CHAPTER IV

RESULTS AND DISCUSSION

The absorption spectra of solutions of chlorzoxazone, paracetamol and their mixture in methanol are shown in Figure 11 over the wavelength range of 200-400 nm. The wavelength of maximum absorbance of chlorzoxazone and paracetamol are 283 and 248 nm, respectively. It can be seen that the absorption spectra of chlorzoxazone and paracetamol are overlapped. Therefore, PCR and PLSR calibration methods are necessary for simultaneous quantitative determination of the two drugs in pharmaceutical preparation without preliminary chemical separation step.

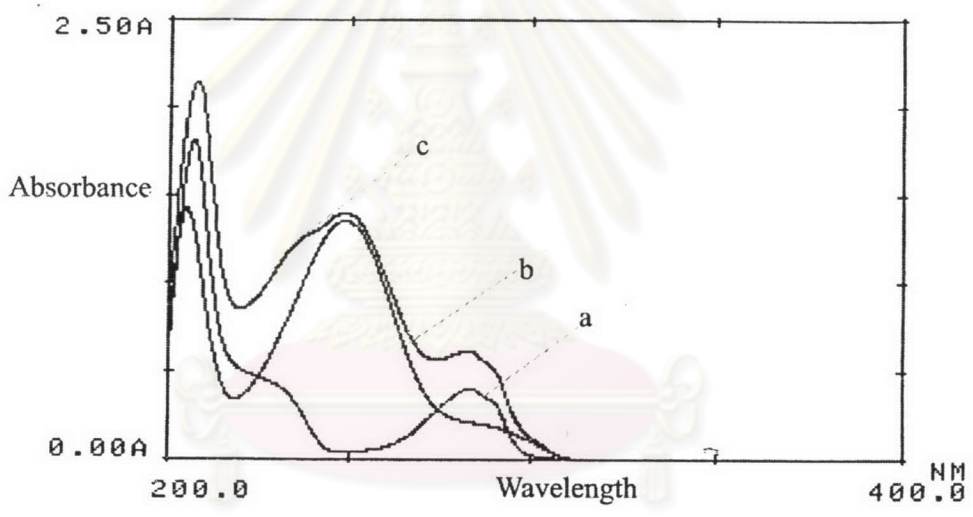

Figure 11 UV absorption spectra of (a) chlorzoxazone (12.5 µg/ml), (b) paracetamol (15 µg/ml) and (c) their mixture in methanol.

The absorption spectra of standard mixture solutions of chlorzoxazone and paracetamol and sample solution of the commercial tablets (product A and B), containing the same concentration of chlorzoxazone and paracetamol in methanol, were also shown in Figure 12 and 13 over the wavelength range of 200-400 nm.

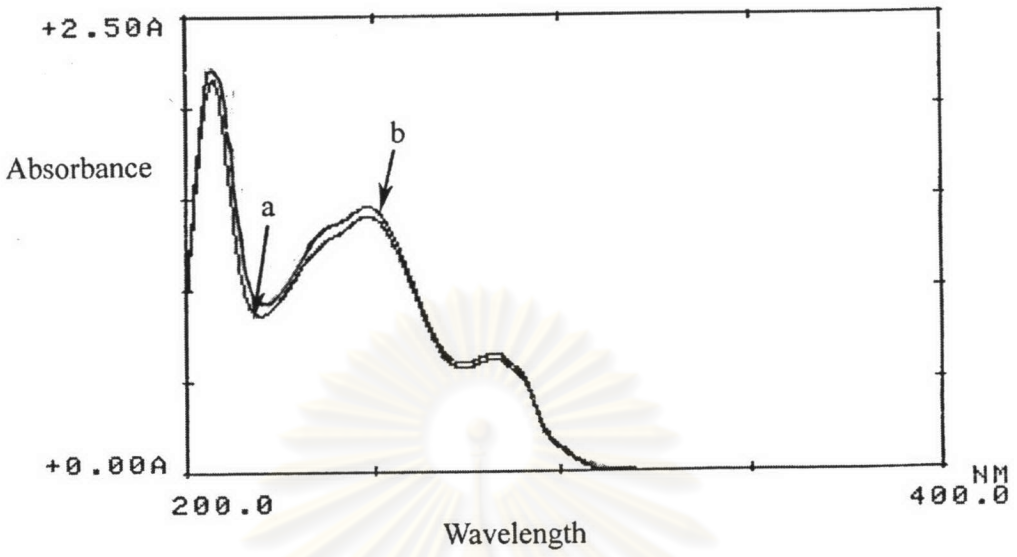

Figure 12 UV absorption spectra of methanolic solution of (a) standard mixture solution of chlorzoxazone (12.5 µg/ml) and paracetamol (15 µg/ml) and (b) sample solution of a commercial product, Cezox®), containing the same concentrations of such drugs.

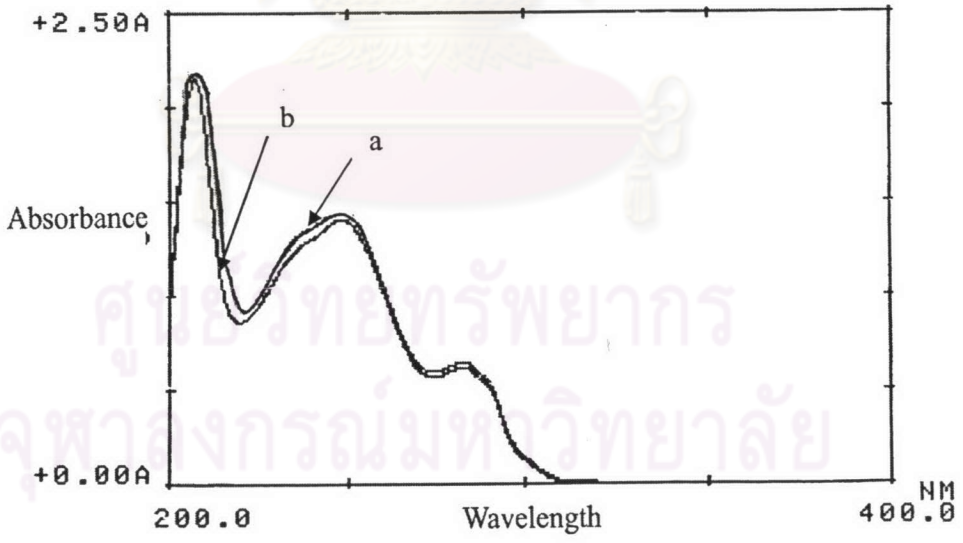

Figure 13 UV absorption spectra of methanolic solution of (a) standard mixture solution of chlorzoxazone (12.5 µg/ml) and paracetamol (15 µg/ml) and (b) sample solution of a commercial product, (Parafon-forte®), containing the same concentrations of such drugs.

1. Determination of the spectrophotometric condition

Determine of the linear range of concentrations.

1.1 Chlorzoxazone

The absorbances and concentrations (µg/ml) of standard chlorzoxazone solutions were listed in Table 4 and a response curve of the absorbance values versus concentrations (µg/ml) was shown in Figure 14. The linear range of chlorzoxazone was found to be 1-50 µg/ml with the coefficient of determination (r^2) of 0.9999.

Table 4 Concentrations and absorbances of chlorzoxazone at 283 nm.

Concentration (µg/ml)	Absorbance at 283 nm
1.09	0.035
2.19	0.072
4.37	0.143
8.75	0.285
17.5	0.565
35	1.125
50	1.582

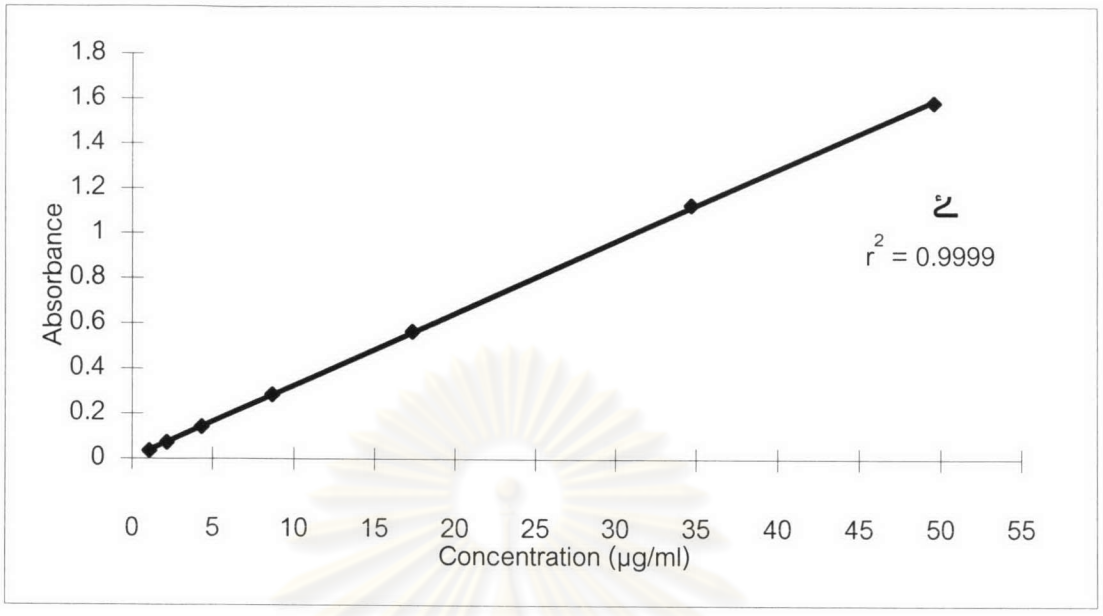

Figure 14 Absorbances at 283 nm versus concentrations (1-50 µg/ml) of standard chlorzoxazone.

1.2 Paracetamol

The absorbances and concentrations (µg/ml) of standard paracetamol solutions were listed in Table 5 and a response curve of the absorbance values versus concentrations (µg/ml) was shown in Figure 15. The linear range of paracetamol was 0.48-25.5 µg/ml (r^2 = 1).

Table 5 Concentrations and absorbances of paracetamol at 248 nm.

Concentration (µg/ml)	Absorbance at 248 nm
0.48	0.050
0.96	0.096
1.91	0.192
3.83	0.372
7.65	0.739
15.3	1.457
25.5	2.404

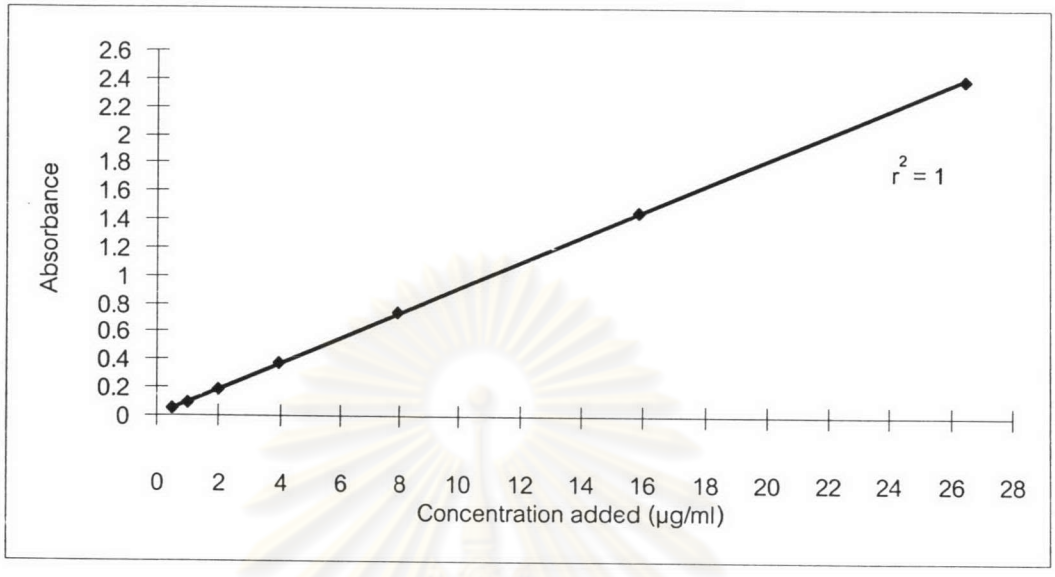

Figure 15 Absorbance values at 248 nm versus concentrations (0.48-30 µg/ml) of standard paracetamol.

2. Establishment of calibration models

2.1 Experimental design of the calibration set

A calibration set of 26 solutions was prepared. Special care was taken to ensure that, in the concentration ranges of all the mixture solutions, the total absorbances did not exceed the linear range of the spectrophotometer and the contribution of each component was additive. The concentrations of chlorzoxazone and paracetamol were varied between 0-18.5925 and 0-23.3250 µg/ml, respectively, through the calibration matrix. The concentration data of the mixtures used as the calibration set for the determination the content of chlorzoxazone and paracetamol was shown to Table 6.

Table 6 Compositions and concentrations of the calibration set.

