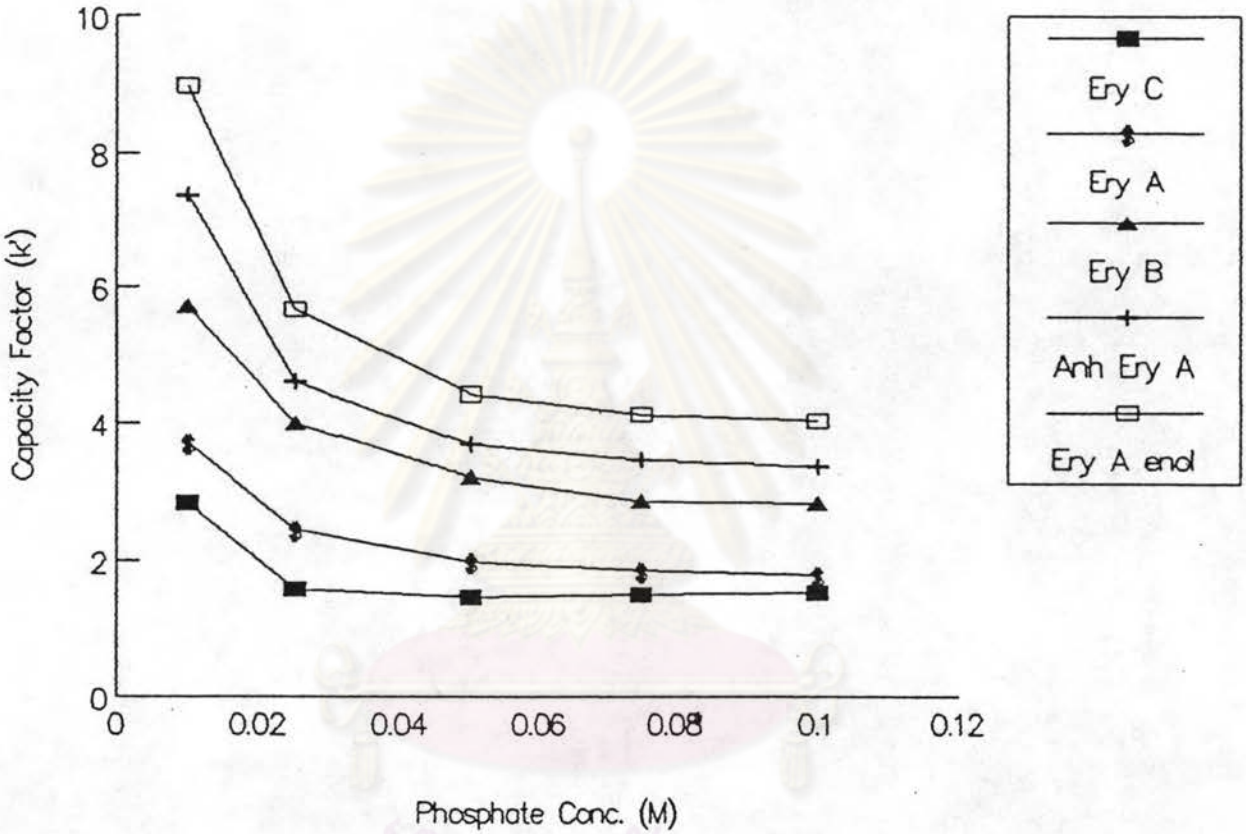

Figure 4. Effect of phosphate buffer concentration on capacity factor (k') for erythromycin, its related substances and degradation products.

