CHAPTER II

EXPERIMENTATION

1. Instruments and Materials

1.1 Apparatus and Instruments

1.1.1 High Performance Liquid Chromatograph, consists of

- Waters model 600 E Multisolvent Delivery System (Milford, MA, USA)
- Waters model 484 Tunable Absorbance Detector (Milford, MA, USA)
- Waters model 746 Data Module (Milford, MA, USA)
- Injection system, Rheodyne 7125 equipped with a 20 μl loop
- Column, μBondapak Phenyl 10 μm, 30 cm x 3.9 mm i.d., Waters (Milford, MA, USA)

1.1.2 Syringe 100 μl, Exmire Microsyringe (Fuji, Japan)

1.1.3 Spectrophotometer, Shimadzu Double-Beam Spectrophotometer UV-180 (Kyoto, Japan)

1.1.4 Analytical balance, Precisa 300 A (Zurich, Switzerland)

1.1.5 pH-Meter, Consort P 307 Microcomputer ionmeter (Germany)

1.1.6 Ultrasonicator, Bransonic 321 (Conn., USA)

1.1.7 Autoclave, Hirayama model HA-3d (Hirayama Manufacturing Corporation, Japan)

1.1.8 Hot air oven, Heraeus Type T 5090 E (Germany)

1.1.9 Incubator, Memmert (Germany)

1.1.10 Micropipet, Pipetman 300 μl (Gilson, UK)

1.1.11 Stainless steel cylinders (OD 8 mm, ID 6 mm, height 10 mm, weight 1.85 g)

1.1.12 Glass petri dishes (20 x 100 mm)

1.2 Materials

1.2.1 Erythromycin, ASEAN reference standard

1.2.2 Erythromycin B, authentic substance (Abbott Laboratories, North Chicago, IL, USA)

1.2.3 Anhydroerythromycin A, authentic substance [Abbott Laboratories (IL, USA) and The Lilly Laboratories (Indianapolis, IN, USA)]

1.2.4 Anhydroerythromycin A - 6, 9 hemiketal enol ether, authentic substance [Abbott Laboratories (IL, USA) and The Lilly Laboratories (IN, USA)]

1.2.5 Glibenclamide, working standard

1.2.6 Erythromycin, raw material [Abbott Thailand (F.E.Zuellig (Bangkok) Ltd.) and Lupin Chemicals (Thailand) Ltd.]

1.2.7 Ery-Tab^{R} enteric-coated tablet, Abbott (each tablet contains Erythromycin base 250 mg)

1.2.8 Acetonitrile, HPLC grade (Baker Analyzed, NJ, USA)

1.2.9 Methanol, HPLC grade (Baker Analyzed, NJ, USA)

1.2.10 Ammonium acetate, AnalaR grade (Merck, Germany)

1.2.11 Phosphoric acid, AnalaR grade (Merck, Germany)

1.2.12 Sodium acetate, AnalaR grade (Merck, Germany)

1.2.13 Sodium dihydrogen phosphate, AnalaR grade (Merck, Germany)

1.2.14 Sodium hydroxide solution, 1 M

1.2.15 Microorganisms, *Micrococcus luteus* (ATCC No. 9341)

1.2.16 Culture media, Antibiotic Medium 1 for incubation and Antibiotic Medium 11 for base layer and seed layer (Difco Laboratories, Detroit, Michigan, USA)

1.2.17 Dibasic potassium phosphate, AnalaR grade (Merck, Germany)

1.2.18 Monobasic potassium phospate, AnalaR grade (Merck, Germany)

1.2.19 Potassium hydroxide, AnalaR grade (Merck, Germany)

2. Method

2.1 Development of chromatographic conditions

2.1.1 Column

Phenyl column was selected in this study. Its polarity is higher than C_8 and C_{18} column. Therefore, large molecular compound like erythromycin should be less retained and also could separate erythromycin A from other components and degradation products.

2.1.2 Detector

UV detector was used, and the UV spectrum of erythromycin A in mobile phase was scanned for selecting the optimum detection wavelength of condition.

2.1.3 Mobile phase

A search for the optimum mobile phase composition when using phenyl column and UV detection, in order to provide the best chromatographic separation of erythromycin A from its related substances and degradation products was investigated as follows:

a) The composition of organic solvents in mobile phase.

Various composition of acetonitrile and methanol in 0.05 M phosphate buffer pH 5.0 were prepared as shown in Table 2.

b) Type and concentration of buffer salts

Three kinds of inorganic salt, ammonium acetate, sodium acetate and sodium dihydrogen phosphate were used to prepare the buffer.

Then, mobile phase consisting of optimum composition of organic solvents from a) and selected buffer salt solution at pH 5.0 were prepared by varying the concentration of buffer in 0.010 to 0.100 M range.

c) The pH of buffer salt solution

Mobile phase consisting of optimum composition of organic solvents from a) and optimum concentration buffer from b) were prepared by varying pH of buffer in pH range of 3.5 to 6.5 by adjusting the pH with phosphoric acid and sodium hydroxide solution.

2.2 Stability of erythromycin solution in optimum mobile phase

In order to investigate the stability of erythromycin in the optimum mobile phase pH obtained from 2.1 c, the stability of erythromycin in mobile

phase at that pH was studied, also at lower and higher pH to observe the degradation of erythromycin at those pH. The solutions were examined at room temperature by analyzing periodically using the optimum HPLC method obtained from 2.1.

2.3 Selection of internal standard

To compensate for various analytical errors, 23 compounds, as shown in Table 11, were randomly screened as internal standard. The solution of substances was injected into the column using the chromatographic condition obtained from 2.1. The selected internal standard must have a completely resolved peak from all interested compounds, must not be present in original sample and must be unrective.