Mixture Number	Chlorzoxazone (μg/ml)	Paracetamol (μg/ml)	Mixture Number	Chlorzoxazone (μg/ml)	Paracetamol (μg/ml)
S1	3.7185	23.3250	S14	11.1555	9.3300
S2	3.7185	18.6600	S15	7.4370	9.3300
S3	3.7185	13.9950	S16	9.2962	11.6625
S4	3.7185	9.3300	S17	3.7185	0
S5	3.7185	4.6650	S18	7.4370	0
S6	7.4370	18.6600	S19	11.1555	0
S7	11.1555	16.9950	S20	14.8740	0
S8	14.8740	9.3300	S21	18.5925	0
S9	18.5925	4.6650	S22	0	4.6650
S10	14.8740	4.6650	S23	0	9.3300
S11	11.1555	4.6650	S24	0	13.9950
S12	7.4370	4.6650	S25	0	18.6600
S13	7.4370	13.9950	S26	0	23.3250

2.2 Selection of wavelength ranges and the number of principal components

In spectrophotometric analysis, the application of the method have involved the use of the whole range of wavelengths (full spectrum method) or the use of the same range of wavelength to quantify each component in the mixture. PCR and PLSR procedures are designated to be full spectrum computational procedures. In situations where the number of wavelengths (variables) is large, the reduction of the number, using only the ones that carry most information, give a safer and easier model to interpret, with fewer factors and with better precision of predictions. Thus, wavelengths with a great deal of noise or irrelevant information are avoided. Several publications indicated that it may be possible to achieve improved performance by selection of sets of wavelengths that exhibit good mean recovery and percentage of RSD for the analyte of interest. Besides a careful selection of wavelength ranges, an appropriate design of

the calibration set to be used in the modeling was taken into account during elaboration of the analytical procedure. The effect of interferences from tablet diluents (placebo) were also investigated as shown in Figure 16-19.

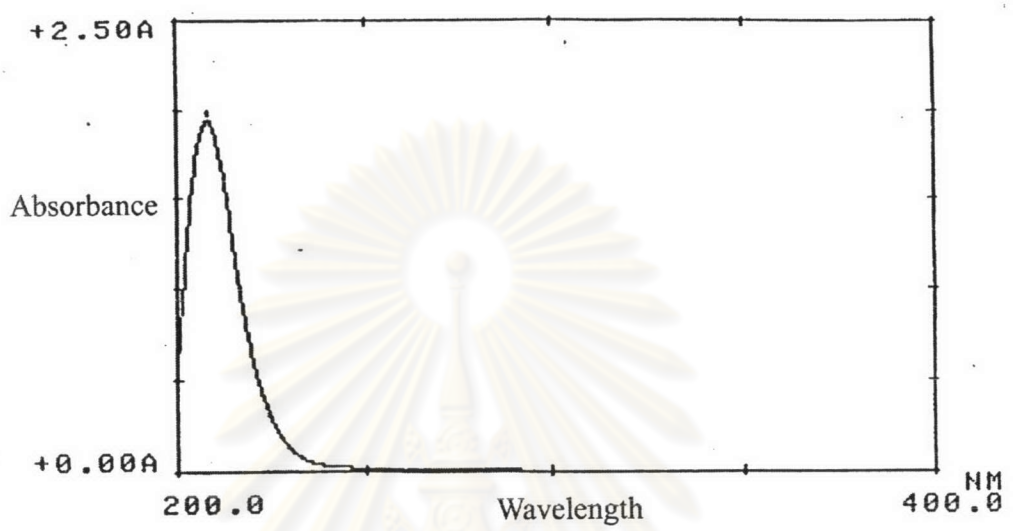

Figure 16 UV absorption spectra of the placebo (equivalent to one tablet) in methanol.

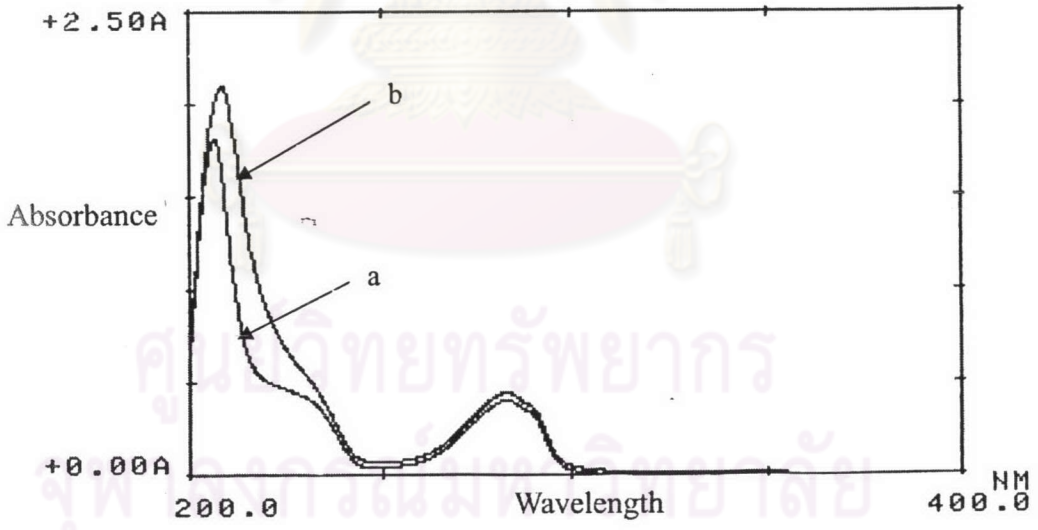

Figure 17 UV absorption spectra of methanolic solution of (a) standard solution of chlorzoxazone (12.5 µg/ml) and (b) chlorzoxazone with placebo.

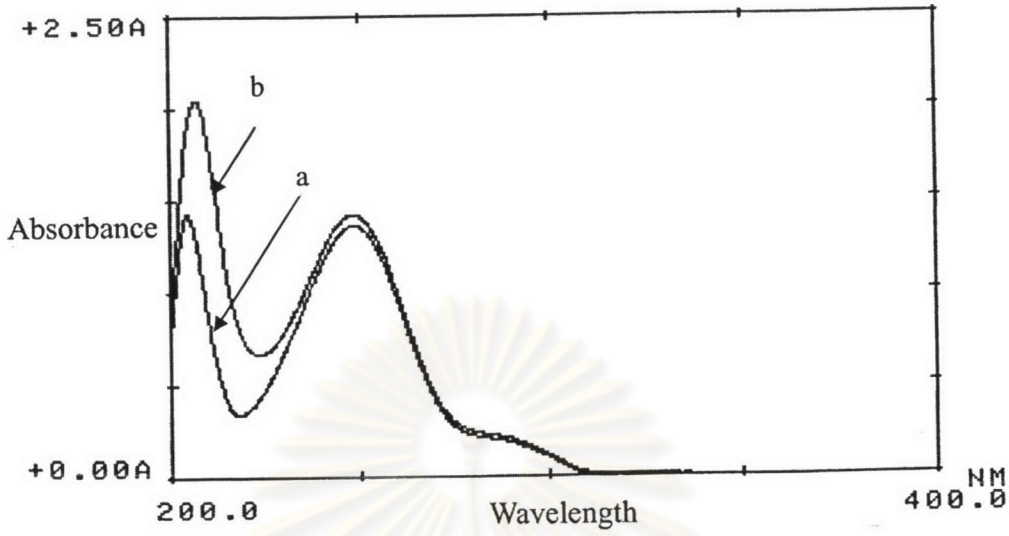

Figure 18 UV absorption spectra of methanolic solution of (a) standard solution paracetamol (15 µg/ml) and (b) paracetamol with placebo.

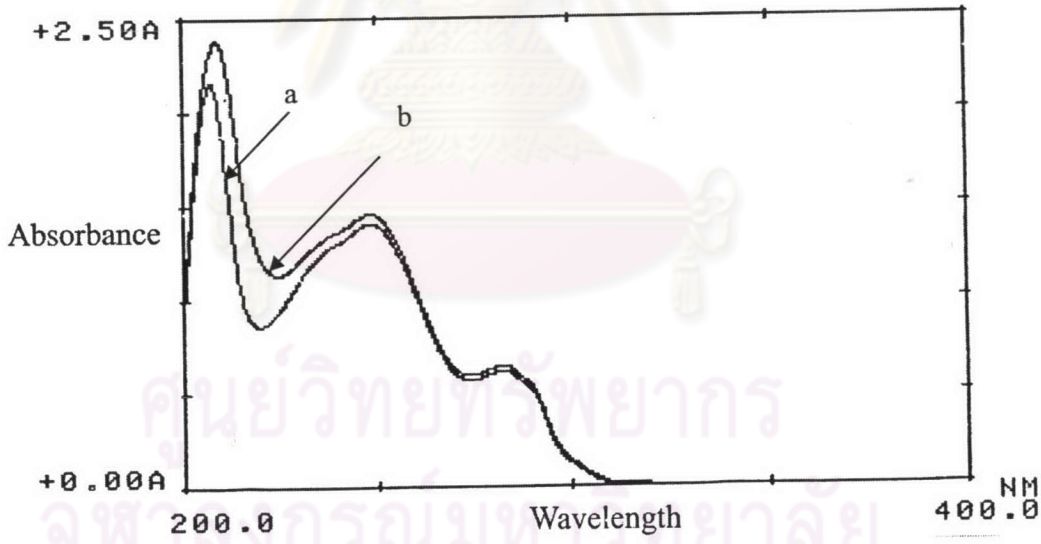

Figure 19 UV absorption spectra of methanolic solution of (a) standard mixture solution of chlorzoxazone (12.5 µg/ml) and paracetamol (15 µg/ml) and (b) their mixture with placebo.

According to the results obtained in Figure 16-19, absorbances at the wavelength range below 220 nm were not included in this study due to interferences from the tablet diluents. Thus absorbances obtained from the wavelength range of 220-350 nm were investigated in establishing the PCR and PLSR models using MINITAB program.

For PLSR, the regression standardize coefficient revealed the sign and magnitude of the relationship between wavelength and absorbance. The standardize coefficient of the wavelength ranges, with the largest positive values, were chosen for establishing the calibration model.

The regression standardize coefficients of chlorzoxazone were presented in Figure 20 and Table 7. The coefficients with positive values were those at wavelengths 220, 270-295, 310-320 and 340-345 nm (Table 7), corresponding respectively to predictors 1, 11-16, 19-21 and 24-25 (Figure 20). The coefficients with the largest values (that were chosen for constructing the model) were those at the wavelength range of 270-295 nm.

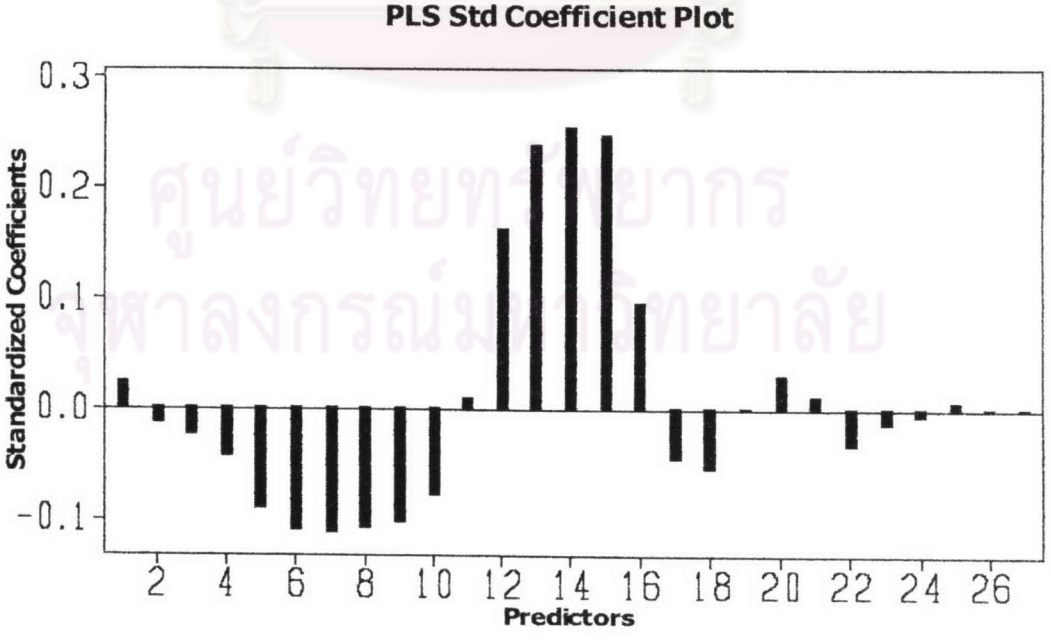

Figure 20 PLSR standard coefficient plot for chlorzoxazone at the wavelength range of 220-350 nm.

Table 7 Regression standardize coefficients of chlorzoxazone and paracetamol.

