CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Analytical method

For rifampicin, the HPLC system was modified from its assay method in USP 24, which does not have an internal standard. Low polar drugs; erythromycin, indomethacin, clotrimazole and spironolactone were tested. Their chromatograms showed retention times at 10.3, 9.1, 5.7 and 11.3 minutes respectively. Indomethacin was selected as having an appropriate retention time to rifampicin (6 mins). The chromatogram of indomethacin and rifampicin were shown in figure 2. In USP 24, sample preparation must be injected to the HPLC system strictly within 30 to 60 seconds. To prove this, we injected the sample preparation eight times consecutively and found that the peak areas were reduced even at the second injection. Therefore it was decided to use only one injection immediately after its preparation.
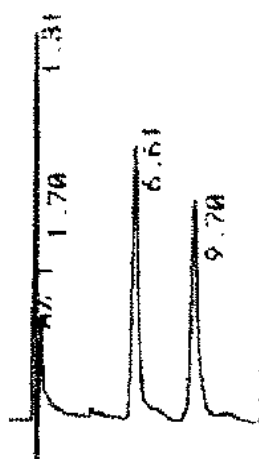
Figure 2 Chromatogram of rifampicin (6.61 mins) and indomethacin (9.70 mins).

For the analysis of pyrazinamide and isoniazid, the method modified from the assay method for pyrazinamide tablets in USP 24 was employed. Mobile phase was prepared by varying the amount of acetonitrile (0, 1, 3 and 5%) in phosphate buffer at pH

3.0. Phosphate buffer was considered to be the mobile phase and showed peaks of pyrazinamide and isoniazid at 8.18 and 9.5 minutes, respectively, as shown in figure 3.
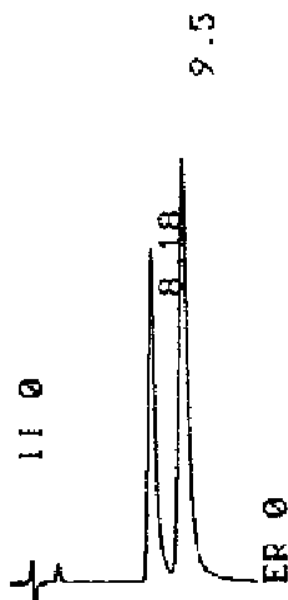

Figure 3 Chromatogram of isoniazid (8.18 mins) and pyrazinamide (9.5 mins).

The assay method for ethambutol in USP and BP has complicated sample extraction and non-aqueous titration. So we modified the identification method for ethambutol in Ethambutol Tablets BP 2001 and validated quantitatively by using spectrophotometric method. A blue complex between ethambutol and copper II ion in a basic medium was formed and the absorbance measured at 268 nm with Diode array UV-visible spectrophotometer (HP 8452 A, USA). The reaction was chelating between copper ion with hydroxy group and amine groups of ethambutol as shown in figure 4.
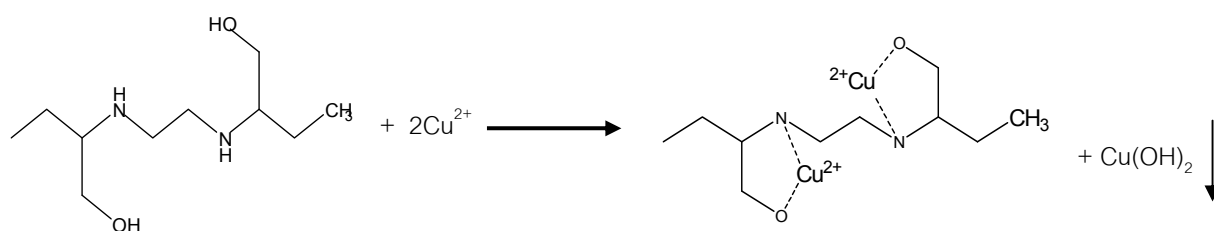

Figure 4 Reaction of ethambutol and cupric ion in basic medium.

3.2 Method validation

3.2.1 Specificity

Stressed degradation products of each drug solution at $80\,^{\circ}$C for 12 and 24 hours were analyzed by the described assay methods compared with the pure compounds. Chromatograms of rifampicin, isoniazid and pyrazinamide showed no peaks as interferences. Spectra of the standard ethambutol and the degraded solutions were the same pattern.

3.2.2 Linearity

The linear regression analysis was obtained by plotting the signals of each drug; peak area ratios for isoniazid, pyrazinamdie and rifampicin and absorbance for ethambutol, versus its concentrations. The results of slopes, intercepts and R^2 are shown in table 2.

Table 2 Results of Linearity from 5 replications of antituberculosis drugs.

Drugs	Slope±SD	Intercept±SD	R^2
Ethambutol	0.0158±0.0004	0.0212±0.116	0.9999
Isoniazid	137986.055±1476.354	−55664.066±4611.021	0.9993
Pyrazinamide	330208.806±3889.579	−2337.04±34714.961	0.9999
Rifampicin	0.1915±0.0130	−0.0635±0.050	0.9935

3.2.3 Accuracy and precision

The accuracy and precision were determined from both intra-day and inter-day analyses. Three levels of concentration in 5 replications were performed on three different days. Accuracy was calculated as %recovery and precision was calculated as %RSD as shown in table 3.

Table 3 Results of accuracy and precision.

Drugs	Conc (μg/ml)	Intra-day			Inter-day		
		Accuracy±SD	%RSD	n	Accuracy±SD	%RSD	n
Ethambutol	19.2	99.376±0.77	0.776	5	99.40±1.38	1.38	3
	36.0	99.02±1.19	1.20	5	98.98±1.07	1.08	3
	60.0	99.74±0.44	0.44	5	99.65±0.54	0.55	3
Isoniazid	2.5	101.35±2.33	2.30	5	103.38±2.23	2.16	3
	7.5	102.16±0.56	0.54	5	100.32±1.21	1.21	3
	12.5	99.83±0.91	0.91	5	100.21±1.10	1.10	3
Pyrazinamide	2.5	101.38±2.47	2.44	5	100.97±2.12	2.10	3
	7.5	99.78±1.32	1.32	5	99.66±0.90	0.91	3
	12.5	100.33±0.06	0.05	5	100.17±1.00	1.00	3

3.3 Non-isothermal stability study

One unit of each drug in 75 %RH at 80, 60, 70 and 50°C was collected at the defined time, analyzed and the drug concentrations reported as shown in figure 5 for isoniazid, pyrazinamide and rifampicin and figure 6 for ethambutol. The concentrations clearly decreased at 80°C for all drugs. Rifampicin also degraded at 70, 60 and 50°C at a faster rate than isoniazid and pyrazinamide. The data showed high variation of the concentrations since only one unit was sampled at each time. Ethambutol concentration decreased continuously although the temperatures changed. However, they still were useful for designing the isothermal stability study. To reduce the high variation of the concentration, ten units of the sample were collected at each time and ground homogeneously before the assay.
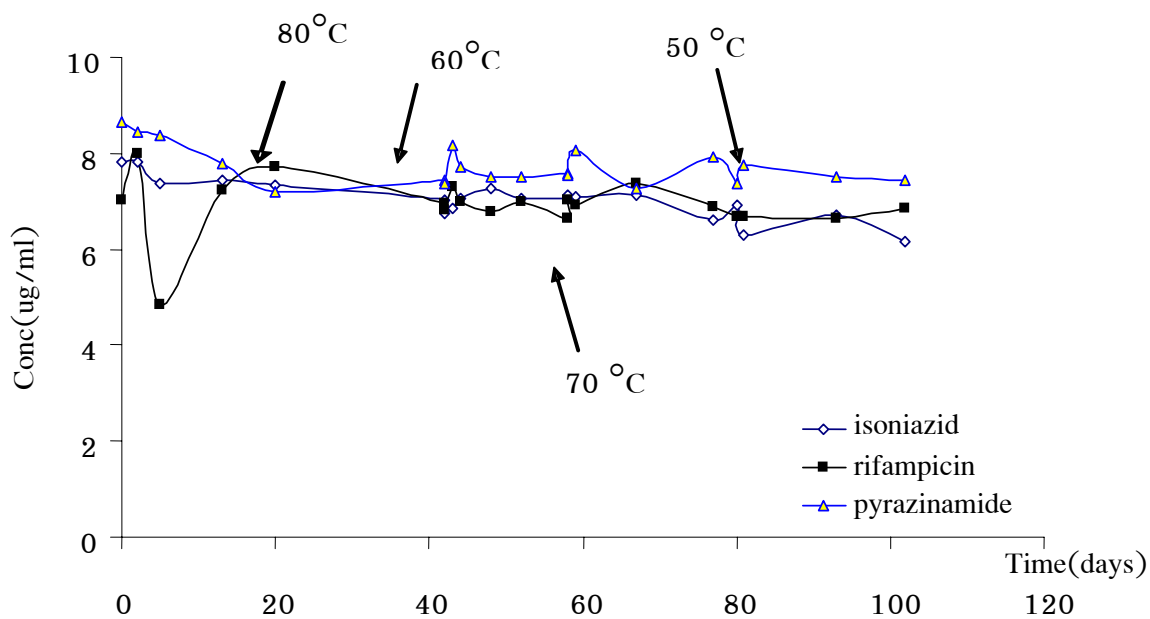
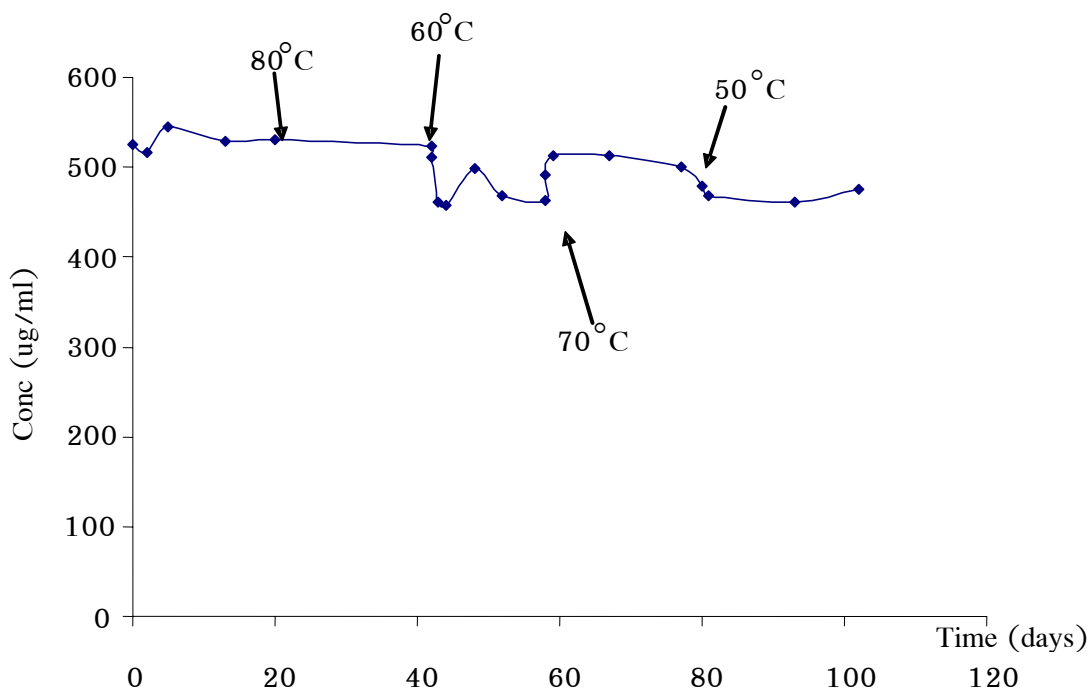Figure 5 Concentration changes of isoniazid, pyrazinamide and rifampicin.