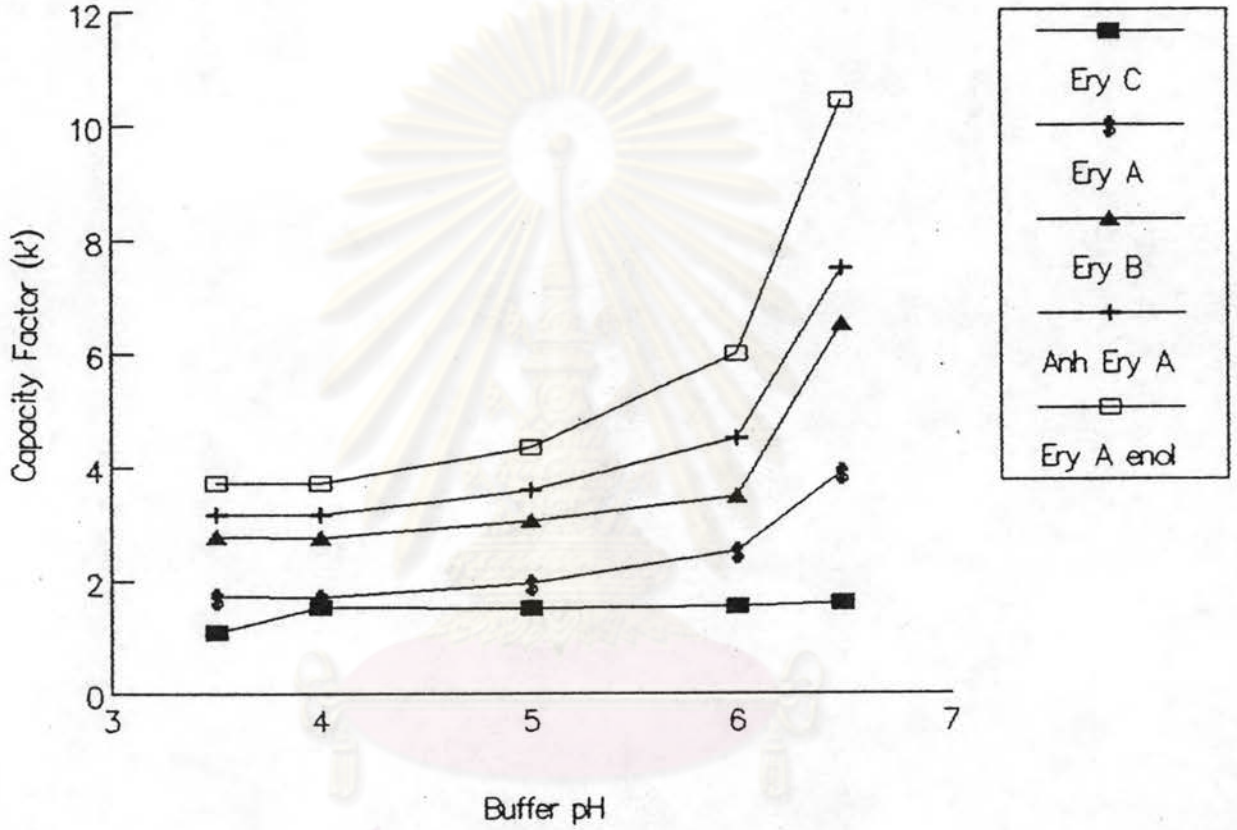

Figure 5. Effect of mobile phase pH on capacity factor (k') for erythromycin, its related substances and degradation products.

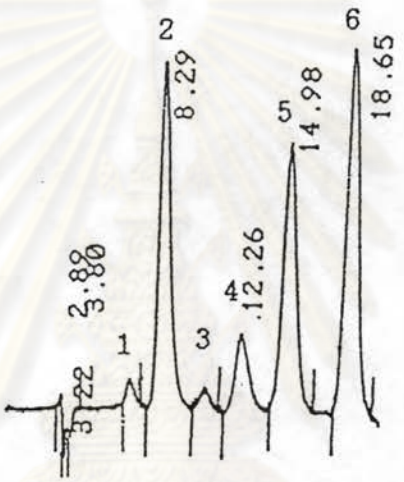

Figure 6. Chromatogram of erythromycin, its related substances, degradation products and internal standard. Peaks: (1)= erythromycin C (2)= erythromycin A (3)= erythromycin B (4)= anhydroerythromycin A (5)= erythromycin A enol ether (6)= glibenclamide (internal standard). Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M phosphate buffer pH 5.0 (15:38:47 v/v); flow rate 1.0 ml/min, monitored at 215 nm.