Wavelength (nm)	PLSR	
	Chlorzoxazone, standardized coefficients	Paracetamol, standardized coefficients
220	0.02035	-0.037124
225	-0.12565	-0.007494
230	-0.024116	0.037984
235	-0.043847	0.078884
240	-0.091662	0.098663
245	-0.111168	0.104628
250	-0.111298	0.104985
255	-0.109194	0.104326
260	-0.103241	0.102302
265	-0.077711	0.094703
270	0.008848	0.064790
275	0.163636	-0.003202
280	0.239180	-0.046302
285	0253930	-0.054287
290	0.247322	-0.047627
295	0.095183	0.036967
300	-0.044908	0.088014
305	-0.053633	0.090255
310	0.001061	0.064332
315	0.031053	0.009317
320	0.010982	-0.009423
325	-0.033024	-0.008070
330	-0.013951	-0.004631
335	-0.006559	-0.003346
340	0.005460	-0.015637
345	0.001474	0.020516
350	-0.001822	-0.011927

For paracetamol, the regression standardize coefficients were presented in Table 7 and Figure 21. The coefficients with positive values were those at wavelengths 230-270, 295-315 and 345 nm (Table 7), corresponding respectively to predictors 3-11, 16-20 and 25 (Figure 21). The coefficients with the largest values, that were chosen for constructing the PLSR model were those at the wavelength range of 230-270 nm.

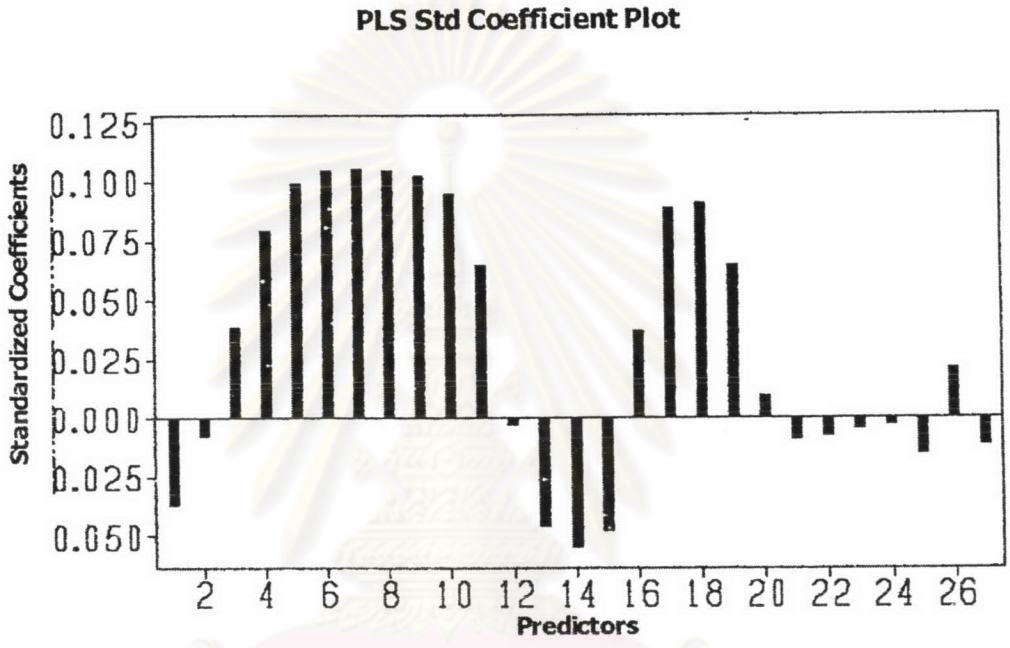

Figure 21 PLSR standard coefficient plot for paracetamol at the wavelength range of 220-350 nm.

The results of the PLSR analysis of chlorzoxazone and paracetamol, using MINITAB program were presented in Table 8, including the number of principal components (PCs), x-variance, R-sq, PRESS, and R-sq (pred). The x-variance is the percentage of variations of x variables that can be explained by the model. The R-sq is the squares of the correlation coefficient (r^2), which indicates the fraction of the total variance explained by the models, resulting in how well the model fits data. PRESS statistic assesses the model's predictive ability. In general, the small the PRESS value, the better the model's predictive ability. Predicted R-sq, R-sq (pred), indicateds how well the model predicts responses for new observations. R-sq (pred) can prevent overfitting of the model, that

is, fitting the model too closely to the data in the current data set. Larger values of R-sq (pred) suggest models of greater predictive ability.

Table 8. Model selection and Validation in the PLSR model.

Chlorzoxazone					Paracetamol				
PCs	X Variance	R-sq	PRESS	R-sq (pred)	PCs	X Variance	R-sq	PRESS	R-sq (pred)
1	0.8303	0.5389	454.502	0.4543	1	0.9460	0.9624	59.2342	0.9560
2	0.9999	0.9997	0.360	0.9996	2	0.9956	0.9927	12.6935	0.9906
3	0.9999	0.9997	0.352	0.9996	3	0.9999	0.9941	10.7373	0.9920
4		0.9998	0.407	0.9995	4		0.9942	12.4406	0.9908
5		0.9998	0.425	0.9995	5		0.9944	12.6479	0.9906
6		0.9998	0.438	0.9995	6		0.9947	17.8493	0.9868
					7		0.9949	15.4234	0.9886
					8		0.9950	15.5602	0.9882
					9		0.9950	16.2387	0.9879

For chlorzoxazone with studied wavelength range of 270-295 nm, the PLSR models with one and two principal components (PCs) could explain the variations of absorbances (x-variance) by 83.03% and 99.99%, respectively. The PLSR model with three PCs was not better than the model with two PCs in explaining the x-variance (99.99%). Comparing the PLSR models with respect to PRESS value, the PLSR model with two PCs had lowest PRESS. the PLSR model with two PCs was finally selected due to the lowest PRESS (Figure 23) and high x-variance, R-sq and R-sq (pred) (Figure 22).

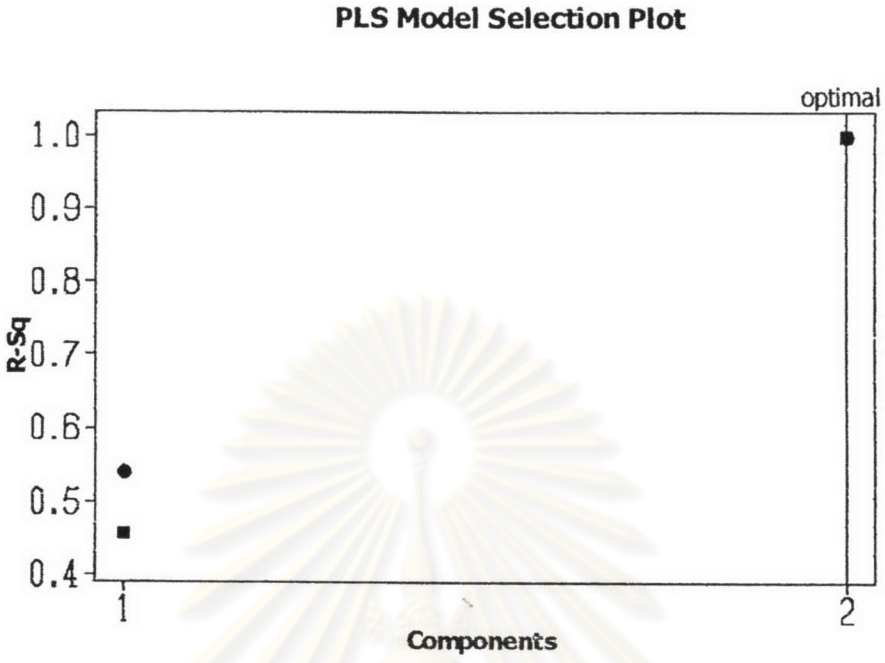

Figure 22 Scatter plot of R-sq (●) and R-sq(pred) (■) versus PCs of chlorzoxazone with studied wavelength range of 270-295 nm.

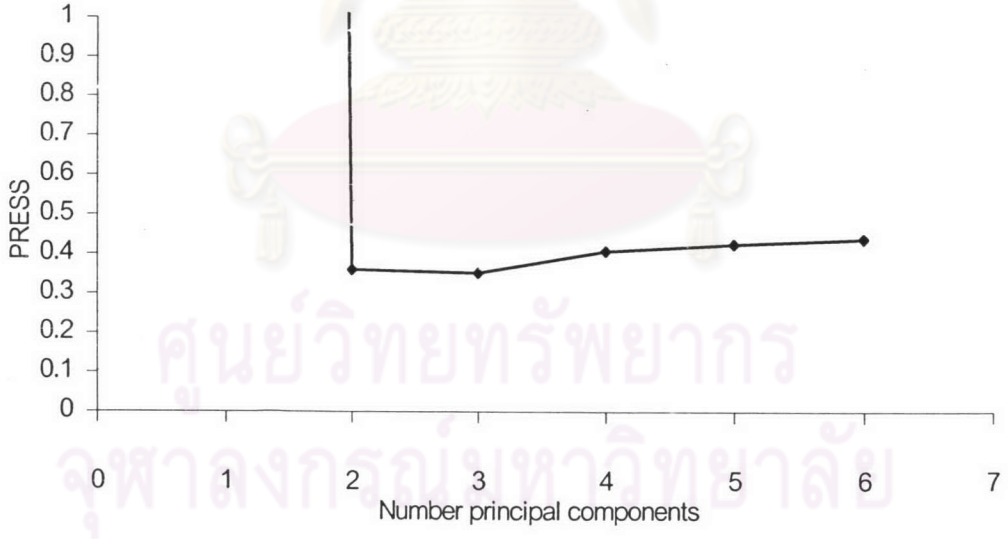

Figure 23 Plot of PRESS against PCs for chlorzoxazone with studied wavelength range of 270-295 nm.

For paracetamol with studied wavelength range of 230-270 nm, the PLSR models with one, two and three principal components (PCs) could explain the variations

of absorbances (x-variance) by 94.60%, 99.56% and 99.99%, respectively. The PLSR model with three PCs was the best in explaining the x-variance (99.99%). Comparing the PLSR models with respect to PRESS value, the PLSR model with three PCs had lowest PRESS. The PLSR model with three PCs was finally selected due to the lowest PRESS (Figure 25) and high x-variance, R-sq and R-sq (pred) (Figure 24).

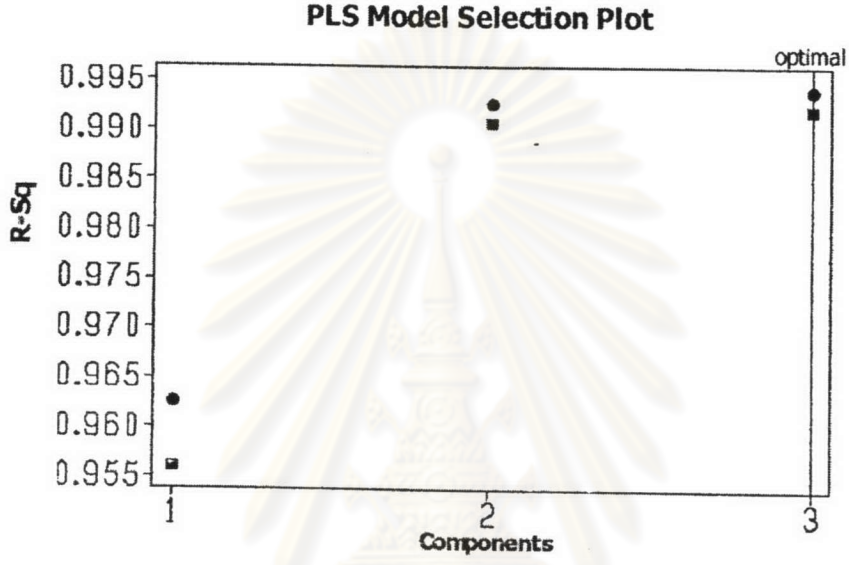

Figure 24. Scatter plot of R-sq (●) and R-sq(pred) (■) versus PCs of paracetamol With studied wavelength range of 230-270 nm.

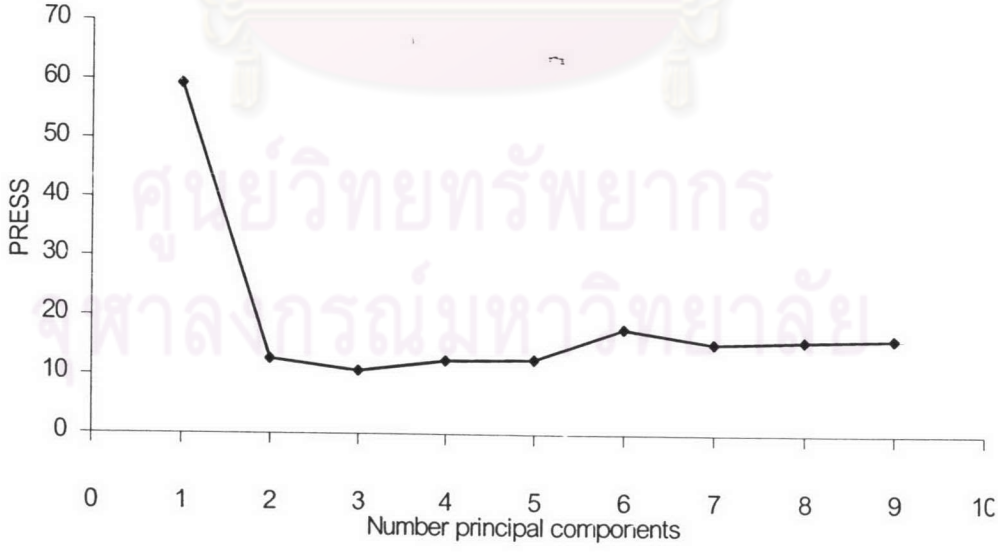

Figure 25. Plot of PRESS against PCs for paracetamol with studied wavelength range of 230-270 nm.

