CHAPTER III

MATERAIALS AND METHODS

Materials

1. Chemicals and reagents

   1.1 Working standards

      1.1.1   Chlorzoxazone, Batch no. B-1720, 99.16% purity, Dy-mach Pharma, India.

      1.1.2   Paracetamol, 99.48% purity; Siam Pharmaceutical Co., Ltd., Thailand.

   1.2 Commercial Preparations

      Two brands of tablets were selected for this study. Product A (Parafon-fort®, Janssen-Cilag) is the imported product. Product B (Cezox®, Pharmasant Lab) is local made product. All products are purchased from drug stores and all have labeled amount of 250 mg chlorzoxazone and 300 mg paracetamol in one tablet.

   1.3. Chemical reagents, all were analytical grade.

      1.3.1   Methanol, E. Merck, Damstadt, Germany.

      1.3.2   Hydrochloric acid, fuming 37%, Lab Scan, Bangkok, Thailand.

      1.3.3   Sodium hydroxide, anhydrous pellets, Mallinckrodt, Mexico.

      1.3.4   Ammonia solution 25%, Lab Scan, Bangkok, Thailand.

      1.3.5   Ethyl acetate, Lab Scan, Bangkok, Thailand.

2   Apparatus

   2.1   UV-Visible Spectrometer (Model UV-160A, SHIMADZU, Japan).

   2.2   Thin layer chromatography plate (Siliga gel GF_{254}, 0.25 mm, 3 x 7 cm) Merck, Germany.

   2.3   MINITAB 14® software, Six Sigma company, U.S.A.

Methods

The experiments were performed in the following sequence

1. Determination of the spectrophotometric condition

2. Establishment of calibration models

3. Method validation

4. Assay of commercial tablets

5. TLC of degradation compounds of chlorzoxazone and paracetamol

1. Determination of the spectrophotometric condition

1.1 Stock solutions

Separately dissolve accurately weighed quantities of chlorzoxazone working standard and paracetamol working standard in methanol to obtain solutions having known concentrations of about 1.0 mg/ml of chlorzoxazone and 1. 2 mg/ml of paracetamol, and mix. Store in a refrigerator at 4 ^{0}C.

1.2 Determination of linear range of concentrations

The solutions of chlorzoxazone containing 1.09, 2.19, 4.37, 8.75, 17.5, 35 and 50 µg/ml and of paracetamol containing 0.48, 0.96, 1.91, 3.83, 7.65, 15.3, 25.5 and 30 µg/ml were separately prepared using methanol as a solvent. The absorbance of each solution was determined at 283 nm for chlorzoxazone and 248 nm for paracetamol, using solvent as a blank. The absorbances of chlorzoxazone and paracetamol solutions were plotted versus their concentrations. The selected concentration range were concentrations that obey Beer's law.

2. Establishment of calibration models

2.1 Experimental design of the calibration and validation sets

A mixture design is used to maximize statistically content information in the spectra. A calibration set of 26 samples was prepared. The concentrations of chlorzoxazone and paracetamol were varied between 3.75-18.75 and 4.5-22.5 µg/ml, respectively, through the calibration matrix. The compositions of the binary mixtures used in the calibration matrix was summarized in Table 2.

Table 2   Compositions and concentrations of the calibration set.

Mixture Number	Chlorzoxazone (µg/ml)	Paracetamol (µg/ml)	Mixture Number	Chlorzoxazone (µg/ml)	Paracetamol (µg/ml)
S1	3.75	22.5	S14	11.75	9
S2	3.75	18	S15	7.5	9
S3	3.75	13.5	S16	9.375	11.25
S4	3.75	9	S17	3.75	0
S5	3.75	4.5	S18	7.5	0
S6	7.5	18	S19	11.25	0
S7	11.25	13.5	S20	15	0
S8	15	9	S21	18.75	0
S9	18.75	4.5	S22	0	4.5
S10	15	4.5	S23	0	9
S11	11.25	4.5	S24	0	13.5
S12	7.5	4.5	S25	0	18
S13	7.5	13.5	S26	0	22.5

A validation set of nine standard mixtures was prepared and their compositions were summarized in Table 3.

Table 3 Compositions and concentrations of the validation set.