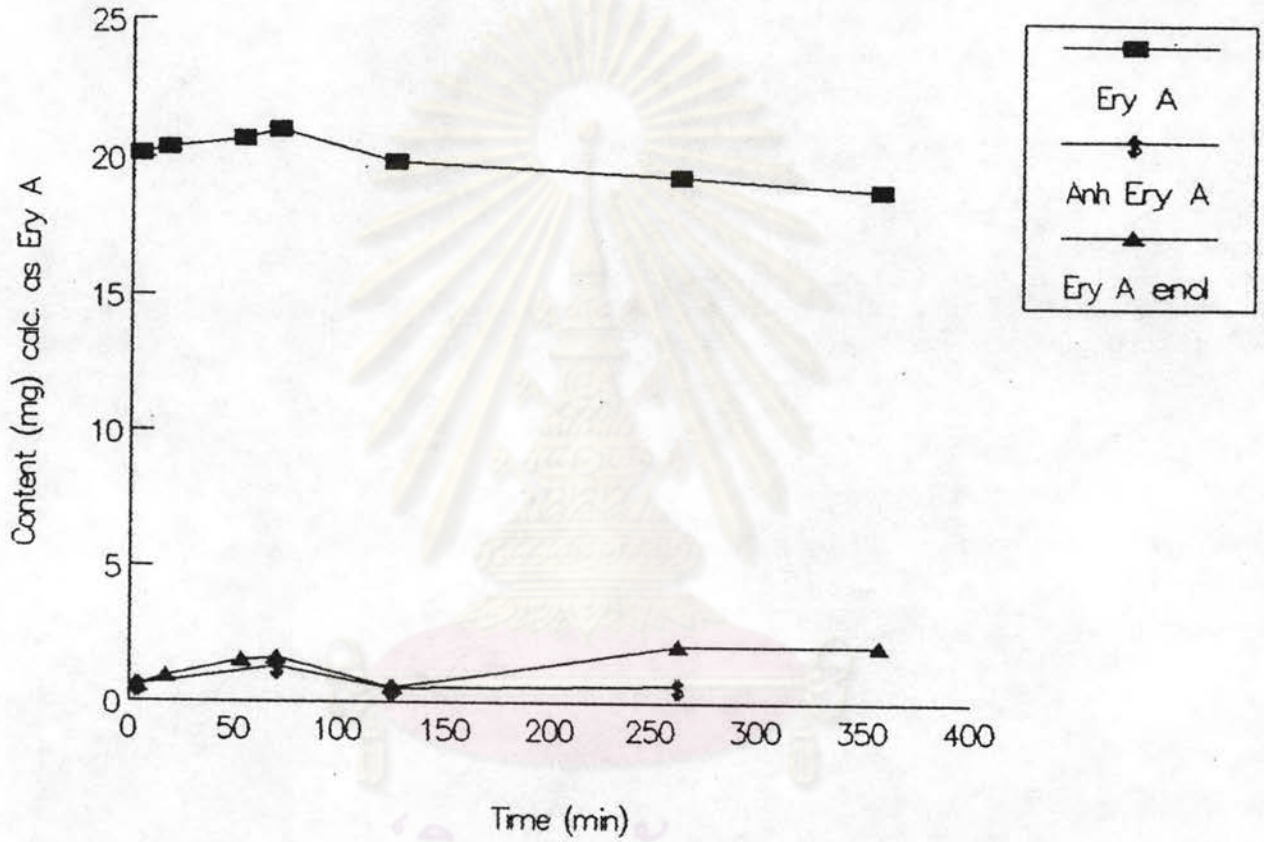

Figure 7. Stability of erythromycin solution in buffer pH 5.0.