For the PCR model. Eigenanalysis of the covariance matrix of chlorzoxazone and paracetamol for the PCR model in Table 9. As the result, for chlorzoxazone, the second principal component has variance 0.017174, account for 15% of the data variability and represent 100% of the total variability. In the same way, for paracetamol, the second principal component has variance 0.1154, account for 5.7% of the data variability and represent 99.60% of the totai variability. Thus, most of the data structure can be captured in two and three underlying dimensions for chlorzoxazone and paracetamol, respectively. The remaining principal components account for a very small proportion of the variability and are probably unimportant. Scree plot displays the eigenvalue associated with a principal component versus the number of the component. Use this plot to judge the relative magnitude of eigenvalues. The scree plot provides this information visually in Figures 26-27.

Table 9. Eigenanalysis of the chlorzoxazone and paracetamol in the PCR model.

Chlorzoxazone				Paracetamol			
PCs	Eigenvalue	Proportion	Cumulative	PCs	Eigenvalue	Proportion	Cumulative
1	0.097003	0.850	0.850	1	1.8941	0.939	0.939
2	0.017174	0.150	1.000	2	0.1154	0.057	0.996
3	0.000006	0.000	1.000	3	0.0083	0.004	1.000
4	0.000001	0.000	1.000	4-24	0.0000	0.000	1.000
5-6	0.000000	0.000	1.000				

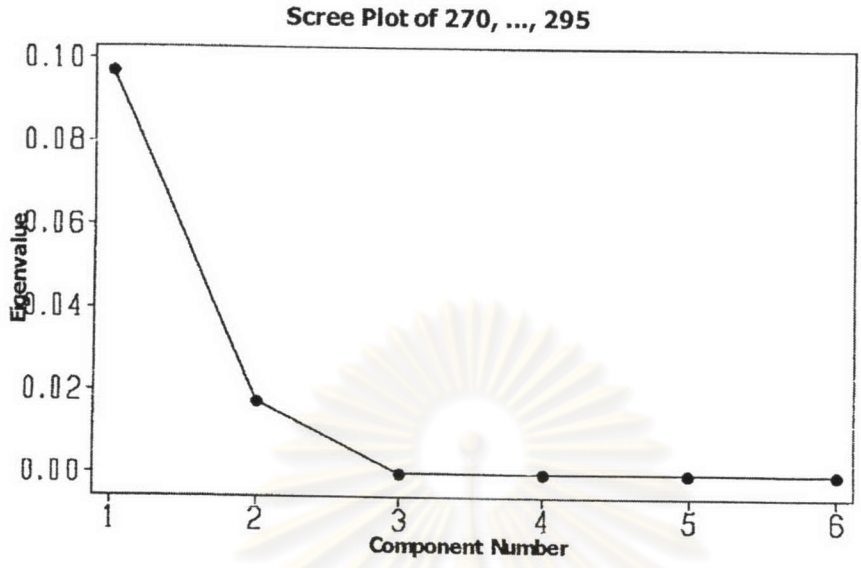

Figure 26 The scree plot expressing the relation between the eigenvalue and the component numbers for chlorzoxazone using the wavelength region 220-350 nm.

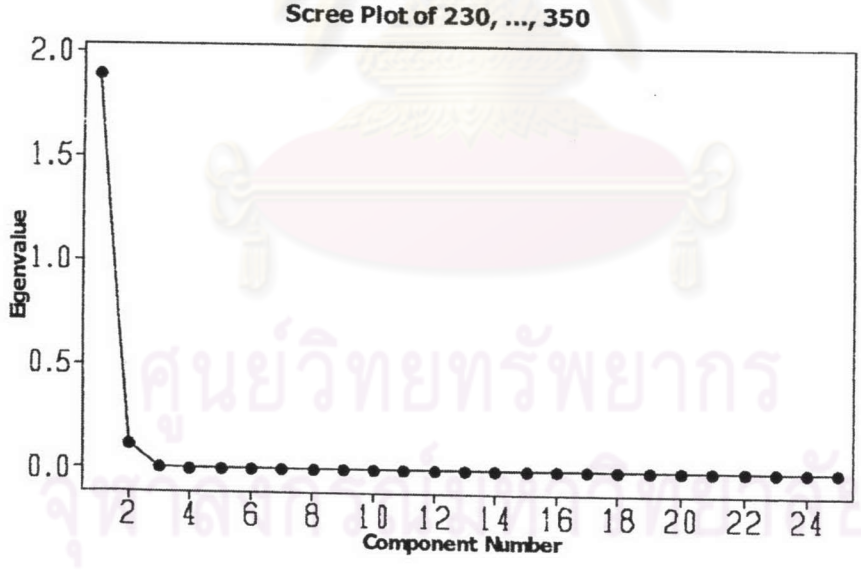

Figure 27 The scree plot expressing the relation between the eigenvalue and the component numbers for paracetamol using the wavelength region 220-350 nm.

Table 10 Statistical parameter of cross-validation for PCR and PLSR from spectral data of calibration set.

Method	Compound	PCs	Wavelength (nm)	PRESS	R-sq
PCR	Chlorzoxazone	2	270-295	0.34	0.9996
	Paracetamol	2	230-350	10.1427	0.9925
PLSR	Chlorzoxazone	2	270-295	0.36	0.9996
	Paracetamol	3	230-270	10.7373	0.9920

Score plot used to display the overall configuration of the data using the principal component and can identify cluster of points. Moreover, this plot corresponds to the representation of sample lead to classification in the PC axes, that is, Eluclidean distances between two samples indicate their likeness. The lower this distance is more the sample are similar. Table 11-12 show X score value of chlorzoxazone and paracetamol from the PCR and PLSR model, respectively.

In Table 11-12 and from the score plot (Figure 28-31) it can be seen the divided data 7 groups. For score plot of chlorzoxazone, ractangles A, B, C, D, E, F and G group objects in the calibration matrix with the concentrations of chlorzoxazone: (A) 0; (B) 3.75; (C) 7.5; (D) 11.25; (E) 15; (F) 18.75; and (G) 9.375 µg/ml. For score plot of paracetamol, ractangles A, B, C, D, E, F and G group objects in the calibration matrix with the concentrations of paracetamol: (A) 0; (B) 4.5; (C) 9; (D) 13.5; (E) 18; (F) 22.5; and (G) 9.375 µg/ml. Moreover, as can be seen the solutions arranged themselves horizontally (i.e. an increase in the first score led to an increase in the concentration of paracetamol in Figure 29 and 31.) and the solutions group vertically according to the concentration of chlorzoxazone, an increase in which decreased (Figure 28) and increased (Figure 30) the second score. From these scores it can be concluded that the scores relate to concentrations of the analytes and the score is important in both PCR and PLSR models.

Table 11. X score value of chlorzoxazone and paracetamol from the PCR model.

No. sample	Chlorzoxazone		Paracetamol	
	PCs 1	PCs 2	PCs 1	PCs 2
1	-1.15017	0.257953	4.90991	0.80871
2	-0.96388	0.198190	3.94747	0.68446
3	-0.78912	0.144067	3.05591	0.63642
4	-0.59827	0.084088	2.08355	0.49905
5	-0.43844	0.032179	1.22352	0.48724
6	-1.19735	0.158321	4.11045	0.96384
7	-1.21732	0.064861	3.31506	1.16766
8	-1.23336	-0.034930	2.45722	1.26951
9	-1.25733	-0.135100	1.60683	1.36979
10	-1.04415	-0.093236	1.50431	1.14045
11	-0.85712	-0.043788	1.51967	1.09297
12	-0.61397	-0.015919	1.24204	0.63176
13	-0.99313	0.102523	3.14935	0.84009
14	-1.03147	0.006574	2.35470	1.01206
15	-0.81986	0.046592	2.24579	0.79961
16	-0.99674	0.050280	2.72026	0.91829
17	-0.23246	-0.032820	0.18899	0.29693
18	-0.44921	-0.065213	0.42410	0.73948
19	-0.64688	-0.113072	0.40642	0.77963
20	-0.87207	-0.146979	0.60402	1.12593
21	-1.06925	-0.197300	0.62117	1.26306
22	-0.20398	0.061436	0.99754	0.12481
23	-0.38264	0.118037	1.92571	0.21814
24	-0.56969	0.178108	2.90833	0.40827
25	-0.75680	0.235548	3.82613	0.47116
26	-0.96973	0.297075	4.80604	0.61977

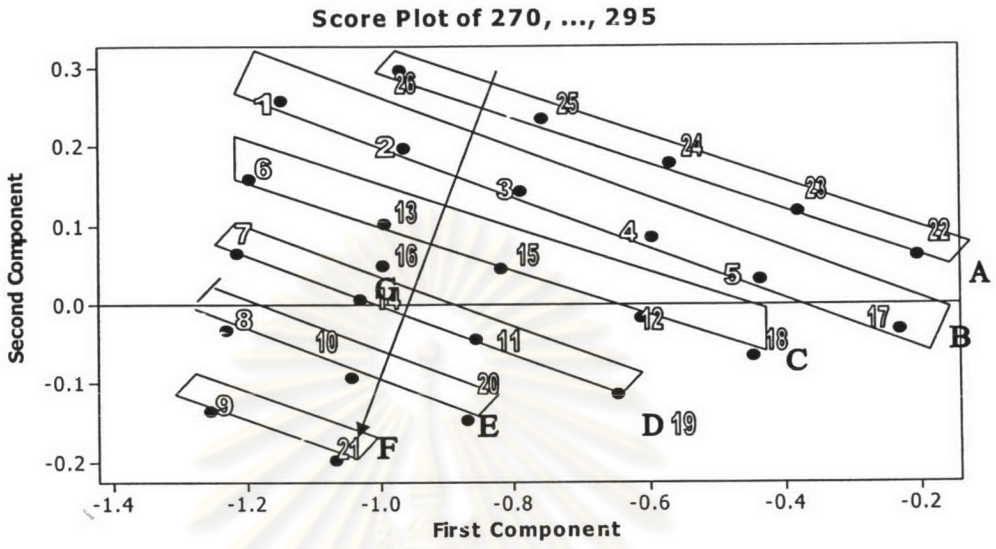

Figure 28. PCR Score plot of chlorzoxazone using the wavelength range of 270-295 nm with 2 PCs

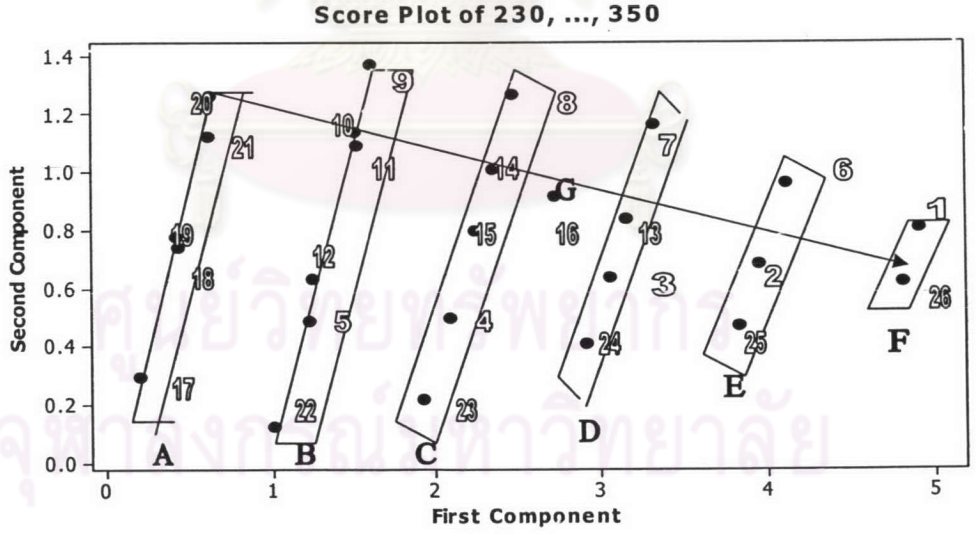

Figure 29. PCR Score plot paracetamol using the wavelength range of 230-350 nm with 2 PCs

Table 12 X score value of chlorzoxazone and paracetamol from the PLSR model.