2.4 Developed HPLC method for analysis of erythromycin in raw material and dosage form

The finally optimum method from 2.1 to 2.3 are as follows:

2.4.1 Chromatographic conditions

Phosphate buffer (0.05 M) was prepared by dissolving sodium dihydrogen phosphate in distilled water and adjusting the pH to 5.0 with sodium hydroxide solution. The mobile phase was prepared by mixing 150 ml of acetoni-

trile, 380 ml of methanol and 470 ml of phosphate buffer and the mixture was filtered through a 0.45 μm membrane filter (Millipore, Type HV) and degassed by sonication. A 30 cm x 3.9 mm i.d. stainless-steel column packed with μBondapak Phenyl 10 μm, was used. The UV detector was set at a wavelength of 215 nm. The mobile phase was used at a flow rate of 1.0 ml per min with a resulting back-pressure of 1,500 p.s.i.

2.4.2 Internal standard solution

Glibenclamide was dissolved in methanol at concentration of 0.03 mg per ml.

2.4.3 Standard preparation

Transfer about 50 mg of erythromycin RS, accurately weighed, to a 50-ml volumetric flask, add 4.0 ml of internal standard solution, dissolve, dilute with mobile phase to volume and mix. The concentration of erythromycin was about 1 mg/ml.

2.4.4 Assay preparations

For raw material, transfer about 50 mg of erythromycin, accurately weighed, to a 50-ml volumetric flask, add 4.0 ml of internal standard solution, dissolve, dilute with mobile phase to volume and mix.

For tablets, weigh and finely powder not less than 20 tablets. Transfer an accurately weighed portion of the powder, equivalent to about 50 mg of

erythromycin, to a 50-ml volumetric flask. Add 35 ml of mobile phase and sonicate for 15 minutes. Add 4.0 ml of internal standard solution, dilute with mobile phase to volume, mix and filter through 0.45 μm membrane filter (Sartorius, Type PTFE).

2.4.5 Assay procedure

Separately inject equal volume (20 μl) of the standard preparation and the assay preparations into the chromatograph, record the chromatograms and measure the responses for the major peaks of erythromycin A. Calculate the quantity of erythromycin A in raw material and tablet using peak height ratios obtained from the assay preparations and standard preparation.

3. Analytical method validation

Validate the obtained method in 2.4 as follows :

3.1 Linearity and range

Linearity was assessed by preparing five standard solutions from 25 to 75 mg of erythromycin RS in 50 ml of mobile phase, which 4.0 ml of internal standard solution has been added, representing the concentration of 0.5 to 1.5 mg per ml of erythromycin. The solutions were then injected into the column using the chromatographic condition described in 2.4. Plot the peak height ratios of

erythromycin and internal standard versus the respective standard concentrations.

3.2 Precision

Intra-day precision was evaluated by repeat analysis of six replicate samples of erythromycin raw material or tablet, using the described HPLC method in 2.4.

Inter-day precision was similarly evaluated for six days. Three replicate samples were used.

The content of erythromycin A and the percentage of relative standard deviation (coefficient of variation) were determined.

3.3 Accuracy

The recoveries of erythromycin were determined using standard addition method. Weigh and finely powder not less than 20 tablets. Transfer accurately weighed portions of the powder equivalent to 35 mg of erythromycin, to 50-ml volumetric flasks. For determining the precision of recovery, add 15.0 mg of erythromycin RS to each flask, five replicates were done. For determining the linearity of recovery, add 5.0, 10.0, 15.0, 20.0 and 25.0 mg of erythromycin RS to each flask. Then, dissolve these samples with mobile phase and

sonicate for 15 minutes. Add 4.0 ml of internal standard solution, dilute with mobile phase to volume, mix and filter through 0.45 μm membrane filter. Calculate the quantity of erythromycin A from calibration curve of 0.48 to 1.28 mg/ml of erythromycin A.

The percentage of recovery was determined by comparing the amount of erythromycin found with the amount added.

3.4 Selectivity or specificity

Selectivity was determined by comparing test results from the analysis of erythromycin tablets as described in 2.4.4 with and without the addition of authentic substances of erythromycin B, anhydroerythromycin A and erythromycin A enol ether.

3.5 Limit of detection

Limit of detection was determined by comparing test results from known concentration of erythromycin substance with those of blank sample (signal-to-noise ratio) and established the minimum level at which erythromycin could be reliably detected. The concentration that gave a signal-to-noise ratio of about 2:1 was accepted.

4. Quantitative analysis of raw material and tablets by HPLC method

The optimum HPLC method in 2.4 was used for analysis of three batches of raw materials and five batches of erythromycin tablets. Six replicate samples of each batch were performed. The amount of erythromycin A in samples was calculated using peak height ratios obtained from assay preparation compared with those obtained from standard solution.

5. Quantitative analysis of raw material and tablets by microbiological assay

The content of erythromycin using the same samples as in HPLC method was determined by the microbiological assay. The current official method of assay for erythromycin (raw material and dosage form) in USP XXII (1990) was used. It employed the cylinder-plate assay using *Micrococcus luteus* (ATCC 9341) as the test organism and Antibiotic Medium 11 as the culture medium. Phosphate buffer (pH 8.0) was used to dilute samples (to about 1.0 μg per ml), as well as for standard curves (from 0.60 to 1.50 μg per ml).

6. Comparison of quantitative analysis of raw material and tablets by HPLC and microbiological assay

The test results obtained from HPLC method were compared with those obtained from microbiological assay by using statistical t-test.