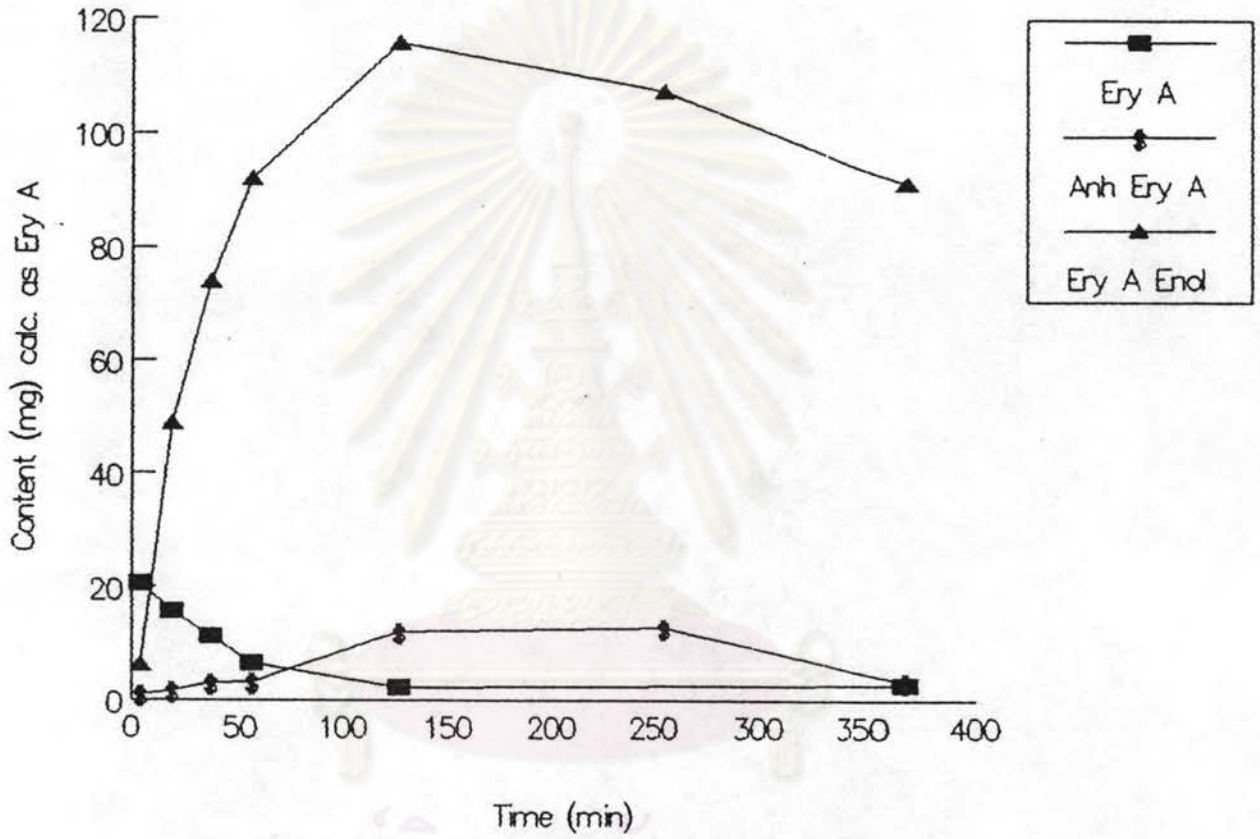

Figure 8. Stability of erythromycin solution in buffer pH 3.0.

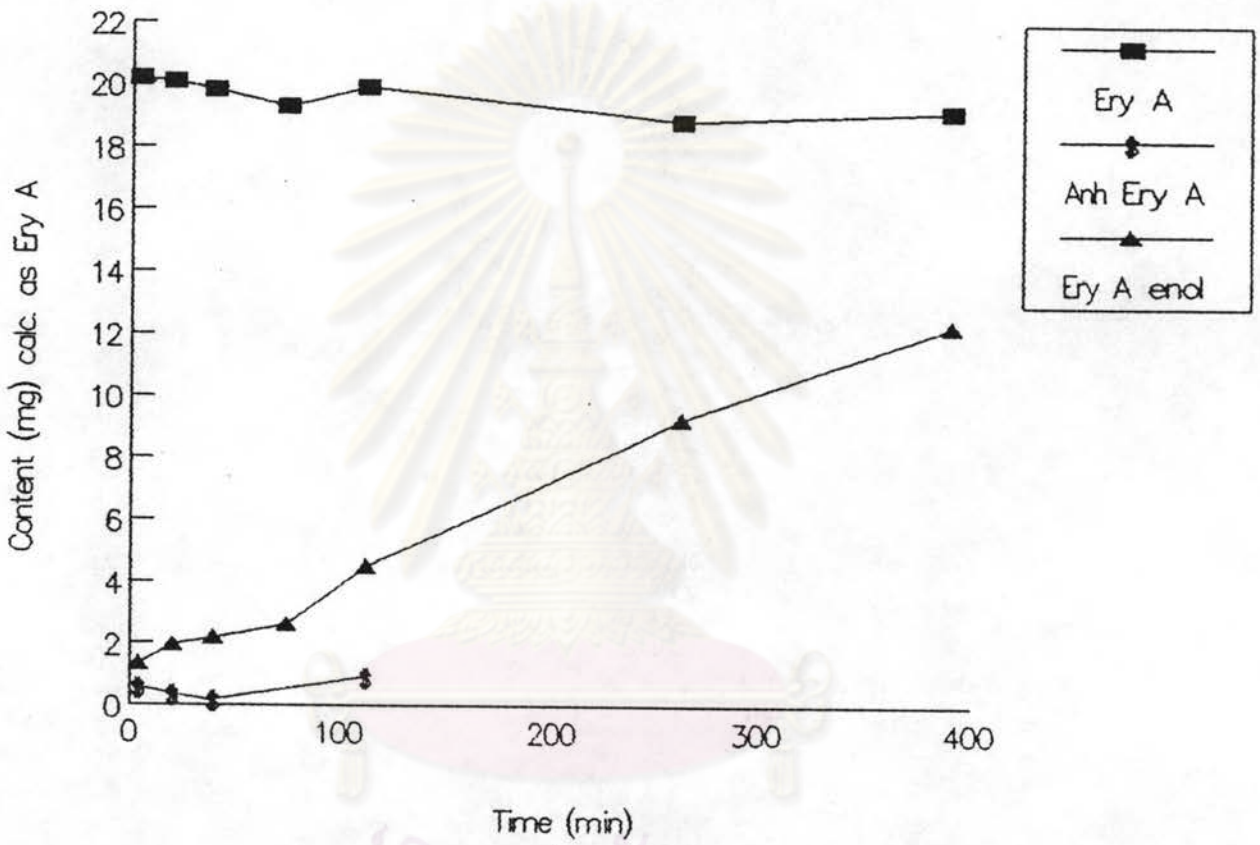

Figure 9. Stability of erythromycin solution in buffer pH 4.0.