No. sample	Chlorzoxazone		Paracetamol		
	PCs 1	PCs 2	PCs 1	PCs 2	PCs 3
1	1.46716	-1.81129	5.66001	0.05279	0.001647
2	0.46631	-1.24412	3.53327	0.32734	-0.067207
3	-0.49755	-0.72380	1.64473	0.25893	0.074227
4	-1.53885	-0.16826	-0.51942	0.58673	-0.019845
5	-2.41675	0.33281	-2.30260	0.38604	0.173445
6	2.07599	-1.09043	4.06213	-0.19992	-.110109
7	2.46990	-0.38153	2.54685	-0.79116	0.061258
8	2.85522	0.35115	0.80205	-0.88530	-0.102138
9	3.31628	1.07061	-0.98454	-0.93426	-0.301715
10	1.83159	0.88321	-1.33616	-0.59087	-0.168773
11	0.48067	0.65675	-1.23298	-1.06094	0.458381
12	-1.14235	0.559974	-2.22378	0.34644	-0.067340
13	0.93386	-0.54959	1.94838	0.00572	-0.091544
14	1.45539	0.15864	0.38907	-0.36389	-0.110535
15	-0.01232	-0.02307	0.03988	-0.08748	0.062984
16	1.10814	-0.15350	1.09143	-0.17691	-0.063510
17	-3.54170	0.93724	-4.65793	0.93988	-0.053064
18	-2.06775	1.07527	-3.77896	-0.45719	0.555732
19	-0.65906	1.29701	-3.89327	0.08444	-0.173380
20	0.87271	1.43460	-3.20367	-0.80791	0.043920
21	2.28079	1.69333	-3.13979	-0.72353	-0.365987
22	-3.99533	0.23975	-3.07364	1.34075	-0.061521
23	-3.01574	-0.29318	-1.05520	1.19416	-0.060979
24	-1.99541	-0.85825	1.18864	0.6O261	0.226825
25	-0.96879	-1.39354	3.15325	0.63081	0.072689
26	0.21756	-1.99955	5.34222	0.32273	0.088638

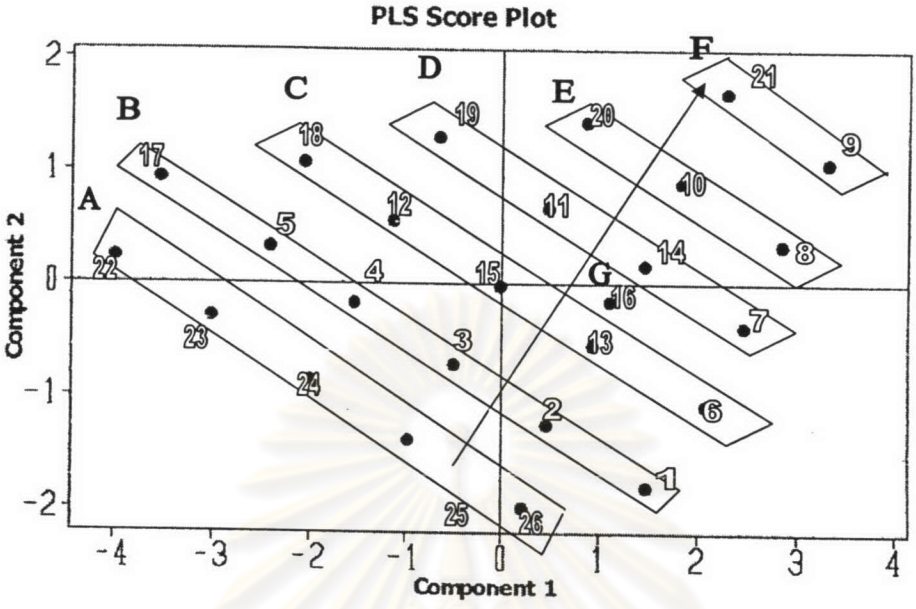

Figure 30 PLSR Score plot of chlorzoxazone using the wavelength range of 270-295 nm with 2 PCs.

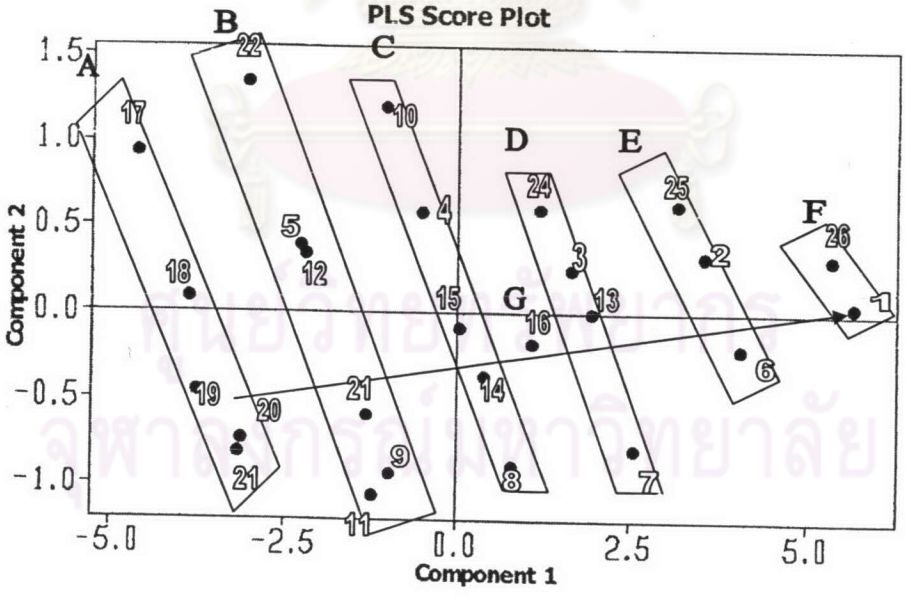

Figure 31 PLSR Score plot paracetamol using the wavelength range of 230-270 nm with 3 PCs.

Loading plot used to display the correlation between the loading of each predictor on the principal components. Compare the important of predictors (wavelength) to the model. In the same way, the loading are directly linked to correlation values between the wavelengths and PCs and added with the wavelengths and response. Generally, a significant wavelength in a PC axe will be far (or long line) to the center of the axe and indicating that it has high loading and is more related to concentration. For the PCR model in Table 13 and Figure 32-33, it can be seen that all wavelengths of chlorzoxazone have moderate or high loading on at least one of the two components while can be seen low loading of paracetamol at the wavelength region above 300 nm. For the PLSR model in Table 14 and Figure 34-35 have explained that the wavelengths of both chlorzoxazone and paracetamol have long lines, indicating that they have high loading and are more related to their concentrations and are significant in the PC axes. From these loading it can be concluded that the selected wavelength region is important in both the PCR and PLSR models.

From this study it can be concluded that both PCR and PLSR models can be graphically showed the relevance and the relation of the original variables to the scores, resulting in less uncorrelated, but more meaningful new variables (26 wavelengths to two or three components), and can also be used for classification.

Table 13 X loading value of chlorzoxazone and paracetamol from the PCR model.

Chlorzoxazone			Paracetamol		
Wavelength (nm)	PCs 1	PCs 2	Wavelength (nm)	PCs 1	PCs 2
270	-0.426028	0.804628	230	0.176979	0.466552
275	-0.431145	0.100450	235	0.273840	0.248328
280	-0.473718	-0.299227	240	0.378382	-0.008397
285	-0.467324	-0.380858	245	0.449448	-0.158550
290	-0.394600	-0.261326	250	0.460836	-0.171523
295	-0.184671	0.199004	255	0.412029	-0.138217
			260	0.318456	-0.070331
			265	0.208940	0.037054
			270	0.115146	0.174341
			275	0.058120	0.315396
			280	0.030357	0.426091
			285	0.022942	0.436735
			290	0.024307	0.356842
			295	0.037663	0.104146
			300	0.031784	0.015092
			305	0.013926	0.004923
			310	0.002683	0.003025
			315	0.000580	0.001975
			320	0.000173	0.000959
			325	0.000097	0.000504
			330	0.000074	0.000227
			335	0.000054	0.000033
			340	0.000048	-0.000056
			345	0.000006	-0.000374
			350	-0.000047	-0.000129

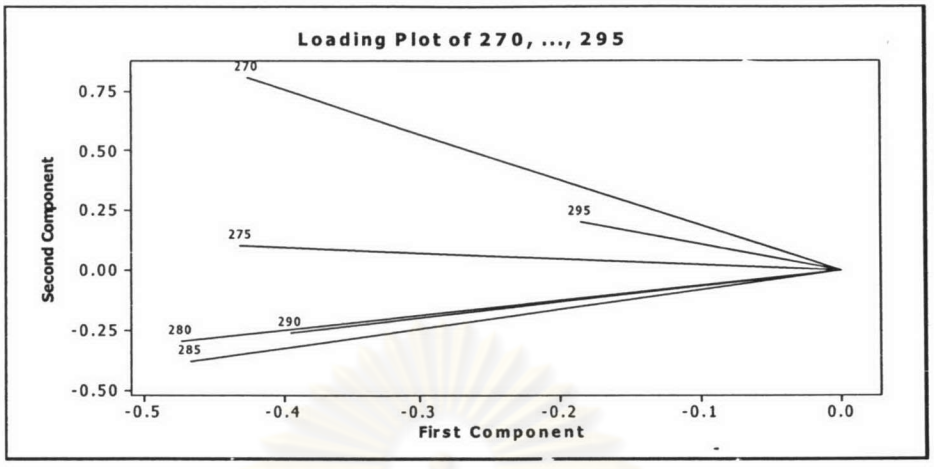

Figure 32 PCR Loading plot of chlorzoxazone using the wavelength range of 270-295 nm with 2 PCs.

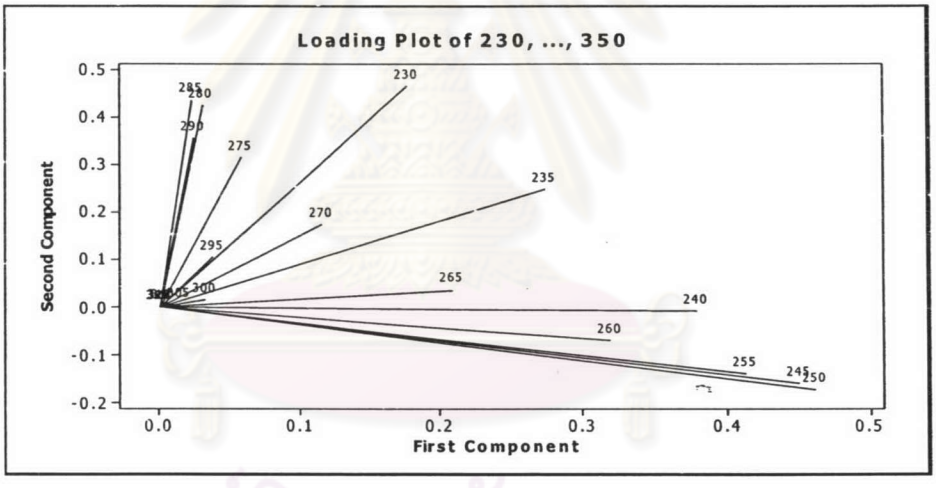

Figure 33 PCR Loading plot paracetamol using the wavelength range of 230-350 nm with 2 PCs.

Table 14 X loading value of chlorzoxazone and paracetamol from the PLSR model.

Chlorzoxazone			Paracetamol			
Wavelength (nm)	PCs 1	PCs 2	Wavelength (nm)	PCs 1	PCs 2	PCs 3
270	0.321869	-0.751047	230	0.291650	-0.566872	0.625444
275	0.473111	-0.279481	235	0.339417	-0.203151	-0.244539
280	0.491876	0.070997	240	0.341738	0.128131	-0.017708
285	0.488173	0.140490	245	0.338391	0.241725	0.131786
290	0.491419	0.082719	250	0.338119	0.248581	0.137742
295	0.402964	-0.571138	255	0.338625	0.235542	0.128117
			260	0.340020	0.195276	0.090016
			265	0.342688	0.195276	-0.089952
			270	0.328707	-0.375613	-0.692690

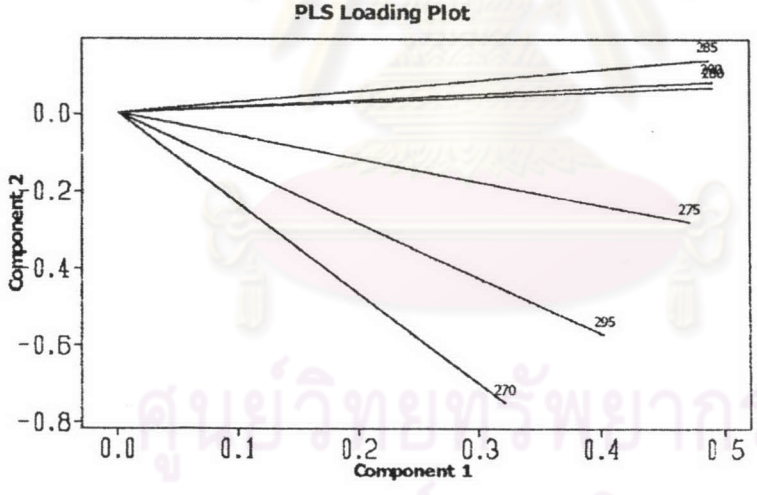

Figure 34 PLSR Loading plot of chlorzoxazone using the wavelength range of 270-295 nm and with 2 PCs.