Mixture Number	Chlorzoxazone (µg/ml)	Paracetamol (µg/ml)
V1	3.75	20.25
V2	5.625	15.75
V3	5.625	11.25
V4	5.625	4.5
V5	9.375	15.75
V6	9.375	6.75
V7	11.25	11.25
V8	13.125	6.75
V9	16.875	6.75

A diagrammatic representation of the mixture design of the calibration set and the validation set was shown in Figure 10.
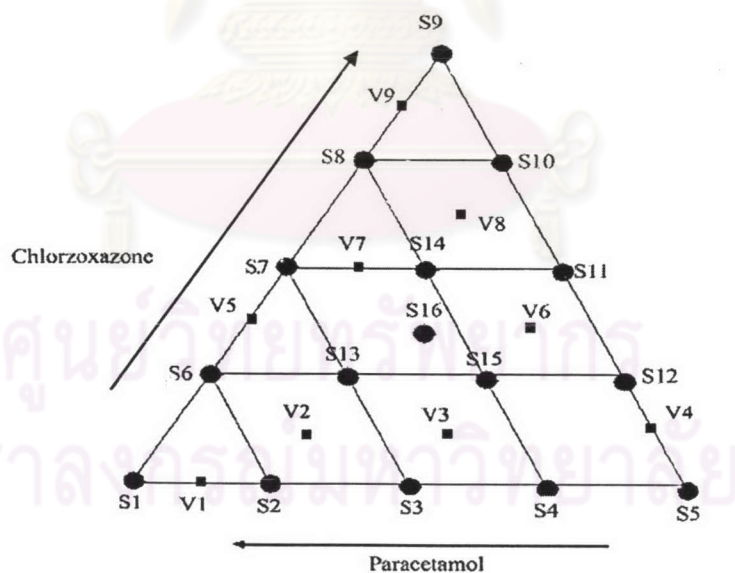

Figure 10 Mixture design for the two-component mixtures used in the calibration set
(●) and the validation set (■).

2.2 <u>Selection of wavelength ranges and the number of principal components</u>

The PCR and PLSR models were constructed from the concentrations and absorbances of the calibration set measuring at five nanometers interval through the wavelength range of 220-350 nm using MINITAB program. Cross validation method, leaving out one sample at a time, was used to select the number of principal components (PCs) in the algorithms. The models were applied to the validation set of standard mixture solutions of chlorzoxazone and paracetamol. The predicted amount of analytes found in the mixtures were obtained from the PCR and PLSR models. The percentage of recovery and relative standard deviation were also calculated.

3. Method validation

The tablet placebo containing 608 mg lactose, 304 mg corn starch, 50 mg polyvinylpyrrolidone K30, 30 mg talcum and 8 mg magnesium stearate was prepared and used for testing in method validation.

3.1 <u>Accuracy</u>

The accuracy of the proposed method was evaluated as the percentage of recovery using the spiked placebo technique. Five different amounts of chlorzoxazone and paracetamol, covering 32-160% of the expected concentrations of drugs, were added to the placebo. The solutions were prepared in triplicate at each concentration. The absorbance data matrix of the spiked placebo solutions was obtained by measuring the absorbance at 26 wavelength points with the intervals of 5 nm ($\Delta\lambda$ = 5 nm) in the spectral region between 220 and 350 nm. Percent recovery was calculated from the ratio of the amount found and the amount added. In order to apply these methods for the analysis of the pharmaceutical dosage form, the influences of commonly used excipients and tablet additives were investigated.

3.2 Precision

Precision of the method was determined in term of percent of relative standard deviation (%RSD), which was determined from the following formula:

$$\%RSD = \frac{SD \times 100}{\overline{X}}$$

where SD and $\overline{X}$ are the standard deviation and mean of the observed data, respectively.

The precision was assessed as follows:

3.2.1 System precision

Absorbances of five standard mixture solutions of chlorzopxazone 4, 8, 12, 16 and 20 µg/ml and paracetamol of 4.8, 9.6, 14.4, 19.2 and 24 µg/ml were measured over the wavelength ranges of 270-295 nm for chlorzoxazone (both PCR and PLSR), 230-350 nm for paracetamol with PCR and 230-270 nm for paracetamol with PLSR analyses. The absorbances were measured in triplicate.

3.2.2 Within-run precision

Five spiked placebo solutions were prepared by adding standard chlorzoxazone (4-20 µg/ml) and paracetamol (4.8-24 µg/ml) to about 120 mg placebo. The solutions were prepared in triplicate at each concentration. Absorbances of the solutions were measured with the same procedure as listed in 3.2.1.

3.2.3 Between-run precision

Absorbances of five spiked placebo solutions, preparing as described in "within-run precision", were measured with the same procedure as listed in 3.2.1. The measurement was performed for three days.