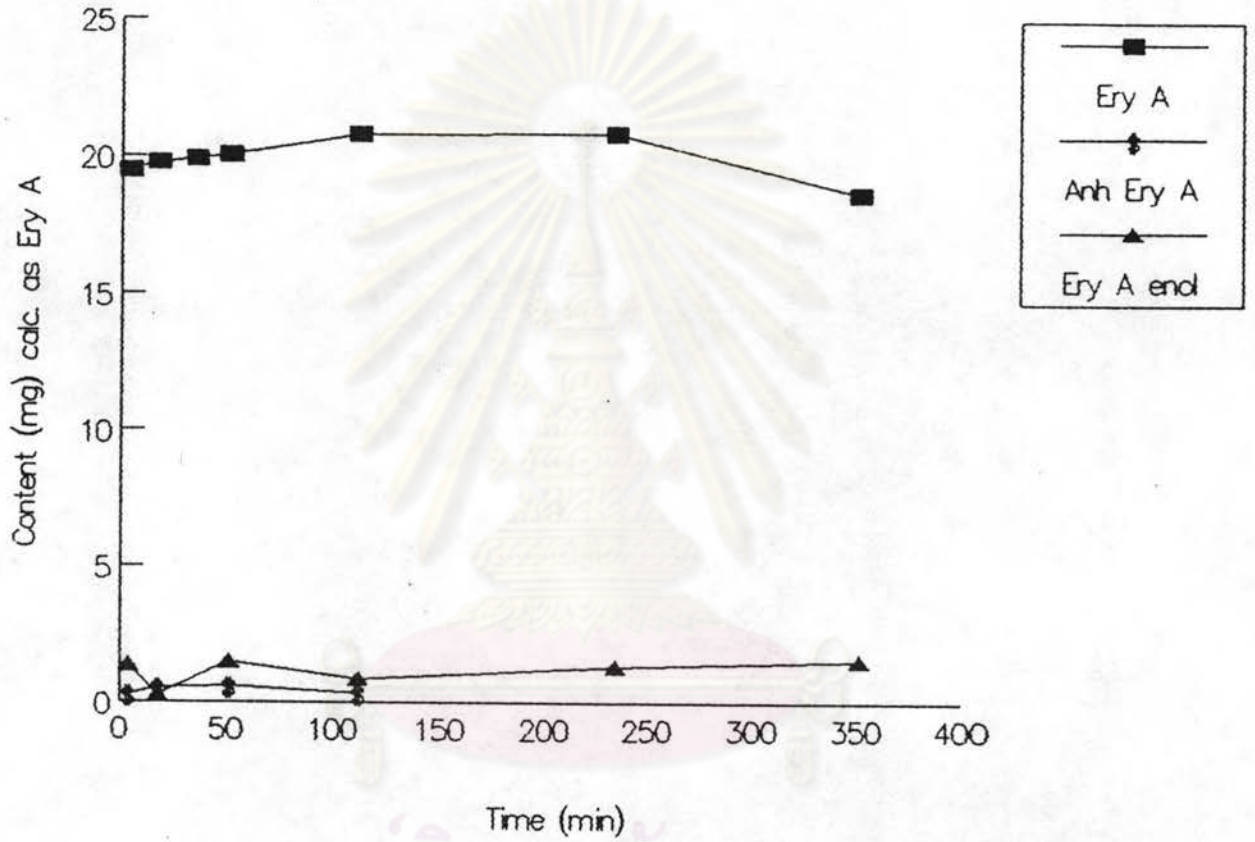

Figure 10. Stability of erythromycin solution in buffer pH 6.0.