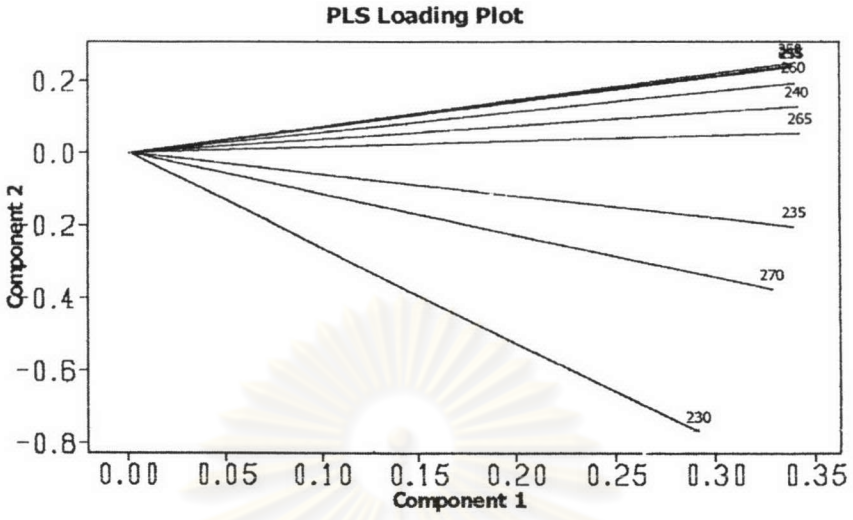

Figure 35 PLSR Loading plot paracetamol using the wavelength range of 230-270 nm with 3 PCs.

The percentage of average recovery and %RSD of chlorzoxazone and paracetamol obtained from the validation set are shown in Table 15. As indicated the above sentence, assay parameter of chlorzoxazone in the PCR model using the wavelength range 270-295 nm and PCs = 2, were resulted in the acceptable percentage of average recovery and percentage of RSD (99.39% and 0.062, respectively), while the PLSR model using the wavelength range 270-295 nm and PCs = 2, were resulted (99.94% and 0.526, respectively). According to paracetamol, the wavelength region was chosen to be 230-350 nm using PCs 2 were given in the PCR model (98.81% and 0.788, respectively) and 230-270 nm using PCs 3 were given in the PLSR model (98.61% and 0.776, respectively).

Therefore, the parameters of the purposed methods were acceptable according to the criteria that the percentage of average recovery in standard mixture obtained between 98-102% and %RSD should not exceed 2%.

Table 15 Statistic parameter of chlorzoxazone and paracetamol using the PCR and PLSR quantitation of nine mixtures on the validation set.

Analytes	PCR*		PLSR*	
	%Mean recovery	%RSD	%Mean recovery	%RSD
Chlorzoxazone	99.36	0.626	99.94	0.526
Paracetamol	98.81	0.778	98.61	0.776

* Mean and RSD of five determinations.

3. Method validation

Validation of and analytical method is a process established in laboratory, to characterize that the methods meet the requirements for the intended analytical application or not. The analytical parameters that should be considered in this validation study were accuracy (%recovery), precision (%RSD), and linearity (r^2) and range.

3.1 Accuracy

The accuracy of an analytical method is the closeness of test result obtained from that method to the true value as the percentage of analyte recovered from the spiked placebo technique. In this study, the experiment was performed by analyzing synthetic standard mixtures (placebo) spiked with known quantities of chlorzoxazone (3.9664, 7.9328, 11.8992, 15.8656 and 19.8320 µg/ml) and paracetamol (4.976, 9.952, 14.928, 19.904 and 24.88 µg/ml), as the demonstrated in Table 16 and 17 for the PCR and PLSR, respectively.

Table 16 Accuracy data for chlorzoxazone using in the PCR and PLSR models.

Concentration (µg/ml)	PCR			PLSR		
	%Recovery*	SD	%RSD	%Recovery*	SD	%RSD
3.9664	109.33	0.408	0.373	109.53	0.375	0.341
7.9328	106.62	0.617	0.579	105.48	0.595	0.566
11.8992	105.64	0.888	0.841	105.52	0.858	0.806
15.8656	106.80	0.976	0.914	106.08	0.936	0.896
19.8320	105.71	0.602	0.569	104.52	0.592	0.570

* Mean and RSD of three determinations.

Table 17 Accuracy data paracetamol using in the PCR and PLSR models.

Concentration (µg/ml)	PCR			PLSR		
	%Recovery*	SD	%RSD	%Recovery*	SD	%RSD
4.976	103.94	0.411	0.395	103.68	0.345	0.333
9.952	101.71	0.423	0.416	101.61	0.403	0.397
14.928	101.65	0.552	0.543	101.52	0.493	0.486
19.904	101.69	0.354	0.348	101.64	0.317	0.312
24.880	101.55	0.429	0.422	101.16	0.359	0.355

* Mean and RSD of three determinations.

3.2. Precision

The precision of an analytical method is the degree of agreement among individual test resulted when the procedure is applied repeatedly to multiple sampling of a homogeneous sample. The precision of an analytical method is usually expressed as the relative standard deviation (RSD) or coefficient of variation (CV). Precision that was considered in this validation study was the measure of the degree of repeatability and intermediate precision. Repeatability refers to the use of the analytical procedure within a laboratory over the shot period of time using the same analyte with the same

equipment. Intermediate precision expresses within-laboratory variation, as on different days and many involve different analysts, equipments, reagents and laboratories.

The within-run and between-run precision were determined by analyzing three replicates of synthetic standard mixtures (placebo) spiked with chlorzoxazone (3.9664, 7.9328, 11.8992, 13.7968 and 19.8320µg/ml) and paracetamol (4.976, 9.952, 14.928, 19.904 and 24.88 µg/ml). As the demonstrated in Table 18 and 19 for the PCR and PLSR, respectively that %RSD of within-run and between-run for chlorzoxazone and paracetamol were less than 2% for all five concentrations.

The results were complied with the USP requirement (% RSD less than 2.0). Therefore, the precision of proposed methods were acceptable according to the criteria.

Table 18 Within-run and between-run precisions of chlorzoxazone in spiked synthetic standard mixtures (placebo) using the PCR and PLSR models.

Concentration (µg/ml)	%RSD (n =3)			
	PCR		PLS	
	Within-run	Between-run	Within-run	Between-run
3.9664	0.452	0.335	0.535	0.515
7.9328	0.451	0.479	0.221	0.225
11.8992	0.671	0.816	0.376	0.489
15.8656	0.497	0.566	0.344	0.462
19.8320	0.256	0.374	0.218	0.348

Table 19 Within-run and between-run precision of paracetamol in spiked synthetic standard mixtures (placebo) using the PCR and PLSR models.

Concentration (µg/ml)	%RSD (n =3)			
	PCR		PLSR	
	Within-run	Between-run	Within-run	Between-run
4.976	1.237	1.581	1.212	1.537
9.952	1.009	1.141	0.978	1.114
14.928	0.414	0.465	0.429	0.481
19.904	0.456	0.416	0.510	0.449
24.880	0.657	0.982	0.513	0.724

3.3 Linearity and range

The linearity of an analytical method is its ability to elicit results that are directly, or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a give range. As a general guide for an assay validation, a method that is linear and unbiased will have a slope of one, a zero intercept and a correlation coefficient of not less than 0.9997. Figure 36-39 showed that correlation coefficients for both compound were satisfactory in PCR and PLSR models.

Range of an analytical method is the interval between the upper and low concentration levels of analyte, covering usually used concentration. In this experiment, linearity was found in drugs concentration range 3.9664-19.8320 and 4.976-24.880 µg/ml for chlorzoxazone and paracetamol, respectively.

A coefficient of determination (r^2) value, an intercept, and slope were calculated as follows.

For chlorzoxazone:

PCR; $Y = 1.0599X + 0.0144$, $r^2 = 0.9997$

PLSR; $Y = 1.054X + 0.0063$, $r^2 = 0.9998$

For paracetamol:

PCR; $Y = 1.0178X + 0.007$, $r^2 = 1$

PLSR; $Y = 1.0168X + 0.0065$, $r^2 = 1$

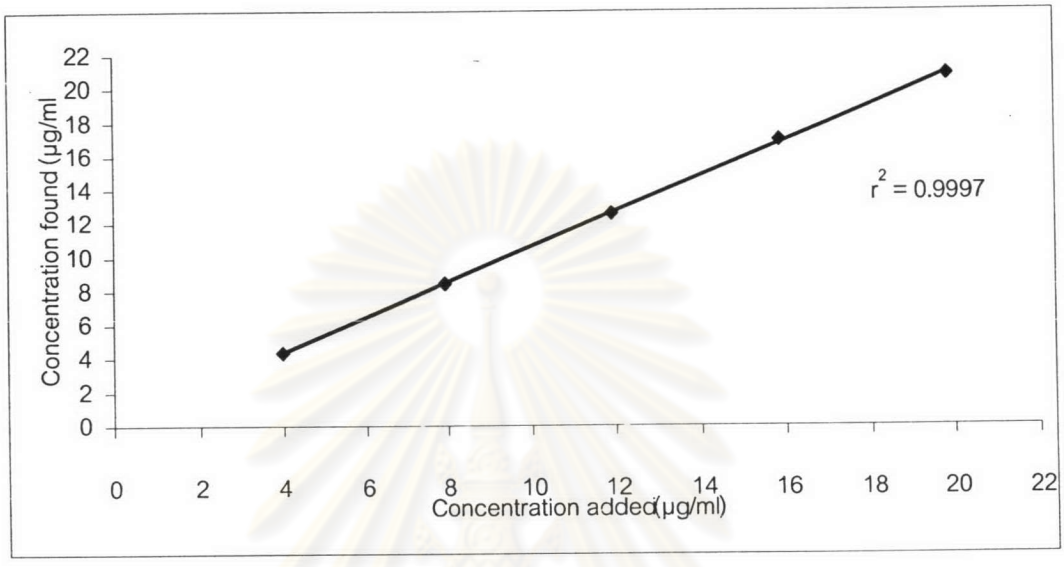

Figure 36 Linear regression line of the concentration added versus the concentration found (µg/ml) of chlorzoxazone in the PCR model.

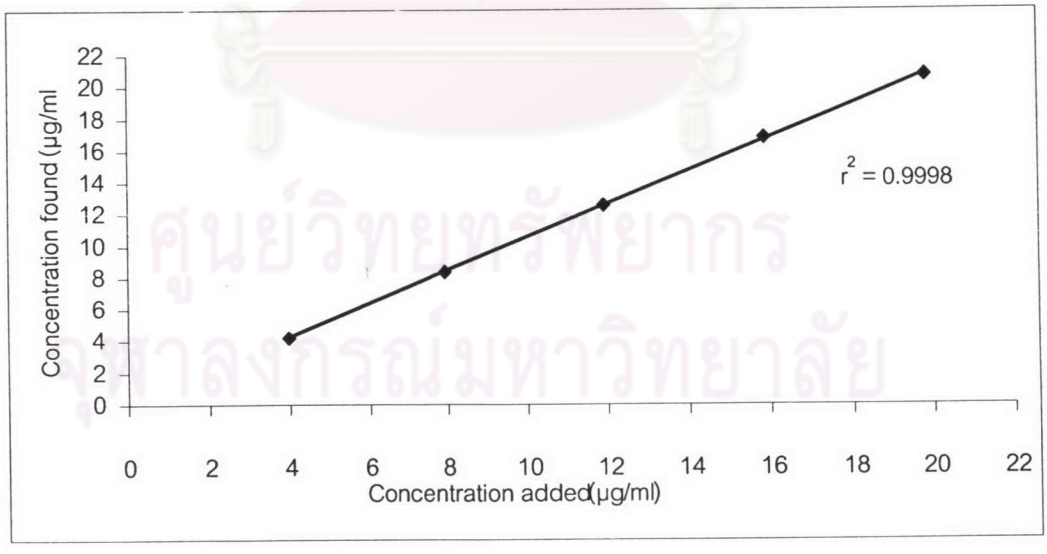

Figure 37 Linear regression line of the concentration added versus the concentration found (µg/ml) of chlorzoxazone in the PLSR model.