Figure 6 Concentration changes of ethambutol.

The drug concentrations were computed by Non-isothermal analyzing data program version 1.0 (W. Wongpoowarak) to give the activation energy and gas constant ratio (E_a/R), initial concentration (C_0) and Arrhenius constant (A). These parameters shown in table 4 were selected from the least sum of squared error model. These parameters were used to calculate rate constant (k). The optimal sampling time and temperature for isothermal stability studies considered from k values were 30, 60, 90,120, 150 and 180 days at 80, 70 and 60°C for isoniazid, pyrazinamide and ethambutol; 70, 60, and 50°C for rifampicin. At high temperature, 80°C, caused the physical change of capsule of rifampicin, therefore the isothermal stability study of rifampicin was started at 70°C.

Table 4 Predicted kinetic parameters from non-isothermal stability at 30°C.

Drugs	C_0 (μg/ml)	E_a/R (K)	A (hours^{-1})
rifampicin	7.0	5899	4.76×10^3
isoniazid	6.6	14411	6.00×10^3
pyrazinamide	8.6	30455	3.90×10^{33}
ethambutol	596	17100	1.20×10^{17}

These parameters were used to calculate rate constant (k). The optimal sampling times and temperatures for isothermal stability studies which were considered from k values were 30, 60, 90, 120, 150 and 180 days at 80, 70 and 60°C for isoniazid, pyrazinamide and ethambutol 70, 60, and 50°C for rifampicin.

3.4 Program validation

The sets of rate constant were calculated by using E_a (20 kcal/mol is the normal values for pharmaceutics), A (120,000) and temperatures at 80, 70, 60 and

50°C. Concentration change data were simulated and then analyzed by the Non-isothermal analyzing data program version 1.0 (W. Wongpoowarak). E_a/R, A and T_{90} obtained from the program were plotted with simulated values shown in figure 7, 8 and 9, respectively.
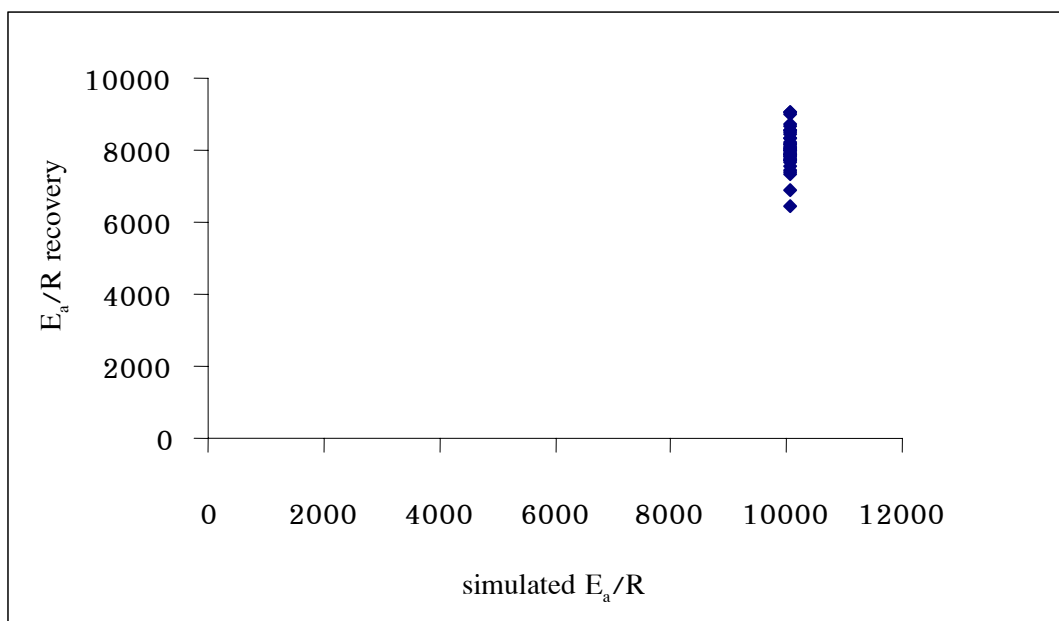

Figure 7 Plot of simulated Ea/R versus Ea/R recovery
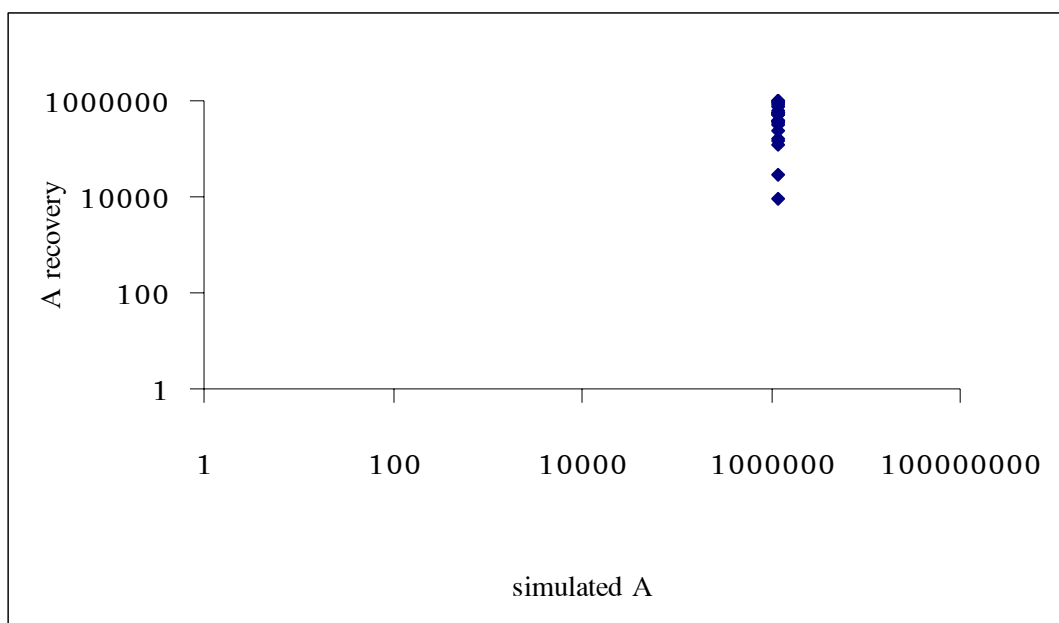
Figure 8 Plot of simulated A versus A recovery (log scale).
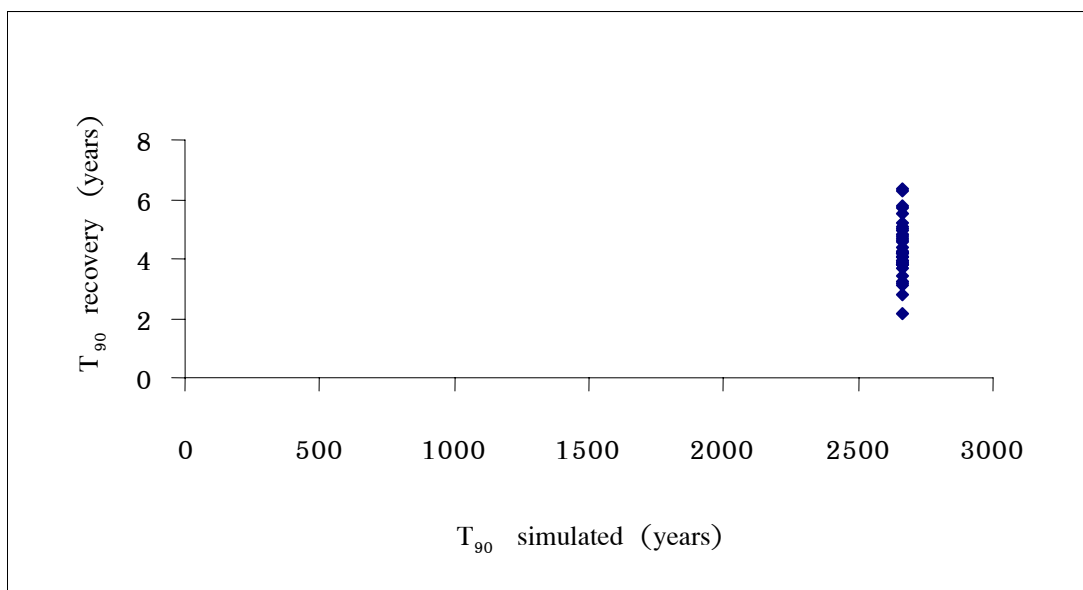
Figure 9 Plot of simulated T_{90} versus T_{90} recovery.