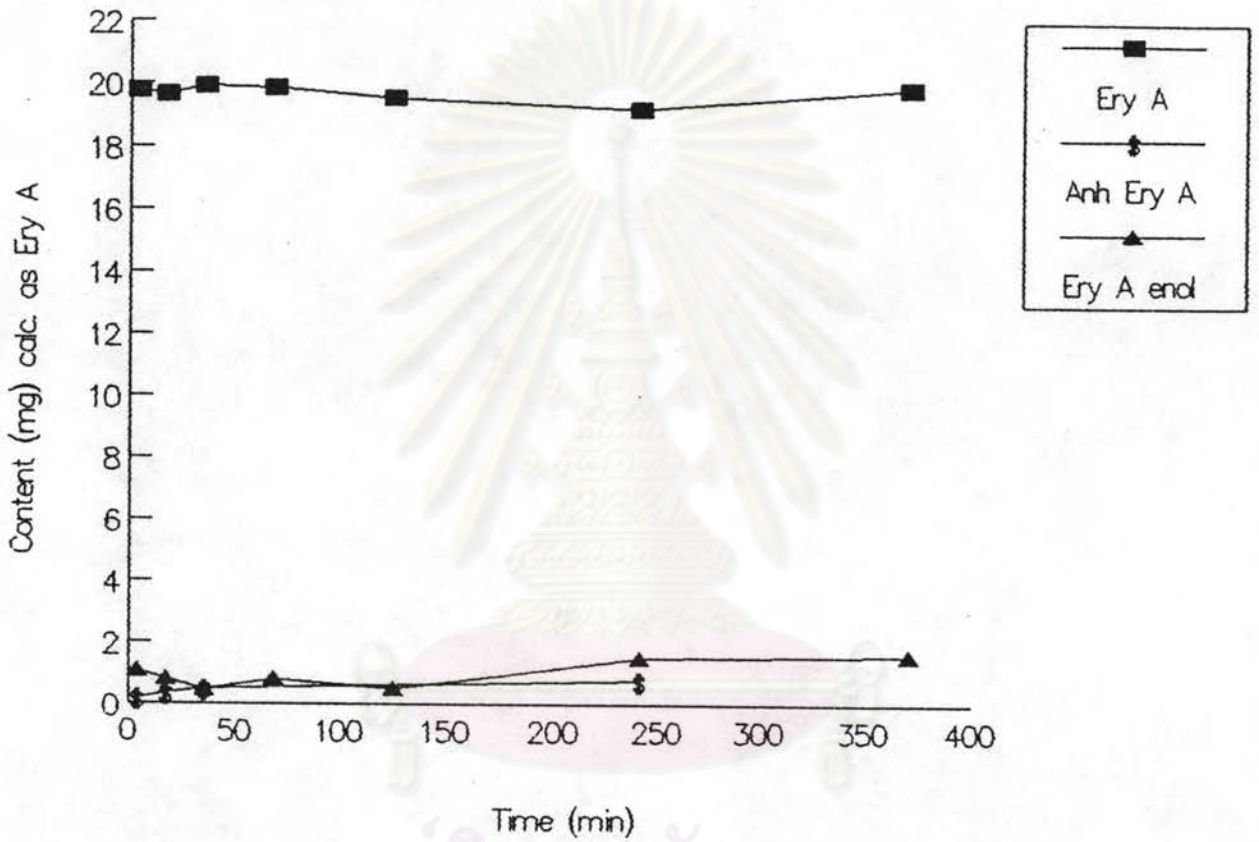

Figure 11. Stability of erythromycin solution in buffer pH 6.5.

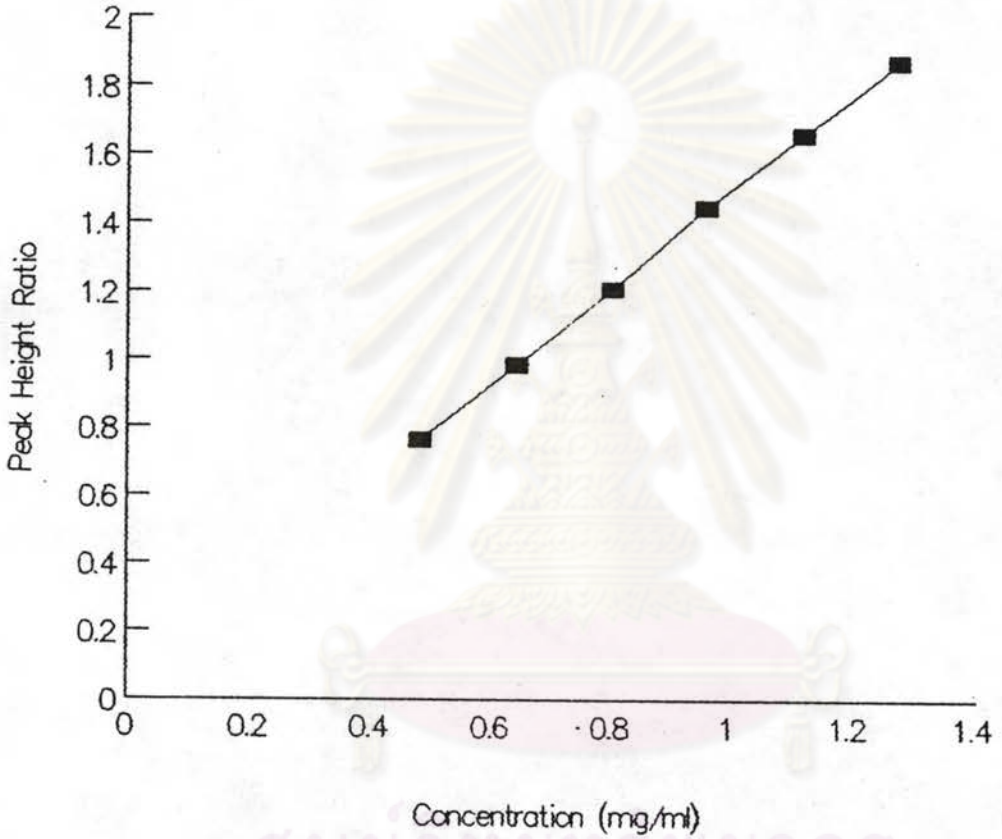

Figure 12. Relationship between concentration and peak height ratio of erythromycin A

