CHAPTER III

RESULT AND DISCUSSION

1. Development of chromatographic conditions

1.1 Column

In several reports, C_8 and C_{18} column was used to separate erythromycin, its related substances and degradation products, and found that erythromycin was too strongly retain in the stationary phase. In addition, heating the column at 70 $^{\circ}$C can accelerate the elution of the compound from the column. However, heating the column can be harmful to the column lifetime.

It is expected that the strong retention of any compounds in the stationary phase like C_8 and C_{18} can be eliminated by using the more polar stationary phase column. Therefore, phenyl column, which is commercially available was selected for this experiment.

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In Table 1, the polarity of the phenyl column is higher than C_8 and C_{18} (N-Alkyl) columns. Other more polar stationary phase like diol, amine and silica are normally considered to be used in normal phase

separation. Therefore, diol, amine or silica column were not selected as stationary phase.

From the experiment, highly efficient separation was achieved from phenyl column with symmetrical peaks even at room temperature.

1.2 Detector

UV spectrum of erythromycin A in mobile phase is shown in Figure 3. The wavelength of maximum absorption was at 288 nm, however the absorptivity was very weak. Detection at this wavelength of major component i.e. erythromycin A can be measured, however, minor components i.e. erythromycin B, erythromycin C, anhydro-erythromycin A and erythromycin A enol ether cannot be detected due to small amount presence and the low absorptivity. At wavelength 200 - 210 nm, the absorptivity is strong but unstable baseline can be observed in the chromatogram which is due to UV cut-off of the acetonitrile (210 nm) and methanol (205 nm). Wavelength at 215 nm was selected since the absorptivity of the compounds were still high and the unstable baseline could be eliminated.

1.3 Mobile phase

The retention times of erythromycin A, erythromycin B, erythromycin C, anhydroerythromycin A and erythromycin A

enol ether were influenced by acetonitrile-methanol composition, buffer concentration and the pH of the mobile phase as follows:

a) <u>The composition of organic solvents in mobile phase</u>

From the experiment, the mixture of acetonitrile and methanol in various proportions were used as shown in Table 2. The influence of acetonitrile and methanol composition in the mobile phase on the capacity factor (k') of erythromycin A, erythromycin B, erythromycin C, anhydroerythromycin A and erythromycin A enol ether are shown. In general k' value for the analysis procedure should not less than 1.0 and not more than 5.0. When acetonitrile was used only at 40% concentration, k' value of erythromycin C was too small and anhydroerythromycin A did not resolved from erythromycin A enol ether. This circumstance can be explained by the fact that the eluting power of acetonitrile is too strong. However, when organic cosolvent was used by reducing the composition of acetonitrile and addition of methanol in various ratio, the well separation between all interested peaks was achieved. The selected optimal composition was acetonitrile and methanol in ratio of 15:38 (v/v), this selection was based on k' value, resolution and analysis time. The retention time of erythromycin C, erythromycin A, erythromycin B, anhydroerythromycin A and erythromycin A

enol ether was 6.7, 8.3, 10.5, 12.3 and 15.0 respectively.

b) <u>Type and concentration of buffer salt</u>

Buffer solution preparing from ammonium acetate, sodium acetate and sodium dihydrogen phosphate were commonly used as a mobile phase composition. For determining the influence of buffer salt, fix organic solvent composition in the mobile phase were evaluated. In Table 3, the effect of buffer salt on plate number and tailing factor was reported, the highest number of theoretical plates and the lowest tailing factor was obtained when sodium dihydrogen phosphate was used as buffer. Therefore, sodium dihydrogen phosphate was selected as buffer salt.

In order to determine the effect of concentration of sodium dihydrogen phosphate on retention of erythromycin, the various concentration of buffer (0.010 to 0.100 M) were prepared with constant organic solvents composition. Table 4 and Figure 4 show the influence of buffer concentration on capacity factor (k'). The capacity factors of all compounds decreased when buffer concentration increased. The variation of buffer concentration at 0.010 and 0.025 M did much affect the capacity factor of all compounds, but the influence at the higher concentration from 0.050 M was small. When buffer concentration at 0.010 to 0.050 M was used, all compounds

were well separated, when increasing the buffer concentration from 0.05 to 0.100 M, peak overlapping of erythromycin C and erythromycin A were noted. Finally, buffer solution at 0.050 M was selected due to well separation and reasonable capacity factor values of all compounds.

c) The pH of buffer salt solution

The effect of mobile phase pH on capacity factor is shown in Table 5 and Figure 5. The capacity factors of all compounds increased when the pH was increased. At pH 3.5 and 4.0, erythromycin C did not resolved from erythromycin A completely. When pH more than 5.0 was used, complete separation was achieved but long retention of compounds were also noted. Therefore, at buffer pH 5.0 was considered to be optimum.

