CHAPTER III

RESULT AND DISCUSSION

1. Development of HPLC Condition

1.1 Optimization of mobile phase

The initial experiments were designed to examine the variation of capacity factor (k´) with the v/v composition of the mobile phase (80-95% methanol) in 20 mM of ammonium acetate (CH_3COONH_4) at pH 7.0 (adjusting the pH of the final eluent).

Table 1 shows the variation of k´ with the volume ratio of methanol and ammonium acetate in the mobile phase. The plot k´ versus percentage of methanol in the mobile phase was also shown in Figure 1.

Condition, a k´ between 1 and 10 is usually desired (Glajch and Kirkland, 1983). For the mobile phase of 80% methanol and 20% ammonium acetate buffer, the k´ values of all compounds seemed to be resonable, but the system did not work well with the sample containing degradation products of promethazine hydrochloride. The system could not resolve the peaks of degradation products of promethazine hydrochloride from that of ephedrine hydrochloride. The same problem

occurred when the mobile phase was composed of 85% methanol and 15% buffer.

When the composition of the mobile phase was 95:05 methanol: buffer, codeine phosphate and promethazine hydrochloride were coeluted and k′ values of all compounds were quite long unsuitable for the analysis.

For the mobile phase 'composition of 90% methanol, the optimum resolution of all drugs and degradation products of promethazine hydrochloride was obtained with appropriate k′ values. Therefore, the system with 90% methanol was chosen.

The next step in the optimization procedure was to determine the optimum pH of the mobile phase (pH 6.0 - 7.5). The results obtained from this investigation were presented in Table 2 as well as in Figure 2.

All analytes seemed to be well resolved from each other at all pH's (6.0-7.5), except at pH 7.5 where the peaks of codeine phosphate and methylephedrine hydrochloride were overlapped and the peaks of codeine phosphate and ethylephedrine hydrochloride were too close. At pH 6.0 and 6.5, k′ values of promethazine hydrochloride and codeine phosphate were quite large and unsuitable for the assay. The mobile phase pH 7.0 was found to be the best, considering both resolution and k′ value of all analytes.

Finally, the concentration of the ammonium acetate buffer was varied between 10-30 mM ammonium acetate. The results of the variation of k′ with the concentration of buffer in mobile phase were presented in Table 3 and Figure 3. Poor resolutions of codeine phosphate and promethazine hydrochloride were obtained when the buffer concentrations were 10, 15, 25 and 30 mM ammonium acetate. The best resolution with resonable k′ values of all analytes was obtained at the buffer concentration of 20 mM ammonium acetate. Therefore, the concentration of 20 mM ammonium acetate buffer was selected.

Thus, the concentration of 20 mM of the ammonium acetate buffer and mobile phase composition of 90% methanol at pH 7.0 was used at a flow rate of 2.0 ml/min. The pressure was found not greater than 2,000 psi. The retention times of ephedrine hydrochloride, codeine phosphate and promethazine hydrochloride were 4, 7 and 9 min, respectively.

1.2 Selection of internal standard

Ethylephedrine hydrochloride was chosen as internal standard, because its peak was sharp, symmetrical and well separated from ephedrine hydrochloride, codeine phosphate and promethazine hydrochloride (Figure 4-6). Furthermore,ethylephedrine was stable and available commercially in high purity. Under the condition described,

the retention time of ethylephedrine hydrochloride was 5 min. The resolution factors of each peak pair were shown in Table 4.

1.3 Selection of wavelength detection

The 265-nm wavelength was used for the detection since it provided suitable response for all compounds under investigation in the mobile phase used in this study.

The UV spectra were shown in Figure 7.

1.4 Photolytic degradation of promethazine hydrochloride

The photolytic degradation study of promethazine hydrochloride was performed with the optimum HPLC conditions established.

After 24 hours, exposing to the neon light, the solution became pink and significant degradation was observed with the appearance of minor peaks eluting before analyte (Figure 8-9).

A principle peak of promethazine hydrochloride was observed at 9 min for both a freshly prepared and a degraded standard solutions. With the presence of degradation products, a decrease in the peak response of promethazine hydrochloride (at 9 min) was noticed.