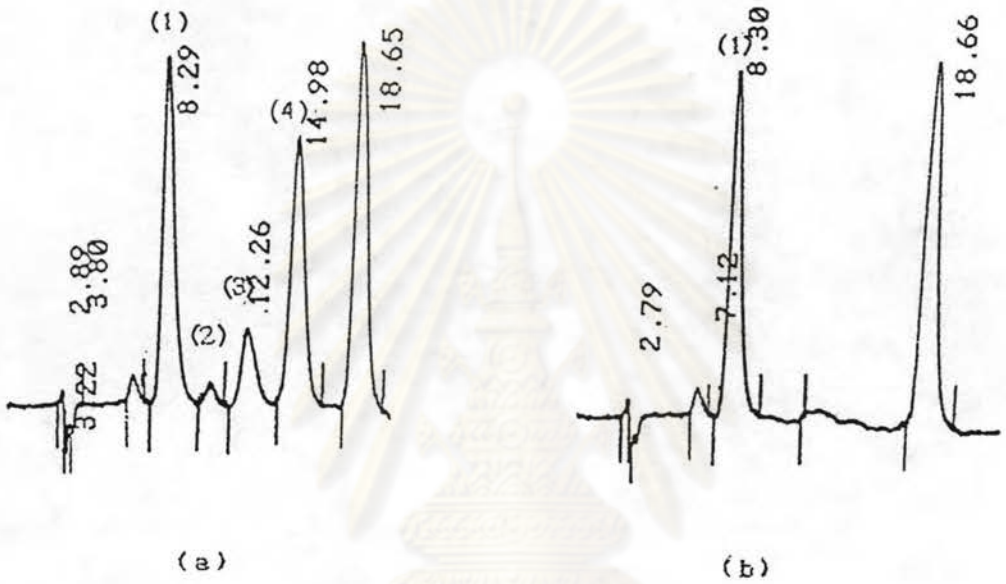

Figure 13. Chromatograms of erythromycin tablets solution with (a) and without (b) the addition of authentic substances erythromycin B, anhydroerythromycin A and erythromycin A enol ether. Peaks: (1)= erythromycin A (2)= erythromycin B (3)= anhydroerythromycin A (4)= erythromycin A enol ether. Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M phosphate buffer pH 5.0 (15:38:47 v/v); flow rate 1.0 ml/min, monitored at 215 nm.