3.5 Drug quality

Ten units of the samples were analyzed individually and showed good uniformity as shown. The average, minimum, maximum, SD and %RSD of the percentage of amount were shown in table 5.

Table 5 Percentage of the amount of drugs before the isothermal stability study.

Drugs	Average	Minimum	Maximum	SD	%RSD
Ethambutol	98.03	96.95	99.57	0.97	0.99
Isoniazid	102.75	100.66	103.90	0.97	0.94
Pyrazinamide	102.94	101.34	105.47	1.38	1.34
Rifampicin	101.48	99.59	102.80	1.09	1.08

3.6 Isothermal stability study

For isothermal stability studies, samples were kept under nine conditions, three levels of %RH, 80, 50 and 20% and three temperatures for 180 days. Ten units of samples were sampled every month and the percentage of the remaining amount of drugs analyzed. The physical change for ethambutol was deliquescent in the strip especially at 80°C and 80%RH. For rifampicin at 70°C and 80%RH, the strip was slightly twisted. The capsules were sticky but the powder did not physically change. For isoniazid at 80°C and 80%RH, the tablets darkened to brown at the third month and later. For pyrazinamide in all conditions, the tablets were harder and had a few orange spots after the fifth month.

The percentage of rifampicin remaining in studied isothermal stability study greatly decreased at 70°C 80%RH and 70°C 50%RH as shown in figure 9. The percentage of rifampicin approximately decreased in the range of 5-45%. At 70°C in all relative humidity levels, the percentage of rifampicin decreased more than the percentage of rifampicin at 60°C and 50°C. ANOVA of percentage of rifampicin remaining data in table 6 showed that the interaction of time and temperature was significant (p<0.001). It implied that the patterns of change in rifampicin amount across time at all three temperatures were different. At 70°C, the rifampicin amount significantly decreased at the first month and decreased furthermore at the later time. At 50 and 60°C, the rifampicin amount significantly decreased at the fifth month and the third month and later as shown in table 7. The interaction of relative humidity and time in table 6 was insignificant (p=0.642) implying that the patterns of change of rifampicin across time at all three relative humidity were similar. The interaction of relative humidity and temperature in table 6 was significant (p=0.002). It implied that the pattern of rifampicin decreased at all three temperatures significantly was different across relative humidity. From table 8 there was no decreased in rifampicin at 60°C on three levels of relative humidity. However at 50 and 70 $^{\circ}$C rifampicin stored at 80%RH showed significant decrease compared to at 20 or 50%RH. The statistical results showed that temperature and humidity significantly affected rifampicin stability.

Table 6 ANOVA of percentage of rifampicin remaining from isothermal stability.

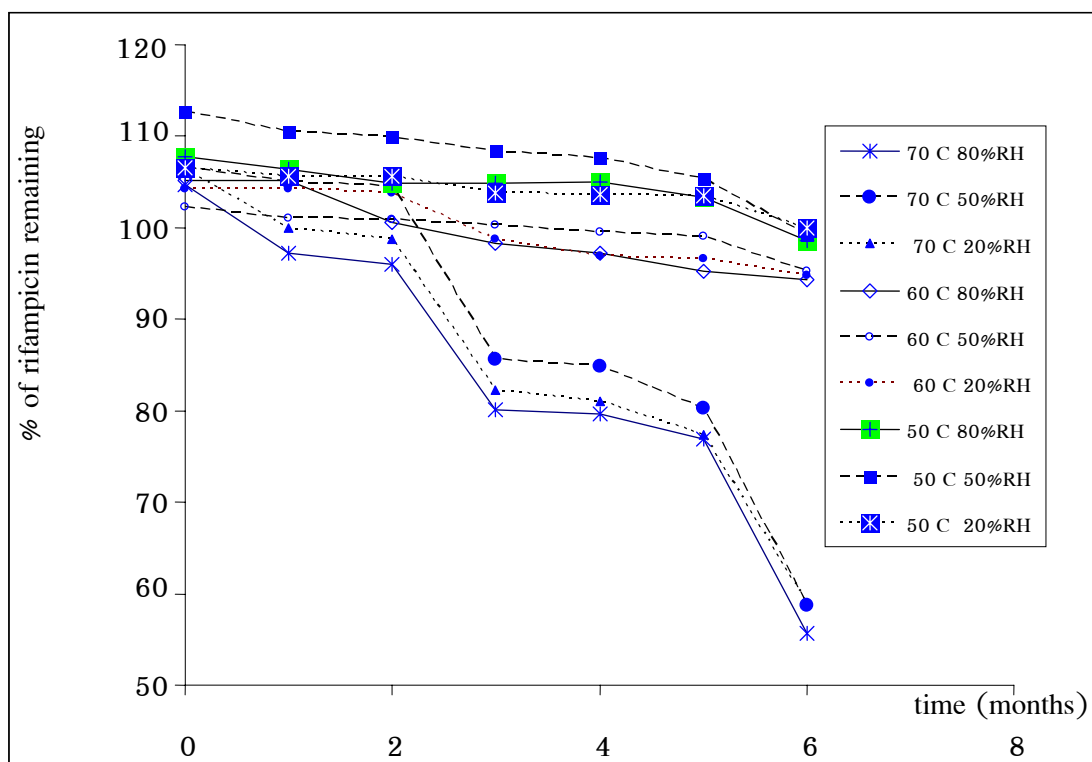| Source	Type III Sum of Squares	df	Mean Square	F	Sig.
RH	94.409	2	47.205	20.699	< 0.001
TEMP	3859.690	2	1929.845	846.218	< 0.001
RH * TEMP	51.394	4	12.848	5.634	0.002
TIME	3223.279	6	537.213	235.563	< 0.001
TIME * RH	22.054	12	1.838	0.806	0.642
TIME * TEMP	2224.586	12	185.382	81.288	< 0.001
Error	54.733	24	2.281		
Total	605432.734	63			

R Squared = 0.994 (Adjusted R Squared = 0.985)

Figure 10 Percentage of rifampicin remaining versus time at nine conditions from
   isothermal stability.

Table 7 Pairwise comparison between temperatures to times (months) of rifampicin.

TEMP	TIME (I)	TIME (J)	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference(a)	
						Lower Bound	Upper Bound
50.00	0.00	1.00	1.477	1.233	1.000	-2.711	5.664
		2.00	2.199	1.233	1.000	-1.988	6.386
		3.00	3.356	1.233	0.250	-0.832	7.543
		4.00	3.571	1.233	0.166	-0.616	7.759
		5.00	4.946(*)	1.233	0.011	0.759	9.133
		6.00	9.772(*)	1.233	<0.001	5.585	13.959
60.00	0.00	1.00	0.457	1.233	1.000	-3.731	4.644
		2.00	2.163	1.233	1.000	-2.025	6.350
		3.00	4.805(*)	1.233	0.014	0.617	8.992
		4.00	6.005(*)	1.233	0.001	1.817	10.192
		5.00	6.912(*)	1.233	<0.001	2.725	11.100
		6.00	9.162(*)	1.233	<0.001	4.974	13.349
70.00	0.00	1.00	-5.113(*)	1.233	0.008	-9.300	-0.926
		2.00	0.980(*)	1.233	0.001	-3.207	5.168
		3.00	18.085(*)	1.233	<0.001	13.897	22.272
		4.00	18.880(*)	1.233	<0.001	14.692	23.067
		5.00	22.587(*)	1.233	<0.001	18.399	26.774
		6.00	42.985(*)	1.233	<0.001	38.798	47.173

Based on estimated marginal means

* The mean difference is significant at the 0.05 level.

a Adjustment for multiple comparisons: Bonferroni.

Table 8 Pairwise comparisons between temperatures to relative humidity of rifampicin

TEMP	RH (I)	RH (J)	Mean Difference (I–J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference(a)	
						Lower Bound	Upper Bound
50.00	20.00	50.00	−3.582(*)	0.807	0.001	−5.659	−1.505
		80.00	−0.341	0.807	1.000	−2.418	1.737
	50.00	80.00	3.241(*)	0.807	0.002	1.164	5.319
60.00	20.00	50.00	0.109	0.807	1.000	−1.969	2.186
		80.00	0.459	0.807	1.000	−1.619	2.536
	50.00	80.00	0.350	0.807	1.000	−1.728	2.427
70.00	20.00	50.00	−3.023(*)	0.807	0.003	−5.101	−0.946
		80.00	2.022	0.807	0.058	−0.055	4.100
	50.00	80.00	5.046(*)	0.807	<0.001	2.968	7.123

Based on estimated marginal means

* The mean difference is significant at the 0.05 level.

a Adjustment for multiple comparisons: Bonferroni.