3.3 Linearity and range

Five standard mixture solutions of placebo were prepared with concentration of chlorzoxazone and paracetamol over the range of 4-20 and 4.8-24 µg/ml, respectively. The concentrations of such drugs in the above solutions were calculated

using the PCR and PLSR models. Method linearity was obtained by plotting the concentration found versus the concentration added (Figure 45-48). The coefficient of determination, using least square analysis, was also determined.

4. Assay of commercial tablets

Weighed and finely powdered not less than 20 tablets of product A and product B. Transferred an accurately weighed portion of the powder, equivalent to about 250 mg of chlorzoxazone and 300 mg of paracetamol, to a 200-ml volumetric flask, added about 120 ml of methanol, and shaked by mechanical means for about 15 minutes. Diluted with methanol to volume and mix. Filtered a portion of this solution, discarding the first 10 ml of the filtrate. Transferred 1.0 ml of the clear filtrate to a 100-ml volumetric flask, diluted with methanol to volume, and mix.

For assaying of chlorzoxazone, using PCR and PLSR with two principal components (PCs), determine the absorbances of the above solutions at the wavelength range of 270-295 nm. For assaying of paracetamol, determine the absorbances of the above solutions at the wavelength ranges of 230-270 and 230-350 nm for PCR with three principal components and PLSR with two principal components, respectively. Methanol is used as the solvent blank.

5. TLC of degradation compounds of chlorzoxazone and paracetamol

5.1 Effect of acid-base catalysis degradation

5.1.1 Effect on standard mixture solution

Acid-base catalysis was studied using the stress condition with th addition of 6N hydrochloric acid and 6N sodium hydroxide (40-43).

Acid catalysis on standard mixture solution was studied by adding three drops of 6N hydrochloric acid to 100 ml methanolic solution containing 0.0625 g chlorzoxazone, 0.075 g paracetamol and 0.03 g of placebo, and mixing

thoroughly. The solution was then kept in the hot air oven at 80 $^{\circ}$C for 0, 1, 2 and 3 hours. Thereafter, the solution was neutralized with 6N sodium hydroxide. Degraded product obtained was analyzed by the thin-layer chromatography. Silica gel GF_{245} was used as the stationary phase for the thin-layer chromatographic (TLC) plate. Develop the chromatogram in the saturated chamber, with a solvent system consisting of a mixture of ethyl acetate, methanol and 25% ammonia (6:3:1) until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Detect the spots on the TLC plate by placing the plate in the iodine-vapor tank.

Base catalysis was performed with the same procedure as acid catalysis, but replacing of 6N hydrochloric acid with 6N sodium hydroxide.

5.1.2 Effect on pharmaceutical preparations

Ten tablet of the products were finely powdered and accurately weighed portion of powder equivalent to one tablet. The experimental procedure was the same as described in 5.1.1.

5.1.3 Effect on the placebo

Accurately weighed the placebo powder equivalent to one tablet. Repeated the procedure described in 5.1.1.

5.2 Effect of oxidative degradation

5.2.1 Effect on standard mixture solution

Oxidative degradation on standard mixture solution was studied by adding three drops of 3% H_2O_2 to 100 ml methanolic solution containing 0.0625 g chlorzoxazone, 0.075 g paracetamol and 0.03 g of placebo, and mixing thoroughly. The solution was then kept 80 $^{\circ}$C in a hot air oven for 0, 1, 2 and 3 hours. Degraded product obtained was analyzed by the thin-layer chromatography with the chromatographic condition as described in 5.1.1.

5.2.2 Effect on pharmaceutical preparation

Ten tablet of the products were finely powdered and accurately weighed portion of powder equivalent to one tablet. The experimental procedure was the same as described in 5.2.1.

5.2.3. Effect on the placebo

Accurately weighed the placebo powder equivalent to one tablet. Repeated the procedure described in 5.2.1.

5.3 Effect of photolysis degradation

5.3.1 Effect on standard mixture solution

The methanolic standard mixture solution containing 0.0625 g chlorzoxazone, 0.075 g paracetamol and 0.03 g placebo was prepared. Photodegradation of the solution was studied by irradiated the solution under sunlight at room temperature for up to one month. At each week, the solution was filtrated and measured the absorbances using UV-Vis spectrophotometer over the wavelength described in 3.2.1. The average percentage of recovery of chlorzoxazone and paracetamol were calculated.

5.3.2. Effect on pharmaceutical preparation

Ten tablet of the products were finely powdered and accurately weighed portion of powder equivalent to one tablet. The experimental procedure was the same as described in 5.3.1.

5.3.3. Effect on the placebo

Accurately weighed the placebo powder equivalent to one tablet. Repeated the procedure described in 5.3.1.