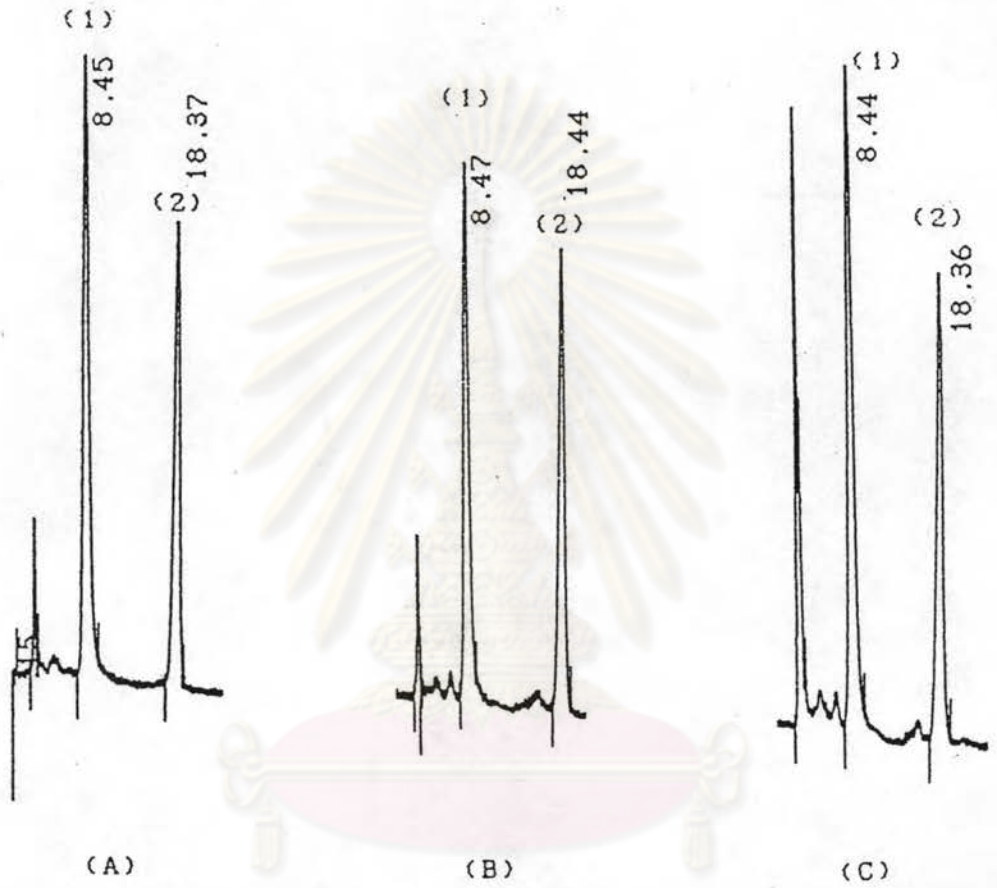

Figure 14. Chromatograms of erythromycin (A) erythromycin RS (B) raw material (C) enteric-coated tablet. Peaks: (1) = erythromycin A, (2) = internal standard (glibenclamide). Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M phosphate buffer pH 5.0 (15:38:47 v/v); flow rate 1.0 ml/min, monitored at 215 nm.

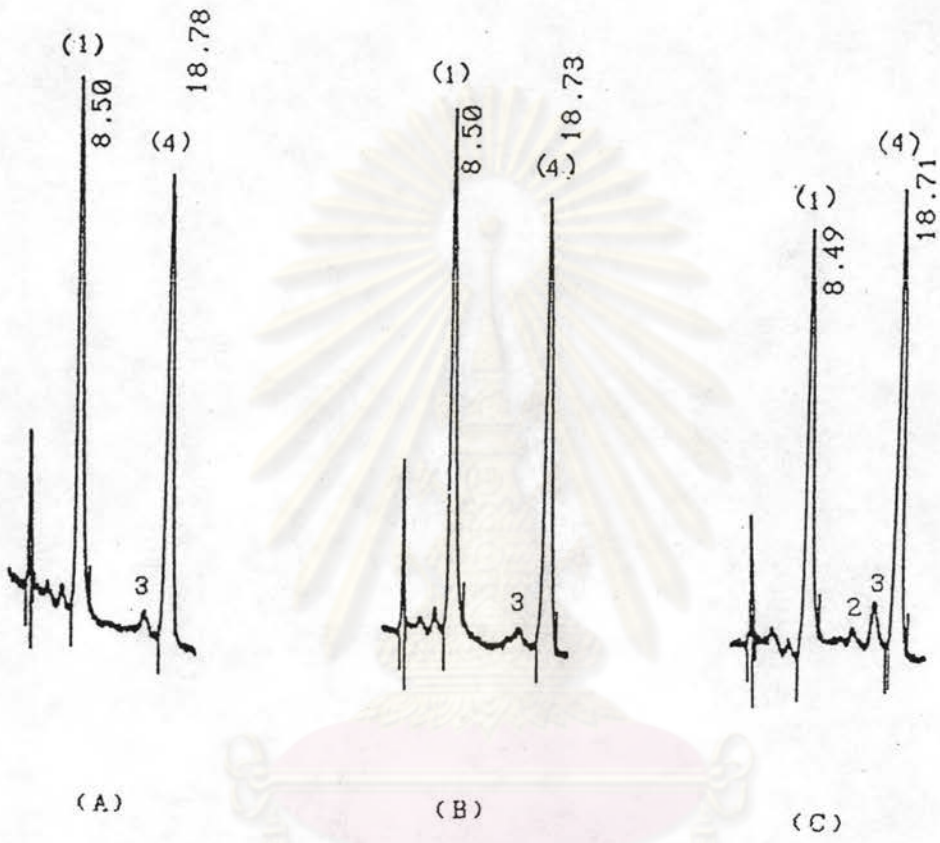

Figure 15. Chromatograms of erythromycin raw material solution (A) RM1 (B) RM2 (C) RM3 .

Peaks: (1)= erythromycin A (2)= anhydroerythromycin A (3)= erythromycin A enol ether (4)= internal standard (glibenclamide).

Column, Phenyl, 10 μm, 30 cm x 3.9 mm I.D.; mobile phase, acetonitrile-methanol-0.05 M phosphate buffer pH 5.0 (15:38:47 v/v); flow rate 1.0 ml/min, monitored at 215 nm.

VITA

Miss Pornlada Krobtong was born on September 5, 1963 in Bangkok. She received her Bachelor of Science in Pharmacy (Second Class Honor) in 1986 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University. From 1986 to 1991, she was employed by the Government Pharmaceutical Organization. Now she is working at Hoechst Thai Limited.