The percentage of ethambutol at 80°C 80%RH and 70°C 80%RH greatly decreased compared to the other condition as shown in figure 12. The percentage of ethambutol approximately decreased in the range of 25–70%. The ANOVA of the percentage of ethambutol remaining in table 9 showed that the interaction of temperature and time was insignificant ($p=0.509$). It implied that the patterns of change of the percentage of ethambutol across time at all three temperatures were similar. From table 9, the interaction of relative humidity and time was significant ($p<0.001$). It implied that the patterns of change of the percentage of ethambutol across all three relative humidity levels were different. From table 11, at 20 and 80%RH; the ethambutol amount significantly decreased at the fourth month and later, while at 50%RH, it significantly decreased at the fifth month and later. The main effect of temperature in table 9 was considered, it was significant ($p<0.001$). From table 10, ethambutol stored at 80°C showed significant decrease compared to at 70 or 60°C. The statistical result showed that temperature and humidity significantly affected ethambutol stability.

Table 9 ANOVA of percentage of ethambutol remaining from isothermal stability.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
RH	883.610	2	441.805	18.500	< 0.001
TEMP	460.303	2	230.151	9.637	< 0.001
RH * TEMP	246.602	4	61.650	2.582	0.063
TIME	5853.478	6	975.580	40.851	< 0.001
TIME * RH	1549.142	12	129.095	5.406	< 0.001
TIME * TEMP	275.383	12	22.949	0.961	0.509
Error	573.148	24	23.881		
Total	399113.991	63			

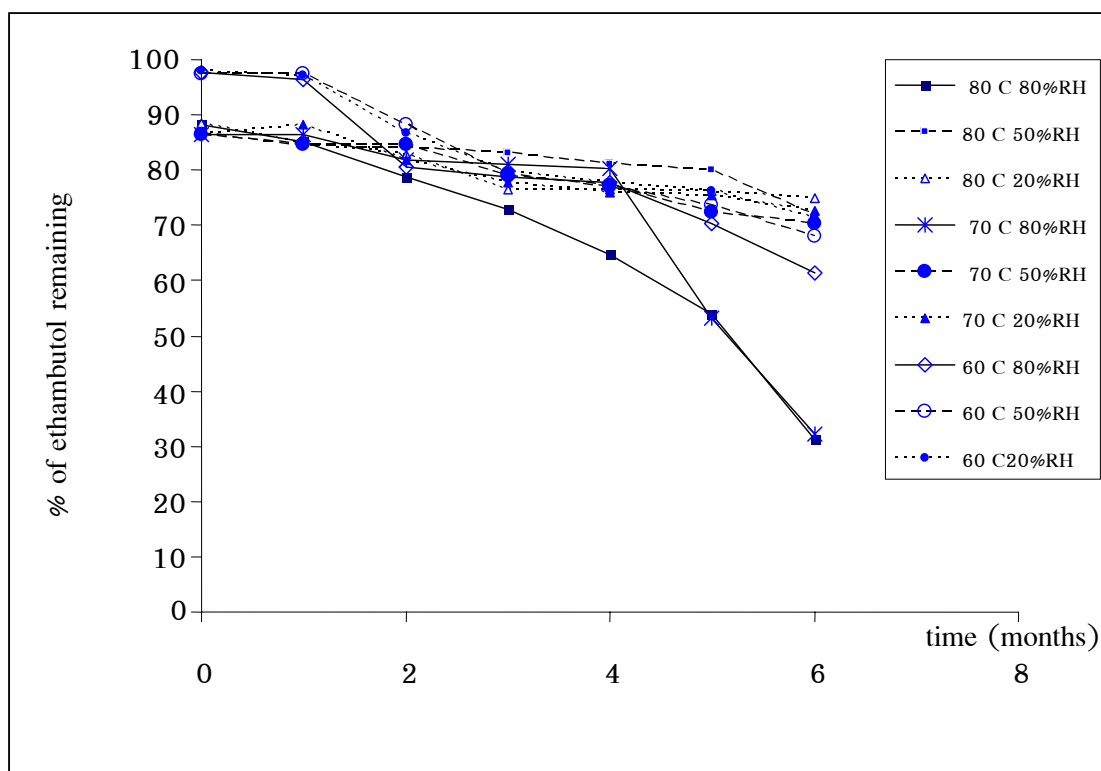R Squared = 0.942 (Adjusted R Squared = 0.85)

Figure 11 Percentage of ethambutol remaining versus time at nine conditions from
isothermal stability.

Table 10 Multiple comparison temperature of ethambutol

Tukey HSD

TEMP (I)	TEMP (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
60.00	70.00	5.5289(*)	1.50811	0.003	1.7627	9.2951
	80.00	5.9192(*)	1.50811	0.002	2.1530	9.6854
70.00	80.00	0.3903	1.50811	0.964	-3.3759	4.1565

Based on observed means.

* The mean difference is significant at the 0.05 level.

Table 11 Pairwise comparisons between relative humidity to time (months) of ethambutol

RH	TIME (I)	TIME (J)	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference(a)	
						Lower Bound	Upper Bound
20.00	0.00	1.00	0.939	3.990	1.000	−12.612	14.489
		2.00	7.150	3.990	1.000	−6.400	20.700
		3.00	12.946	3.990	0.072	−0.605	26.496
		4.00	14.243(*)	3.990	0.033	0.692	27.793
		5.00	15.046(*)	3.990	0.020	1.496	28.596
		6.00	17.962(*)	3.990	0.003	4.412	31.512
50.00	0.00	1.00	1.517	3.990	1.000	−12.034	15.067
		2.00	4.726	3.990	1.000	−8.825	18.276
		3.00	9.859	3.990	0.440	−3.691	23.410
		4.00	11.891	3.990	0.137	−1.660	25.441
		5.00	14.918(*)	3.990	0.021	1.367	28.468
		6.00	20.238(*)	3.990	0.001	6.687	33.788
80.00	0.00	1.00	1.492	3.990	1.000	−12.058	15.043
		2.00	10.442	3.990	0.317	−3.108	23.992
		3.00	13.198	3.990	0.062	−0.352	26.748
		4.00	16.642(*)	3.990	0.007	3.092	30.192
		5.00	31.619(*)	3.990	<0.001	18.068	45.169
		6.00	49.212(*)	3.990	<0.001	35.662	62.762

Based on estimated marginal means

* The mean difference is significant at the 0.05 level.

a Adjustment for multiple comparisons: Bonferroni.

The percentage of pyrazinamide remaining after isothermal stability greatly decreased at 80°C 80%RH and 70°C 80%RH, compared to other conditions as shown in figure 12. The percentage of pyrazinamide approximately decreased in the range of 5-25%. The ANOVA of the percentage of pyrazinamide remaining in table 12 showed that the interaction of temperature and time was significant ($p<$0.001). This implied that the pattern of change in the percentage of pyrazinamide remaining was different at 80, 70 and 60°C. At 70°C and 80°C, the percentage of pyrazinamide decreased significantly at the fourth and the first month, respectively ($p<$0.001). At 60°C, pyrazinamide remained stable for six months as shown in table 14. The interaction of relative humidity and time in table 13 was insignificant ($p=$0.060). It implied that the patterns of change in the percentage of pyrazinamide remaining across time at all three relative humidity were similar. The interaction of relative humidity and temperature in table 12 was significant ($p=$0.004). It implied that the pattern of pyrazinamide decrease at all three temperatures across relative humidity significantly was different. Form table 14, there was no decrease in pyrazinamide at 60°C on three levels of relative humidity. However at 70 $^{\circ}$C, pyrazinamide stored at 20%RH showed significant decrease compare to at 50 or 80%RH. At 80°C, pyrazinamide stored at 80%RH showed significant decrease compared to those at 20 and 50%RH. The statistical result showed that temperature and humidity significantly affected pyrazinamide stability.

Table 12 ANOVA of percentage pyrazinamide remaining from isothermal stability.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
RH	316.233	2	158.117	11.120	< 0.001
TEMP	23.111	2	11.556	0.813	0.456
RH * TEMP	285.981	4	71.495	5.028	0.004
TIME	2469.350	6	411.558	28.943	< 0.001
TIME * RH	357.234	12	29.769	2.094	0.060
TIME * TEMP	1407.008	12	117.251	8.246	< 0.001
Error	341.270	24	14.220		
Total	595930.001	63			

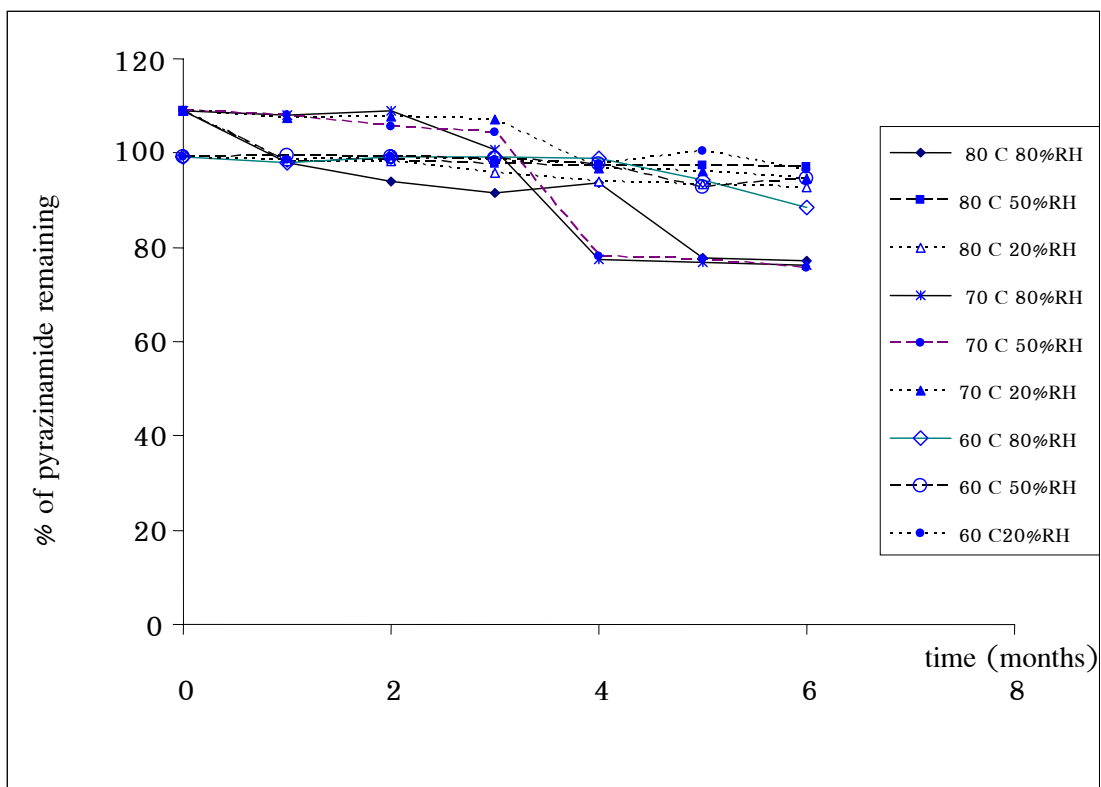R Squared = 0.934 (Adjusted R Squared = 0.830)

Figure 12 Percentage of pyrazinamide remaining versus time at nine conditions from

isothermal stability.