Thus, the optimum chromatographic conditions of phenyl column, UV detector at 215 nm and the mobile phase of acetonitrile, methanol and 0.05 M sodium dihydrogen phosphate at pH 5.0 (15:38:47, v/v) were used.

Chromatograms of erythromycin A, its related substances (i.e. erythromycin B, erythromycin C), degradation products (i.e. anhydroerythromycin A, erythromycin A enol ether) and internal standard (glibenclamide) are illustrated in Figure 6. The eluting order was erythromycin C, erythromycin A, erythromycin B, anhydro-

erythromycin A, erythromycin A enol ether and glibenclamide with the retention time of 6.7, 8.3, 10.5, 12.3, 15.0 and 18.6 minutes respectively.

2. Stability of erythromycin solution in optimum mobile phase

Erythromycin solution in buffer pH 3.0, 4.0, 5.0, 6.0 and 6.5 were examined at room temperature for 2 weeks. The results are presented in Table 6-10 and Figure 7-11. The content of anhydroerythromycin A and erythromycin A enol ether shown in these Tables were calculated as erythromycin A. At the optimal buffer pH of 5.0, the content of erythromycin A was quite stable. However slightly degradation was observed (as shown in Table 6 and Figure 7), confirming the need to make a fresh solution for each analysis.

At buffer pH of 3.0, erythromycin A degraded rapidly and erythromycin A enol ether was greatly found, as shown in Table 7 and Figure 8.

At buffer pH of 4.0, the content of erythromycin A slightly decreased but erythromycin A enol ether was quite clearly detected, the results presented in Table 8 and Figure 9.

This experiment confirm the report of Atkins,

Herbert and Jones (1986) that erythromycin degraded in acid medium.

At buffer pH of 6.0 and 6.5, erythromycin A was stable (as shown in Table 9-10 and Figure 10-11) but, as mentioned above, these pH were not selected because long elution time was obtained.

Thus, the stability of erythromycin in the optimal mobile phase was quite good.

3. Selection of internal standard

Table 11 shows the relative retention times of the various substances to erythromycin A enol ether. From relative retention time in this table, glibenclamide was chosen as internal standard since it was well separated from all interested compounds under the optimum condition and elution time was not too long, as shown in Figure 6.

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

The optimal method has already summarized in 2.4 of Chapter II and the obtained chromatogram is shown in Figure 6.

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5. Analytical method validation

Three batches of erythromycin raw material and five batches of erythromycin enteric-coated tablets were used in this experiment. Detail of the sample used is shown in Table 12.

5.1 Linearity and range

Five standard solutions of 0.48, 0.64, 0.80, 0.96, 1.12 and 1.28 mg per ml of erythromycin A were used in the linearity determination. When plotting the peak height ratio of erythromycin A to that of the internal standard versus the erythromycin A concentration, the calibration curve was found to be linear over the range of concentration observed. A slope of 1.3975, and y-intercept of 0.0943 was obtained by linear regression analysis and correlation coefficient was 0.9999 as shown in Table 13 and Figure 12. This result proved that the concentration of about 1 mg/ml of erythromycin used in standard and assay preparations was in linearity range.

5.2 Precision

Intra-day and inter-day precision data for the analysis of erythromycin raw material and tablets are summarized in Table 14-17.

For the intra-day precision of raw material RM1, the contents of erythromycin A were 903.92 to 912.10 μg/mg with a relative standard deviation of 0.34% (n=6), as shown in Table 14. For inter-day precision of the same sample, the mean contents of erythromycin A were 898.61 to 908.29 μg/mg with a relative standard deviation of 0.39% (n=6), as shown in Table 15.

The intra-day precision of tablets FP4, the contents of erythromycin A were 225.05 to 232.84 mg/tab with a relative standard deviation of 1.21% (n=6), as presented in Table 16. For the inter-day precision of the same sample, the mean contents of erythromycin A were 225.96 to 234.85 mg/tab with a relative standard deviation of 1.34% (n=6), as shown in Table 17.