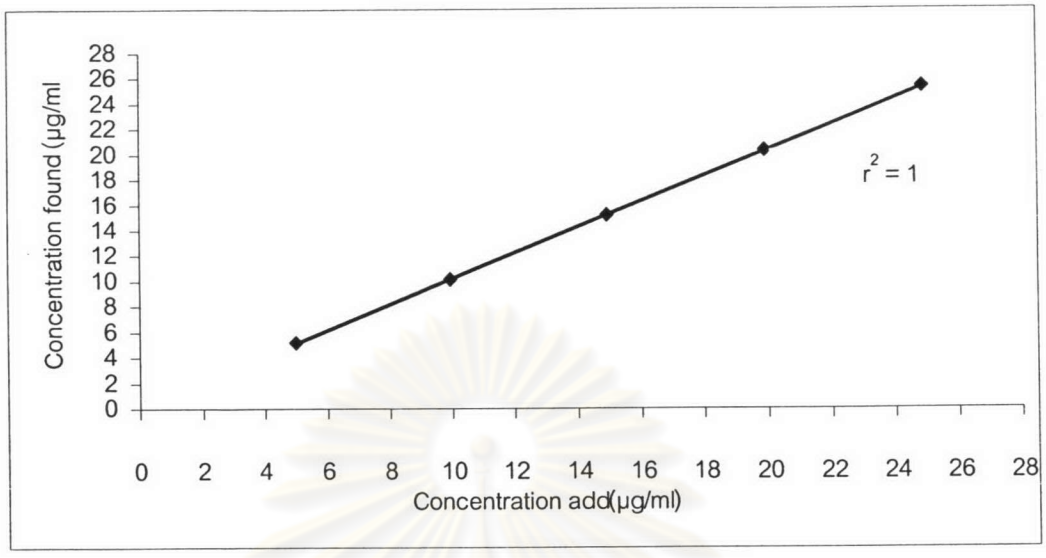

Figure 38 Linear regression line of the concentration added versus the concentration found (µg/ml) of paracetamol in the PCR model.

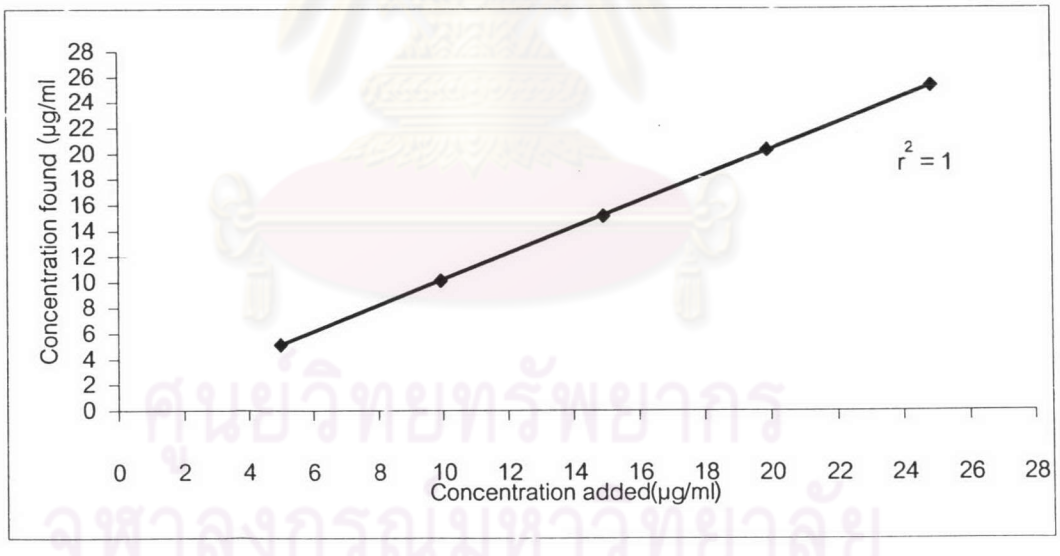

Figure 39 Linear regression line of the concentration added versus the concentration found (µg/ml) of paracetamol in the PLSR model.

4. Assay of pharmaceutical formulations

The proposed method was applied to the determination of chlorzoxazone and paracetamol in tablets for two formulations (Product A and B). The assay data were given in Table 20. Assay content of chlorzoxazone was found to be in product A (105.39% and 105.74%) and product B (106.30% and 106.21%) for the PCR and PLSR models, respectively. Assay content of paracetamol was found to be in product A (101.26% and 101.15%) and product B (101.374% and 101.73%) for the PCR and PLSR models, respectively.

Table 20 Assay results for chlorzoxazone and paracetamol of product A and B using the PCR and PLSR models.

Method	Parameter (n = 5)	Product A		Product B	
		Chlorxozaxone	Paracetamol	Chlorxozaxone	Paracetamol
PCR	%Label amount	105.39	101.26	106.30	101.74
	%RSD	0.417	0.758	0.302	0.471
PLSR	%Label amount	105.74	101.15	106.21	101.73
	%RSD	0.349	0.761	0.093	0.479
t(cal)*		0.373	2.342	0.354	1.464

**t* (crit) = 2.7764, p=0.05

Comparison of the mean recovery of chlorzoxazone and paracetamol in spiked synthetic standard mixtures (Table 20) and percentage of the labeled amount of products (Table 21). Further, after the amount of active into the placebo we found that no different these compounds between synthetic mixture and two products. This shows that the excipients present in the commercial preparation selected did not interfere in quantitation of chlorzoxazone and paracetamol in these methods. All the results

obtained by using the methods described above were compared with each other and no significant differences was observed between the amount of drugs found as theoretical values for t at p = 0.05 level (t = 2.7754, n = 5) for commercial formulation in results were obtained when PCR and PLSR models were applied to the prediction of synthetic mixtures, thus providing a high resolving power for both chemometric methods in the analysis of multicomponent complex mixtures. Moreover, both procedures gave results in agreement with the labeled drugs content when applied on pharmaceutics.

PLSR seemed to be little more sensitive in the case of an extensive spectral overlap. Actually, chlorzoxazone determination, which were present in a low concentration compared to the larger amount of paracetamol, showed slightly better result, in terms of accuracy and precision, by using the PLSR model.

PCR accounts for all the spectral data simultaneously and then, in second step of multiple regressions, correlates these with the components data. On the other hand, PLSR provides to individually analyze each component by correlating in variation in the component information with respect to the spectral data. Therefore, even a slight difference in the variable data, like an absorbance of the components present in small amounts, is taken into account for producing a more robust model with greater predictive power than the calibration model constructed using PCR.

Table 21 Mean recovery of chlorzoxazone and paracetamol in spiked synthetic standard mixtures (placebo).

Method	Parameter (n = 5)	Synthetic standard mixtures	
		Chlorzoxazone	Paracetamol
PCR	%Mean recovery	106.34	101.89
	%RSD	0.278	0.433
PLSR	%Mean recovery	106.29	101.81
	%RSD	0.063	0.428
t(cal)*		0.342	1.822

* *t* (crit) = 2.7764, p=0.05

5. TLC of degradation compounds of chlorzoxazone and paracetamol

5.1 Effect of acid-base catalysis degradation on standard mixture solution, pharmaceutical preparation and the placebo

Chlorzoxazone (*32*) contains a benzoxazolone ring system, which is highly unstable due to presence of both lactam and lactone functional groups in the fused ring system. Both groups are subject to base catalysis. Paracetamol (*32*) contains an imine functional group, the degradation in aqueous solution appears to be both an acid catalysed and base cataysed hydrolysis.

Upon studying acid-base catalysis hydrolysis, it was found that chlorzoxazone and paracetamol in standard mixture solution and product exhibited incomplete hydrolysis products as revealed by thin layer chromatography (Figure 40-43). Many new spot formed of standard mixture solution and product whereas a new spot of the placebo were found in difference solvent with various time on silica gel GF_{254} with iodine detection.

5.2 Effect of oxidation degradation on standard mixture solution, pharmaceutical preparation and the placebo

The results were reported as earlier described in acid-base catalysis degradation as illustrated by TLC (Figure 44).

Figure 40 TLC of methanolic solution 0.35 mm, 3 x 7 cm Silica gel GF_{254} plates with iodine detection.

(A) chlorzoxazone (1), standard solution of chlorzoxazone and paracetamol (2), paracetamol (3)

(B) standard mixture solution (1), chlorzoxazone (2), product (3)

(C) standard mixture solution (1), paracetamcl (2), product (3)

(D) standard mixture solution (1), standard solution of chlorzoxazone and paracetamol (2), product (3)

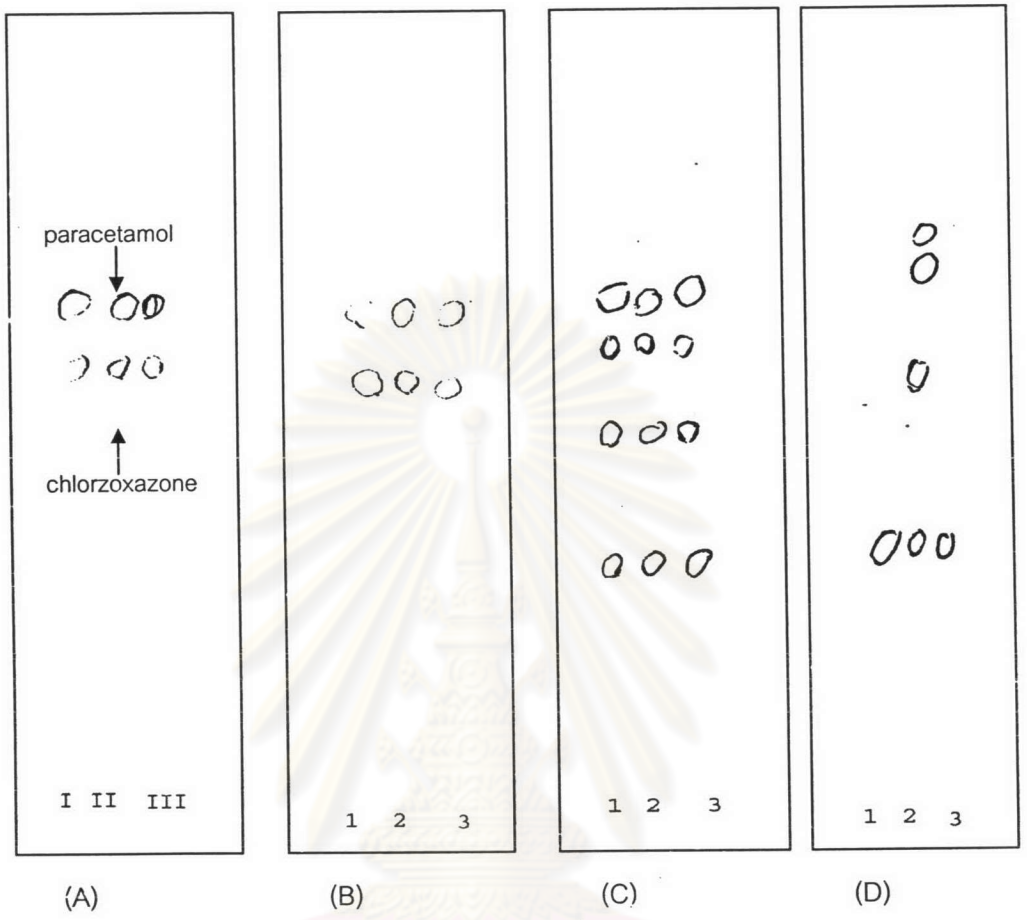

Figure 41 TLC of hydrolysis of standard mixture solution on 0.35 mm, 3 x 7 cm Silica Gel GF_{254} plates with iodine detection.

A = in methanol (I), acid (II) and base (III) at t = 0 hr.

B = methanolic solution.

C = acid-catalysis hydroiysis.

D = base-catalysis hydrolysis.

1, 2, 3 = time at 1, 2 and 3 hr., respectively.

Figure 42 TLC of hydrolysis of product on 0.35 mm, 3 x 7 cm Silica gel GF_{254} plates with iodine detection.

A = in methanol (I), acid (II) and base (III) at t = 0 hr.

B = methanolic solution.

C = acid-catalysis hydrolysis.

D = base-catalysis hydrolysis.

1, 2, 3 = time at 1, 2 and 3 hr., respectively.

Figure 43 TLC of hydrolysis of the placebo on 0.35 mm,3 x 7 cm Silica gel GF_{254} plates with iodine detection.

A = the methanol (I), acid II) and base (III) at t = 0 hr.

B = methanolic solution.

C = acid-catalysis hydrolysis.

D = base-catalysis hydrolysis.

1, 2, 3 = time at 1, 2 and 3 hr., respectively.