Table 13 Pairwise Comparisons between temperature to time (months) of pyrazinamide

TEMP	TIME (I)	TIME (J)	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference(a)	
						Lower Bound	Upper Bound
60.00	0.00	1.00	0.567	3.079	1.000	-10.053	10.859
		2.00	1.434	3.079	1.000	-10.429	10.483
		3.00	1.612	3.079	1.000	-10.299	10.613
		4.00	1.879	3.079	1.000	-9.406	11.506
		5.00	2.046	3.079	1.000	-7.256	13.656
		6.00	2.384	3.079	1.000	-4.569	16.343
70.00	0.00	1.00	0.452	3.079	1.000	-9.333	11.579
		2.00	1.574	3.079	1.000	-8.966	11.946
		3.00	2.382	3.079	1.000	-5.519	15.393
		4.00	9.416(*)	3.079	<0.001	14.524	35.436
		5.00	9.939(*)	3.079	<0.001	15.047	35.959
		6.00	11.764(*)	3.079	<0.001	16.391	37.303
80.00	0.00	1.00	2.934(*)	3.079	0.035	0.434	21.346
		2.00	5.376(*)	3.079	0.013	1.684	22.596
		3.00	7.826(*)	3.079	0.003	3.474	24.386
		4.00	9.279(*)	3.079	<0.001	3.597	24.509
		5.00	9.900(*)	3.079	<0.001	9.077	29.989
		6.00	10.370(*)	3.079	<0.001	9.597	30.509

Based on estimated marginal means

* The mean difference is significant at the 0.05 level.

a Adjustment for multiple comparisons: Bonferroni.

Table 14 Pairwise comparison between temperature to relative humidity of pyrazinamide.

TEMP	RH (I)	RH (J)	Mean Difference (I–J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference(a)	
						Lower Bound	Upper Bound
60.00	20.00	50.00	1.154	2.016	1.000	−4.033	6.342
		80.00	1.864	2.016	1.000	−3.323	7.052
	50.00	80.00	0.710	2.016	1.000	−4.477	5.897
70.00	20.00	50.00	8.724(*)	2.016	0.010	3.537	13.912
		80.00	8.817(*)	2.016	0.010	3.630	14.005
	50.00	80.00	0.093	2.016	1.000	−5.095	5.280
80.00	20.00	50.00	−2.056	2.016	0.954	−7.243	3.132
		80.00	5.776(*)	2.016	0.026	0.588	10.963
	50.00	80.00	7.731(*)	2.016	0.002	2.644	13.019

Based on estimated marginal means

* The mean difference is significant at the 0.05 level.

a Adjustment for multiple comparisons: Bonferroni.

Isoniazid greatly decreased at $80^{\circ}C$ 80%RH as shown in figure 13. The percentage of isoniazid decreased approximately in the range of 11–77%. The ANOVA of percentage isoniazid remaining in table 15 showed that interaction of temperature and time was insignificant (p=0.134). It implied that the patterns of change of the percentage of isoniazid across times at all three temperatures were similar. The interaction of relative humidity and time in table 14 was insignificant (p=0.319). It implied that the patterns of change of the percentage of isoniazid across time in all relative humidity were similar. The interaction of relative humidity and temperature in table 15 was significant (p<0.001). It implied that the pattern of isonaizid decrease at all three temperatures was different across three relative humidity levels. From table 16, there was no decrease on isoniazid at 60 or $70^{\circ}C$ on three levels of humidity. However at $80^{\circ}C$, isoniazid at 80%RH showed significant decrease compared to those at 20 or 50%RH. The statistical result showed that temperature and humidity significantly affected isoniazid stability.

Table 15 ANOVA of percentage isoniazid remaining from isothermal stability.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
RH	3607.203	2	1803.601	18.863	< 0.001
TEMP	10471.410	2	5235.705	54.758	< 0.001
RH * TEMP	5897.662	4	1474.415	15.420	< 0.001
TIME	4458.325	6	743.054	7.771	< 0.001
TIME * RH	1413.555	12	117.796	1.232	0.319
TIME * TEMP	1931.996	12	161.00	1.684	0.134
Error	2294.770	24	95.615		
Total	465269.682	63			

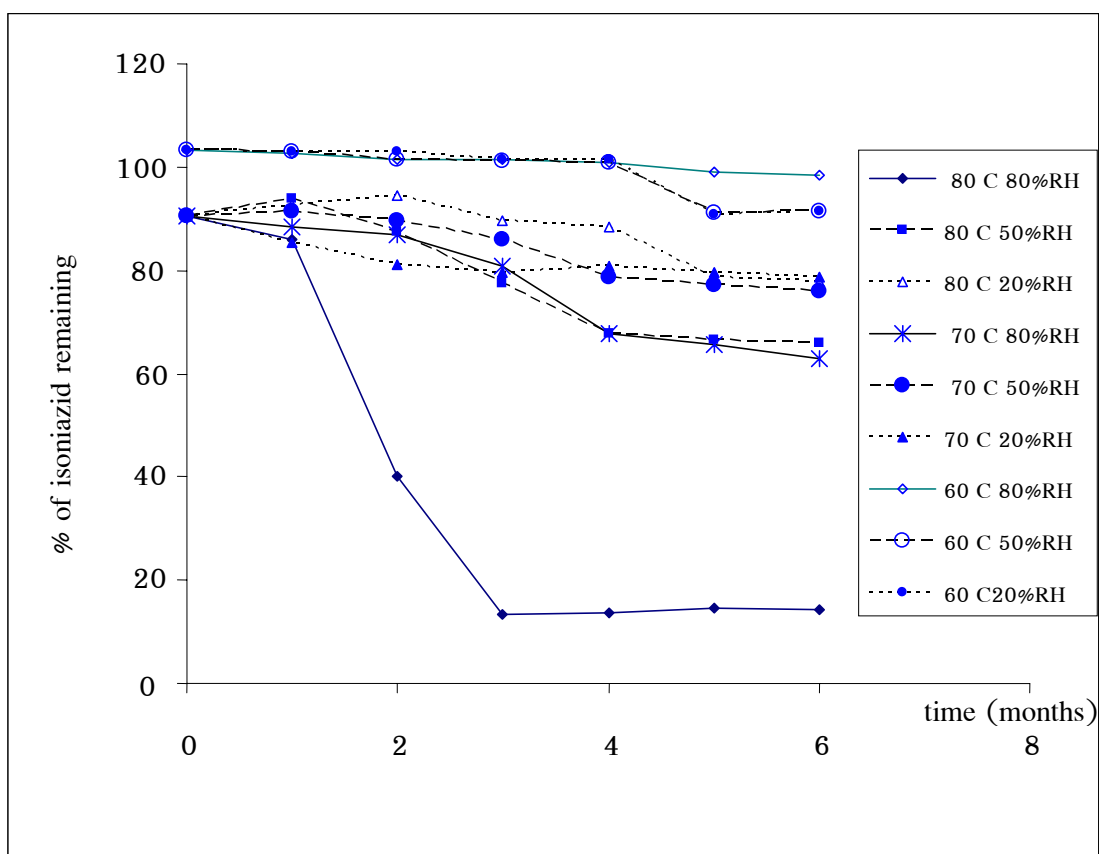R Squared = 0.924 (Adjusted R Squared = 0.803)

Figure 13 Percentage of isoniazid remaining versus time at nine conditions from

isothermal stability.

Table 16 Pairwise comparisons between temperature to relative humidity of isoniazid

TEMP	RH (I)	RH (J)	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference(a) Lower Bound	Upper Bound
60.00	20.00	50.00	0.314	5.227	1.000	-13.137	13.766
		80.00	-1.824	5.227	1.000	-15.276	11.628
	50.00	80.00	-2.138	5.227	1.000	-15.590	11.314
70.00	20.00	50.00	-1.877	5.227	1.000	-15.329	11.575
		80.00	4.752	5.227	1.000	-8.700	18.203
	50.00	80.00	6.629	5.227	0.651	-6.823	20.080
80.00	20.00	50.00	8.943	5.227	0.300	-4.509	22.395
		80.00	48.491(*)	5.227	<0.001	35.040	61.943
	50.00	80.00	39.549(*)	5.227	<0.001	26.097	53.000

Based on estimated marginal means

* The mean difference is significant at the 0.05 level.

a Adjustment for multiple comparisons: Bonferroni.