From these results, the observed relative standard deviations are quite low, maximum value is 1.34%, so the precision of this method is satisfactory.

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5.3 <u>Accuracy</u>

For precision of recovery, three sets of five replicate standard addition method were performed as shown in Table 18. The percentage of recoveries of 97.86, 103.12 and 98.15% were found with relative standard deviations of 2.13, 4.12 and 3.51% respectively. The observed recoveries are nearly 100% which is acceptable value.

However, the percentage of relative standard deviation was quite high, the variation may occured from the coating material of enteric-coated tablets.

For linearity of recovery, the relationship between the added erythromycin and the erythromycin found was linear with correlation coefficient of 0.9997, 0.9983 and 0.9989. The results are shown in Table 19.

5.4 Selectivity or specificity

Table 20 shows peak response from the analysis of erythromycin tablets with and without the addition of erythromycin B, anhydroerythromycin A and erythromycin A enol ether. Chromatograms are also shown in Figure 13. The results show that these substances do not interfere, both position and peak response, with erythromycin A peak.

Therefore, this chromatographic condition is able to measure the analyte specifically in the presence of its related substances and degradation products.

5.5 Limit of detection

The limit of detection, of this method was 9.26 ppm of erythromycin based on peaks that could be easily manually measured (signal to noise ratio was about 2:1). Precision data were obtained by the repeated analysis and

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resulted in relative standard deviation of 5.06% (n=10) as shown in Table 21.

The obtained detection limit is sufficiently sensitive for detection.

6. <u>Quantitative analysis of raw material and tablets by HPLC method</u>

With the optimum chromatographic condition, a number of commercial erythromycin raw material and enteric-coated tablets samples, as shown in Table 12, were analysed. The chromatograms are shown in Figure 14.

The mean content of erythromycin in term of erythromycin A obtained by analysis of three batches of raw material were 911.07, 909.71 and 840.60 μg/mg on anhydrous basis with relative standard deviation of 1.18, 0.81 and 1.16% (n=6) respectively, as shown in Table 22.

The USP content limit of erythromycin in raw material is not less than 850 μg per mg, calculated on the anhydrous basis (USP,1990). The results are all in good precision, but the content of erythromycin in RM3 is lower than the limit. This could be explained by the degradation of erythromycin during production processing or during storage, as shown by the chromatograms, in Figure 15, compared with those obtained from the other manufacturer.

The mean content of erythromycin in term of erythromycin A obtained by analysis of five batches of enteric-coated tablets were 247.95, 238.68, 230.94, 229.36 and 227.86 mg/tablet with relative standard deviation of 1.98, 1.90, 1.90, 1.23 and 0.97% (n=6) respectively, as shown in Table 23.

The USP (USP, 1990) content limit of erythromycin in tablets is not less than 90.0 and not more than 120.0 percent of labeled amount, in this case, equivalent to erythromycin 225 to 300 mg/tablet. The results are all within the limit with good precision, however, the amount of three batches (FP3, FP4 and FP5) were quite low.

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

The mean content of erythromycin of the three batches of raw material were 893.11, 896.65 and 834.49 μg/mg on anhydrous basis with relative standard deviation of 7.63, 4.74 and 8.17% (n=2) respectively, as shown in Table 22.

The mean content of erythromycin of the five batches of enteric-coated tablets were 244.72, 238.65, 235.07, 234.04 and 229.86 mg/tablet with relative standard deviation of 6.24, 6.24, 4.29, 6.23 and 3.04% (n=2) respectively, as shown in Table 23.

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

For comparison purpose, the microbiological assay was performed in parallel with this HPLC method on the same samples of erythromycin raw material and erythromycin enteric-coated tablets, as reported in 6 and 7. The results obtained indicated good correlation between the HPLC and microbiological assays significantly at 95% confidential limit by using the statistical t-test, as shown in Table 24. However, the relative standard deviation obtained from microbiological assay were 4.74 - 8.17% (n=2) for raw material and 3.04 - 6.24% (n=2) for tablets which were higher than those obtained from HPLC method, 0.81 - 1.18% (n=6) for raw material and 0.97-1.98% (n=6) for tablets. The variation may be from small sample size used (n=2) and other factors such as many steps procedure in assay preparation, precision in inoculum preparation and technical skill etc.

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