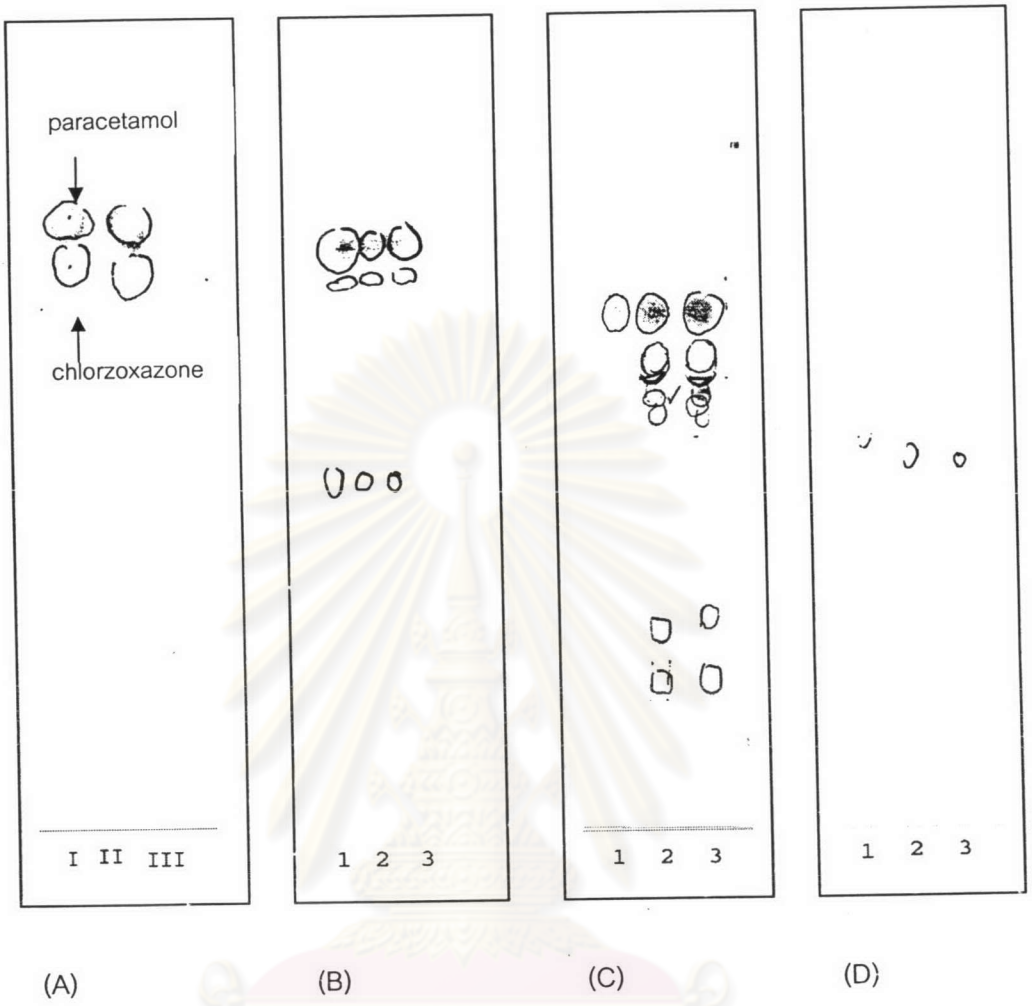

Figure 44 TLC of oxidation on standard mixture solution, product and the placebo degradation on 0.35 mm,3 x 7 cm Silica gel GF_{254} plates with iodine detection.

A = standard mixture solution (I), the placebo (II), product (III) and at t = 0 hr.

B = standard mixture solution.

C = product.

D = the placebo.

1, 2, 3 = time at 1, 2 and 3 hr., respectively.

5.2. Effect of photolysis degradation

The photo-induced catalysis method was determine simultaneous in the absorption spectra of standard mixture solution of chlorzoxazone and paracetamol, product and the placebo in methanol by UV-Visible. The purposed method was evaluated as the percentage of recovery each week (except the placebo). The results of standard mixture solution and product of chlorzoxazone and paracetamol were given in Table 22. The obtained results were compare and gave rise nearly each other to percentage of recovery values. The range of 103.59-106.28% and 101.06-104.5% in the PCR model, as well as, the range of 104.18-106.27% and 101.60-104.54% in the PLSR model for standard mixture solution of chlorzoxazone and paracetamol, respectively. The range of 104.93-107.44% and 100.34-104.76% in the PCR model, as well as, the range of 105.1-107.69% and 100.96-105.28% in the PLSR model for product of chlorzoxazone and paracetamol, respectively.

No statistically significant difference of the percentage of recovery of standard mixture and their product were observed between condition of daylight and dark as theoretical values for t-test at P = 0.05 level (t = 3.1824, n = 4). The result were confirmed by bright (daylight) and dark lines were shown parallel likely in trend upper for chlorzoxazone and down for paracetamol as illustration in Figure 45 and Figure 46, respectively.

Table 22 Effect of photolysis on standard mixture solution and product (chlorzoxazone: paracetamol = 12.5: 15 µg/ml).

Condition solution	Week	%Recovery							
		Standard mixture solution				Product			
		PCR		PLSR		PCR		PLSR	
		C	P	C	P	C	P	C	P
Daylight	1	104.15	101.56	104.71	101.4	105.02	100.35	105.6	100.17
	2	104.76	101.78	105.07	101.76	104.93	100.68	105.16	100.54
	3	105.09	103.41	105.04	103.28	105.72	102.59	105.47	102.49
	4	106.28	104.5	106.27	105.28	107.16	104.76	106.92	104.54
Dark	1	104.30	101.49	104.64	101.34	105.1	100.37	106.12	100.16
	2	103.59	101.06	104.18	100.96	104.78	100.34	105.1	100.2
	3	104.58	102.34	104.46	102.27	107.44	102.97	107.65	102.65
	4	105.13	103.54	105.7	103.39	106.83	103.85	107.69	103.61
t (cal)*		2.183	2.425	3.11	2.496	0.701	0.773	1.793	1.165

**t* (crit) = 3.1824, p = 0.05

C = chlorzoxazone, P = paracetamol

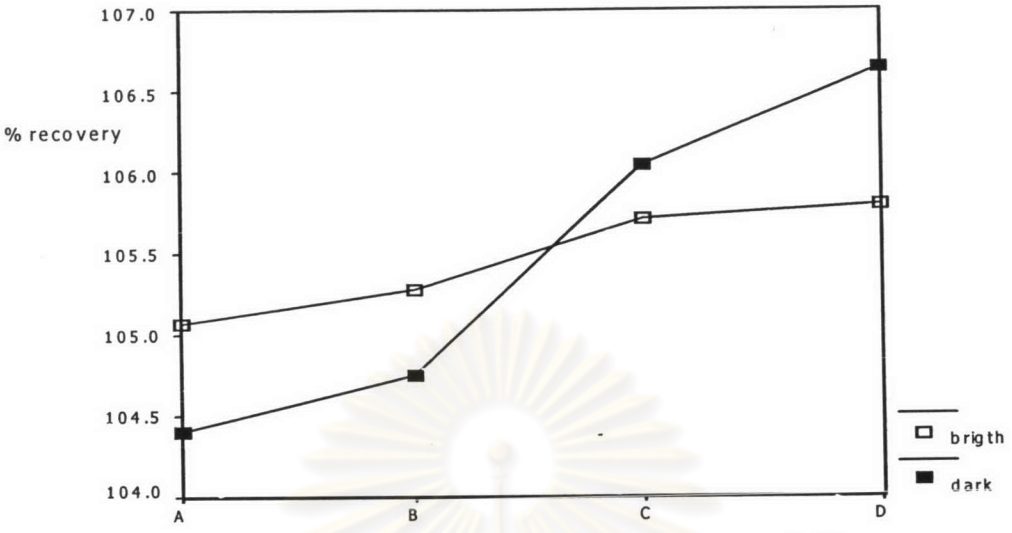

Figure 45 Estimated mean average of the percentage recovery of chlorzoxazone in standard mixture solutions and commercial tablets.

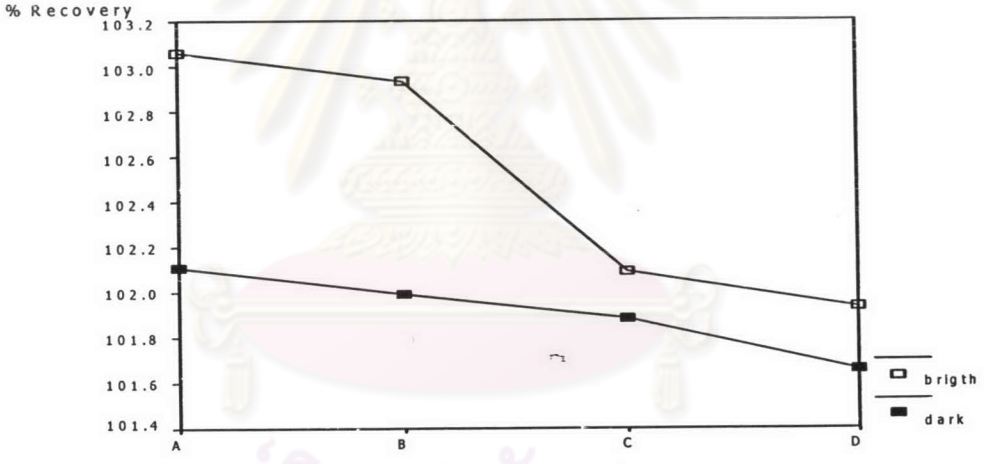

Figure 46 Estimated mean average of the percentage recovery of paracetamol in standard mixture solutions and commercial tablets.

A = standard mixture in PCR model

B = standard mixture in PLSR model

C = product in PCR model

D = product in PLSR model

Additionally, it can be seen that the absorption spectra of standard mixture solution, product and the placebo, storing in the dark and exposing to daylight for four weeks, were slightly different as shown in Figure 47, 48 and 49, respectively. Finally, No statistically significant photolysis was observed upon exposing the standard mixture solution, product and the placebo to daylight for a month.

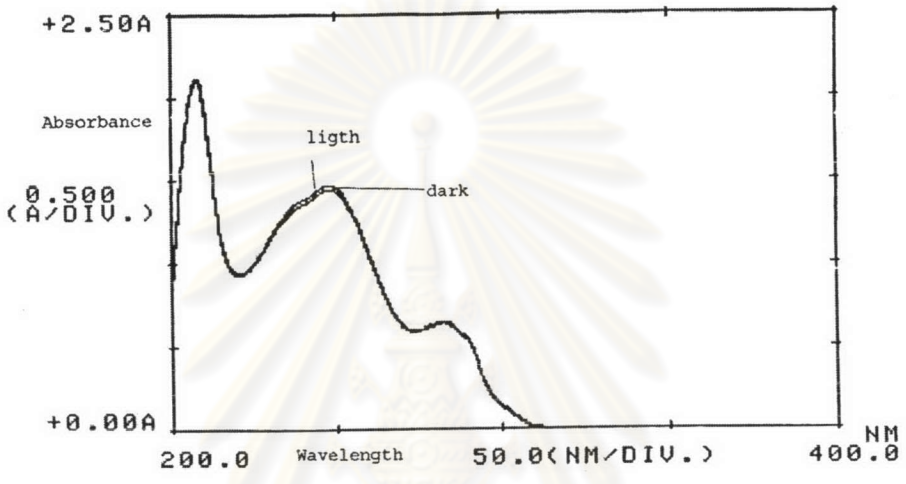

Figure 47 Comparison of UV absorption spectra of standard mixture solutions, after four weeks of storing in the dark and exposing to daylight.

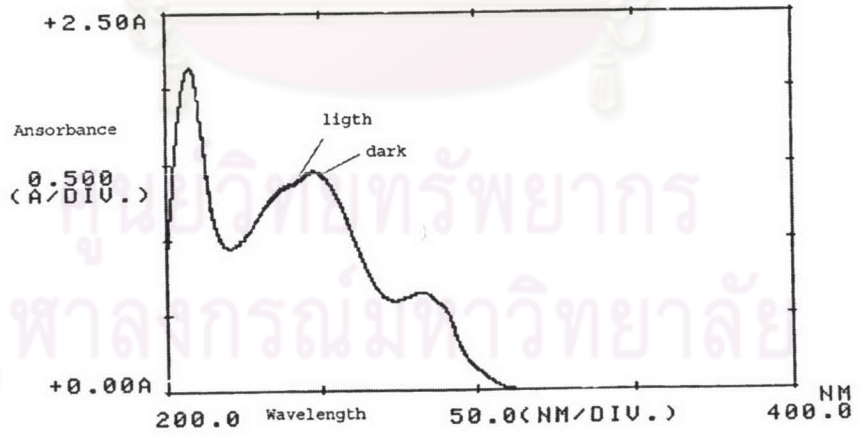

Figure 48 Comparison of UV absorption spectra of commercial tablets, after four weeks of storing in the dark and exposing to daylight.

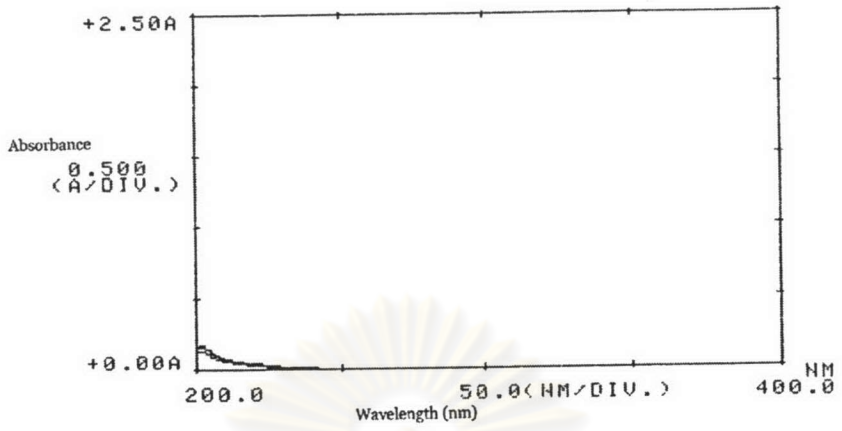

Figure 49 Comparison of UV absorption spectra of placebo, after four weeks of storing in the dark and exposing to daylight.