Predicted kinetic parameters consisting of the activation energy and gas constant ratio (E_a/R) and the Arrhenius constant (A) at and 30°C from isothermal stability study showed in table 17. These two parameters were compared with the predicted parameters from the non-isothermal stability method shown in table 4. These predicted parameters values from non-isothermal stability study were more than the predicted parameters values from isothermal stability study. The Arrhenius parameters from non-isothermal stability were not acceptable for predicting shelf-lives. The non-isothermal stability method was unacceptable (Vyazovkin *et al.*, 1999 and Galway, 2003) for the reason that it may be unknown reaction model and decomposition involved several step (Vyazovkin *et al.*, 1999). However, it can be useful for planning isothermal stability testing which was more acceptable.

From the k 25°C and k 30°C, drugs shelf-lives predicted by the Arrhenius method were shown in table 18. Ethambutol had shelf-life less than three years while isoniazid and rifampicin had shelf-lives more than three years except isoniazid in 20%RH. Pyrazinamide had shelf-life more than five years except pyrazinamide in 50%RH. The shelf-lives of ethambutol, isoniazid, rifampicin and pyrazinamide were reported 5, 3, 3 and 3 years respectively (Bureau India, 2006 and IPCH Homepage). The shelf-life of ethambutol, isoniazid at 20%RH and pyrazinamide at 50%RH were quite atypical.

The Arrhenius method gave atypical E_a/R values of ethambutol, so its shelf-life is not reasonable. Therefore we used another extrapolation technique by the Yoshioka for shelf-life calculating (Yoshioka and Cartensen, 1990). This method was used for stability associated with both humidity and temperature. The humidity and temperature were changed in terms of vapor pressure. The method required that the humidity condition at each temperature be below the critical relative humidity (CRH); the condition at which the solid dissolved. The fraction, x (%), of drug decomposed after storage time, t (day), a temperature, T (Celsius), and a vapor pressure, P (mmHg), was given by the following formula:

$$x = kP^s t^n$$

where P was water vapor pressure, t was storage time and k, s and n were constants. The k, s and n constants were found by non-linear regression, which consist of seven estimated methods, Quai-newton, simplex, simplex and Quai-newton, hooke-Jeeves pattern moves, hooke-Jeeves and Quai-newton, Rosenbrock pattern search and Rosenbrock and Quai-newton. The constants from the estimated method, which gave the smallest SSE, were

selected to extrapolate the shelf-life. The k, s and n constants of ethambutol, isoniazid, rifampicin and pyrazinamide were estimated by nonlinear regression by hooke-Jeeves and Quai-newton estimated methods as shown in table 18. The shelf-lives of ethambutol, isoniazid, rifampicin and pyrazinamide extrapolated by using the constants of each drug at 25 and 30°C and 20, 50 and 80% RH as shown in Table 18. Ethambutol and rifampicin had shelf-life less than three years except for rifampicin at 25°C and 20%RH. Isoniazid had shelf-life more than three years except at 30°C and 80 and 50%RH and 25°C 80%RH. Pyrazinamide had shelf-life less than five years.

The Arrhenius method was suitable for predicting homogenous system such as solution. Sometime the Arrhenius behavior was not linear because of phase transition, temperature and complex reaction mechanism (Waterman *et al.*, 2005). For this stability study, the drugs are in solid dosage form. It might be not suitable for predicting with the Arrhenius method because there were some interfering factors such as packaging, heterogeneous system. Also the drugs were studied in the packaging, ethambutol in aluminum foil, rifampicin in blister pack. The condition inside packaging was different from the experimental conditions. Therefore the reaction and mechanism of drugs decompositions occurred might be different from unpackaged dosage form.

The role of packaging is to protect pharmaceutical dosage forms from environment. In this study, blister pack and aluminum foil were used. The literature reported that, moisture sensitive compound tablets (PEG-7762928) in polyvinyl chloride blister, cyclic olefin blister and aclar blister stored at 40°C and 75%RH for six months were determined 84-97% active ingredient, respectively while aluminum foil could protect extremely all active ingredient (Allinson *et al.*, 2001). The polyvinyl chloride with laminate of polymonochlorotrifluooethylene protected pharmaceutical dosage form from moisture more than the polyvinyl chloride blister (Amidon, 1988). Blister pack and aluminum foil laminated with high-density polyethylene, polystyrene and polyvinyl chloride could resist the heat (high temperature) at the fair to poor level.

The predicted shelf-lives of ethambutol tablets in aluminum foil at 25 and 30°C by the Arrhenius and the Yoshioka methods were less than three years while the commercial pharmaceutical was five years. Ethambutol might be not suitable for predicting with two methods because it had phase transition and the condition study may be above critical relative humidity that did not meet for the Arrhenius method and the Yoshioka

method, respectively. The 80°C and 80%RH condition for ethambutol may be not appropriate because deliquescence occurred and did not fit the requirement of Arrhenius and the Yoshioka methods.

The important limits for this study were sampling time interval and number of sample. Total sampling time for some conditions were not long enough to observe deterioration moreover the changing of drugs may be in lag time. Therefore isothermal stability study should be longer at least one half-life in order to accurately predict shelf-life. Using tablets/capsules in/out package might be a limit of the non-isothermal stability study since variation of content in each unit therefore powder should be used instead.

Table 17 Predicted kinetic parameters of antituberculosis drugs from isothermal stability at 25°C and 30°C by using Arrhenius equation.

Drugs	%RH	Ea/R (K)	A	k at 25°C (months^{-1})	$t_{90\%}$ 25°C (years)	k at 30°C (months^{-1})	$t_{90\%}$ 30°C (years)
ethambutol	80	4258.50	2.88×10^{4}	1.79434×10^{-2}	< 3	2.27152×10^{-2}	< 3
	50	-5380.61	5.93×10^{-9}	4.11858×10^{-1}	< 3	3.05737×10^{-1}	< 3
	20	-3961.00	3.55×10^{-7}	2.10220×10^{-1}	< 3	1.6882×10^{-1}	< 3
isoniazid	80	22503.45	1.88×10^{27}	3.01512×10^{-6}	>3	1.0483×10^{-5}	>3
	50	6673.10	1.07×10^{7}	2.00756×10^{-3}	>3	2.9050×10^{-3}	>3
	20	1710.06	3.5114026	1.13058×10^{-2}	< 3	1.2429×10^{-2}	< 3
pyrazinamide	80	7648.06	1.92×10^{8}	1.37169×10^{-4}	> 5	2.0950×10^{-3}	< 5
	50	1711.14	3.1871855	1.02247×10^{-2}	< 5	1.1241×10^{-2}	< 5
	20	14322.35	1.49×10^{16}	2.0029×10^{-5}	> 5	4.4271×10^{-5}	> 5
rifampicin	80	11357.42	1.83×10^{13}	5.12347×10^{-4}	>3	9.6094×10^{-4}	>3
	50	8834.65	8.40×10^{9}	1.11909×10^{-3}	>3	1.8253×10^{-3}	>3
	20	12598.49	6.75×10^{14}	2.94200×10^{-4}	>3	5.9105×10^{-4}	>3

Table 18 Constants from nonlinear regression (hooke-Jeeves and Quai-newton estimated
method) and extrapolated shelf-lives at 25 and 30°C.

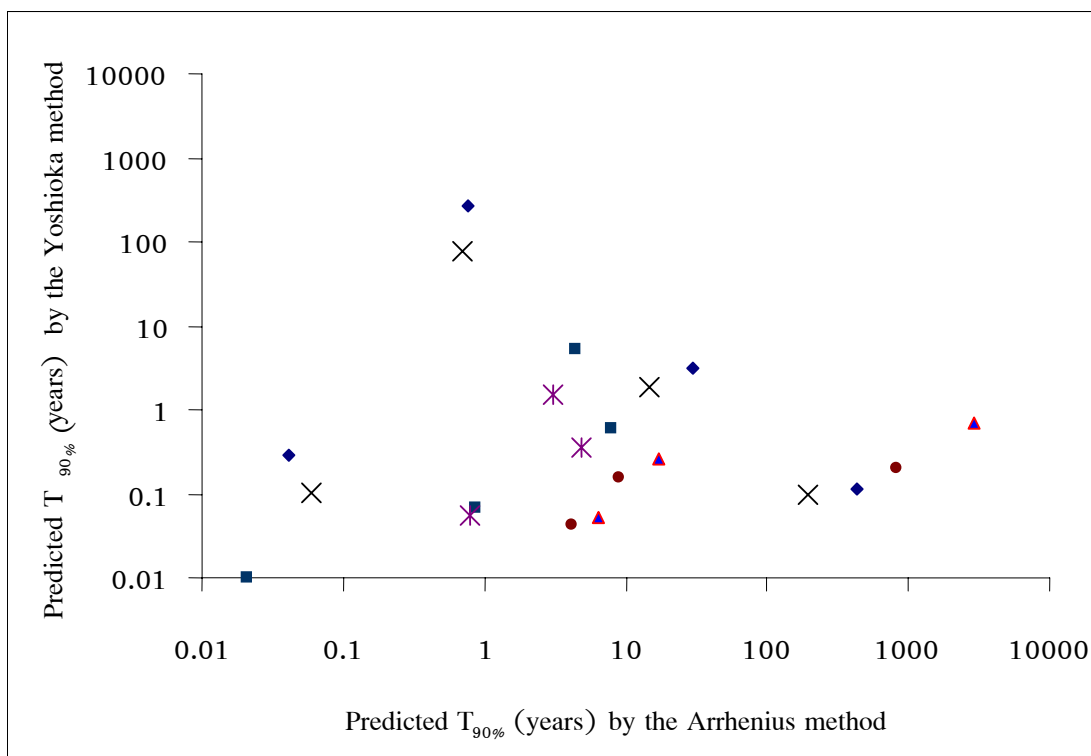| Drugs	%RH	Constants			Shelf-lives (years)	
		k (day^{-1})	n	s	at 25°C	at 30°C
Ethambutol	80	3.010x10^{-4}	0.4853	1.7864	< 3	<3
	50				< 3	< 3
	20				< 3	<3
Isoniazid	80	1.392x10^{-7}	0.6892	2.9449	< 3	< 3
	50				>3	< 3
	20				>3	>3
Pyrazinamide	80	1.009x10^{-1}	0.6622	0.3759	<5	<5
	50				<5	<5
	20				<5	<5
Rifampicin	80	5.260x10^{-4}	0.7605	1.3691	<3	<3
	50				<3	< 3
	20				>3	<3

Figure 14 Correlation of the predicted shelf-lives of antituberculosis drugs ($T_{90\%}$) at 25
and 30°C by the Arrhenius method and the Yoshioka method.

Corrrelation of the predicted shelf-lives of antituberculosis drugs at 25 and 30°C by the Arrhenius method and the Yoshioka were shown in figure 14. The Yoshioka model is more generalizes than the Arrhenius model, for it include relative humidity into relationship.

The recovery of ethambutol, isoniazid, pyrazinamide and rifampicin by two methods, the Arrhenius method and the Yoshioka method was shown in figure 15, 16, 17 and 18 respectively. In figure 15, at 80, 70 and 60°C the Arrhenius method gave better prediction for ethambutol at all conditions than the Yoshioka method. At 80°C and 20%RH and at 70°C and 50%RH, two methods could predict ethambutol closely. In figure 16, at 80 and 70°C the Arrhenius method gave better prediction for isoniazid than the Yoshioka method did. At 60°C the Yoshioka method gave better prediction for isoniazid than the Arrhenius did expect for 50%RH. In figure 17, at 80 and 70°C, the Arrhenius method gave better prediction for pyrazinamide than the Yoshioka method did. At 60°C the Yoshioka method gave better predicted for pyrazinamide than the Arrhenius method did except for 20%RH. In figure 18, at 70, 60 and 50°C the Arrhenius method

gave better prediction for rifampicin at all conditions than the Yoshioka method did. At 70°C and 80%RH and at 60°C and 50%RH, two methods could predict closely.
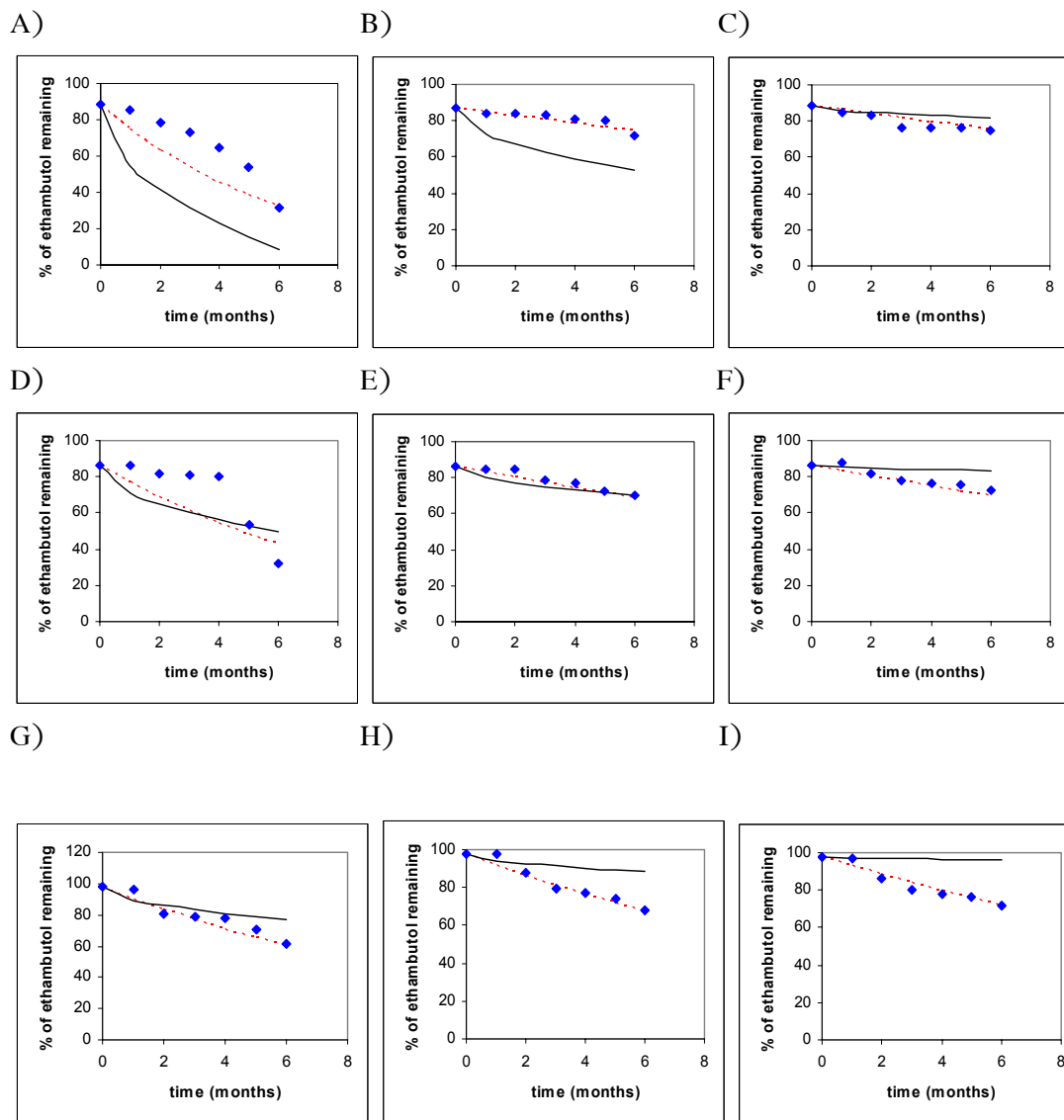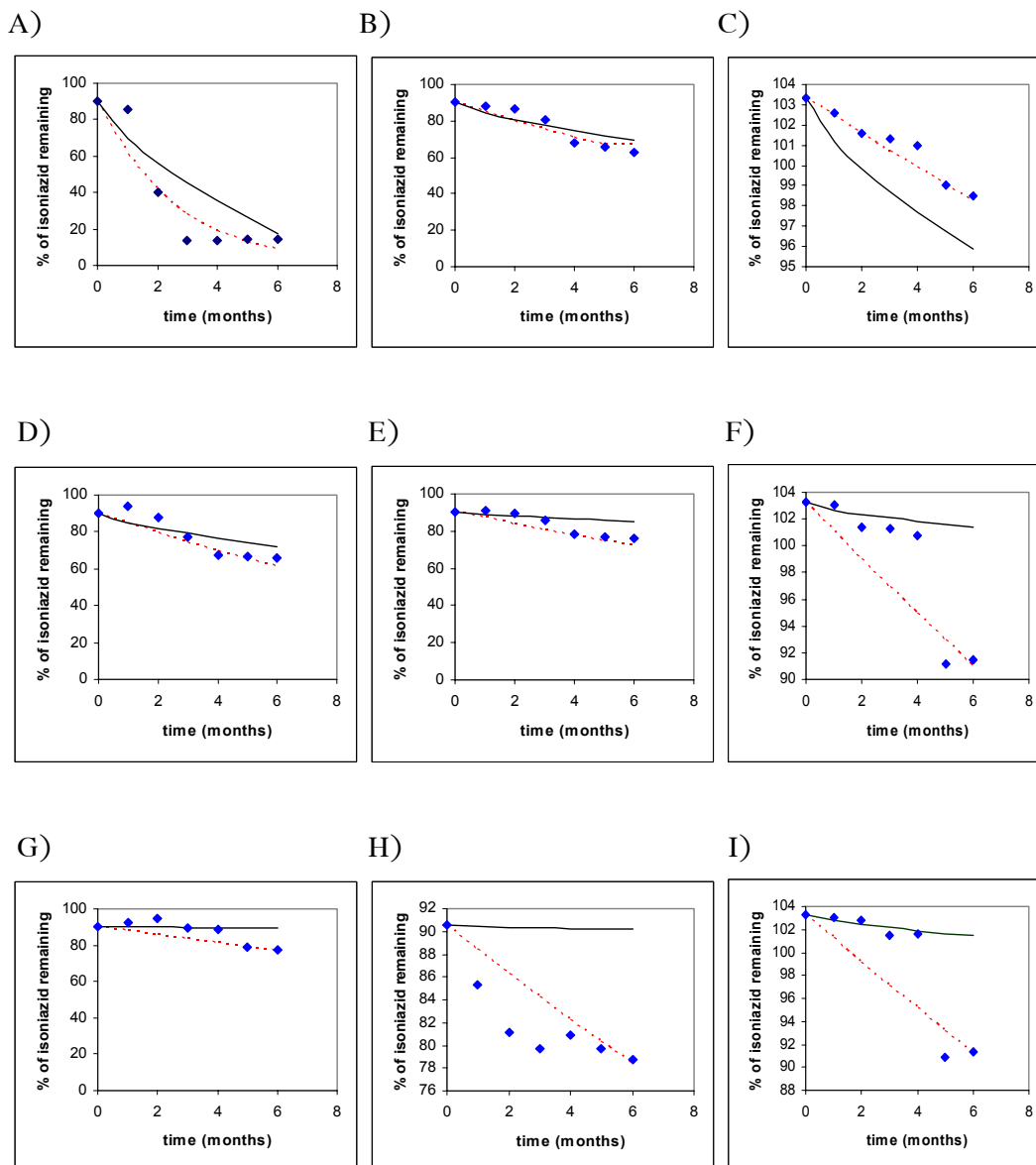
Figure 15 Compared Arrhenius method (········)   and Yoshioka method (——) for
predicting percentage of ethambutol remaining after isothermal-stability study at
nine conditions (A) at 80°C and 80%RH, B) at 70°C and 80%RH,
C) 60°C and 80%RH, D) at 80°C and 50%RH, E) at 70°C and 50%RH,
F) 60°C and 50%RH, G) at 80°C and 20%RH, H) at 70°C and 20%RH,
I) 60°C and 20%RH).

Figure 16 Compared Arrhenius method ($\cdots\cdots$) and Yoshioka method (——) for predicting percentage of isoniazid remaining after isothermal-stability study at nine conditions (A) at 80°C and 80%RH, B) at 70°C and 80%RH, C) 60°C and 80%RH, D) at 80°C and 50%RH, E) at 70°C and 50%RH, F) 60°C and 50%RH, G) at 80°C and 20%RH, H) at 70°C and 20%RH, I) 60°C and 20%RH).
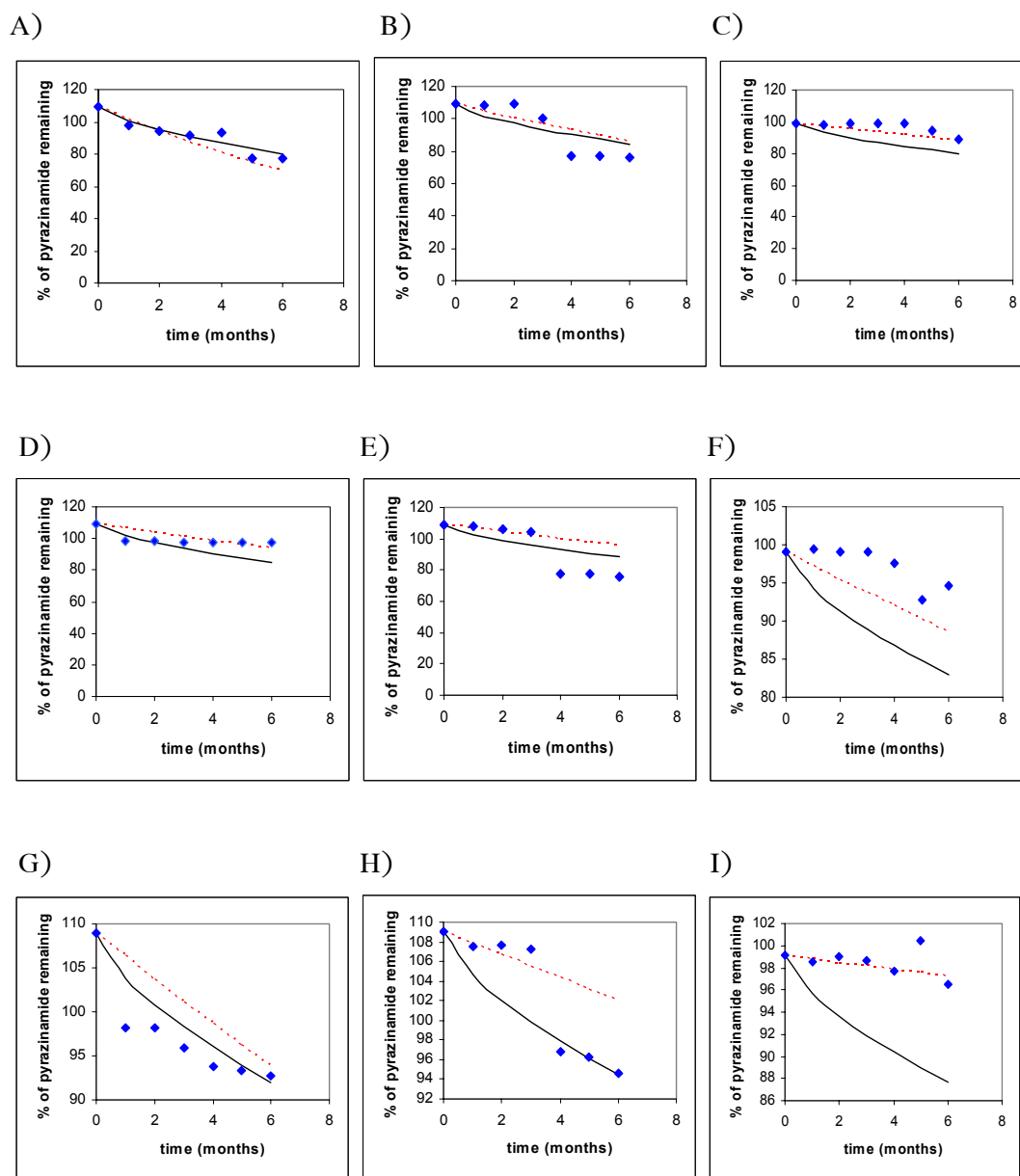
Figure 17 Compared Arrhenius (........) method and Yoshioka method (——) for
predicting percentage of pyrazinamide remaining after isothermal-stability study
at nine conditions (A) at 80°C and 80%RH, B) at 70°C and 80%RH,
C) 60°C and 80%RH, D) at 80 and 50%RH, E) at 70°C and 50%RH,
F) 60°C and 50%RH, G) at 80°C and 20%RH, H) at 70°C and 20%RH,
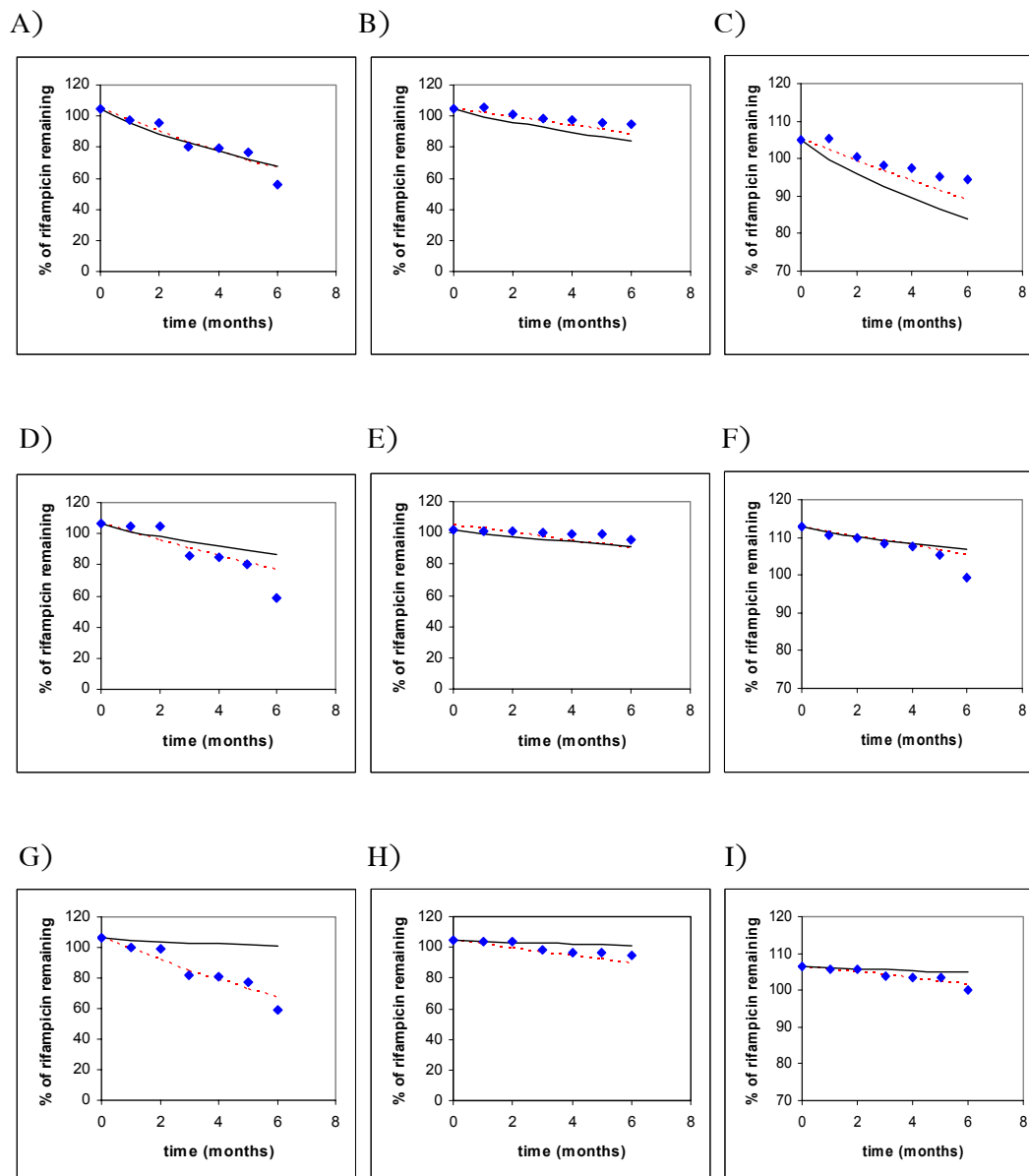I) 60°C and 20%RH).

Figure 18 Compared Arrhenius method (.........)   and Yoshioka method (———) for predicting percentage of rifampicin remaining after isothermal stability study at nine conditions. (A) at 70°C and 80%RH, B) at 60°C and 80%RH, C) 50°C and 80%RH, D) at 70°C and 50%RH, E) at 60°C and 50%RH, F) 50°C and 50%RH, G) at 70°C and 80%RH, H) at 60°C and 50%RH, I) 50°C and 20%RH).