After 7 days, the aqueous solution was violet and turbid. The solution became clear but still violet in color upon diluting with mobile phase. Degradation was marked with the appearance of several minor peaks eluting before the analytes and at long retention time after the analytes, as seen in Figure 10.

2. Development of Solid Phase Extraction Procedure

The first step in the optimization of SPE procedure was to determine an appropriate amount of the washed and dried Amberlite XAD-2 resin. The amount of the resin was varied ranging from 500 mg upto one gram. For analyzing 1.0 ml of syrup sample, about one gram (7-8 cm height) of the resin was found to be sufficient. The analytes totally adsorbed onto the resin with none were found in the waste as evidenced by the TLC result.

After the resin was dry-packed into a glass column, the column was conditioned by washing with dichloromethane folllowed by pure methanol. This serves two purposes (McDowall, 1989) which are :

1. To open up the hydrocarbon chains and hence increase the surface area available for interaction with the analytes.

2. To remove residues from the packing material that might interfere with the analysis, failure to carry

out this stage effectively will result in poor recoveries of analytes due to reduced retention on the column and interference peaks on the chromatogram which are unrelated to the original sample.

The next step of conditioning was to wash the sorbent bed with distilled water followed by suitable buffer. This should remove excess methanol and prepare the surface for the sample.

The carbonate buffer solution pH 11.0 was used because at this pH most basic amine drugs including ephedrine hydrochloride, codeine phosphate and promethazine hydrochloride were present primarily as unionized forms and therefore exhibited strong interaction with the Amberlite XAD-2 resin.

The second step of SPE procedure was sample loading. The sample volume loaded should not be too large. Otherwise the column over loading occurred, resulting in a reduction in recovery. In this study, 1.0 ml syrup sample was used. After 5-10 minutes, the column washing or rinsing was taken place. Washed the column with distilled water to remove selectively interferences from the sample syrup which might interfere with the subsequent chromatography.

Finally, the elution step was performed in order to separate the adsorbed sample molecules from the adsorbent

with the aid of a suitable solvent. Various organic solvents were tried and dichloromethane was found suitably, providing the best elution strenth that the analytes could be eluted with the lowest possible volume. Besides, the boiling point of dichloromethane is quite low therefore speeding up evaporation process of the eluting fraction. Fifteen millitres of dichloromethane was used for eluting the analytes.

The eluting efficiency of organic solvents were given in Table 5.

The TLC chromatogram for examined the presence of analytes was seen in Figure 11.

3. Method Validation

3.1 Standard Linearity

Standard curves were constructed for each compound by plotting peak area ratios versus standard concentrations (Table 6 and Figures 12-14). The standard curves were linear over the range studies (40-72 mcg/ml for ephedrine hydrochloride, 48-87 mcg/ml for codeine phosphate and 20-36 mcg/ml for promethazine hydrochloride), with correlation coefficients of 0.997 or higher. Regression equations of standards were listed below.

For ephedrine hydrochloride

$y = 0.0029x - 0.00086$

$n = 5, \quad r = 0.9973$

For codeine phosphate

$y = 0.0190x - 0.0696$

$n = 5, \quad r = 0.9979$

For promethazine hydrochloride

$y = 0.0994x - 0.0272$

$n = 5, \quad r = 0.9993$

where

y = peak area ratio

x = standard concentration

n = the number of concentration levels

r = correlation coefficient

3.2 Precision Analysis

The result of the intra-day and inter-day precision were presented in Table 7-8.

For the intra-day precision (Table 7), the relative standard deviation were 1.22%, 1.86% and 1.64% for ephedrine hydrochloride, codeine phosphate and promethazine hydrochloride, respectively.

The relative standard deviation (n = 3) of the inter-day presicion (Table 8) were 1.37%, 1.99% and 2.04%

for ephedrine hydrochloride, codeine phosphate and promethazine hydrochloride, respectively.

3.3 Extraction recovery

The extraction recovery was examined by comparing the amount recovered with the amount added using the proposed method. The mean recoveries were 99.65% at all concentrations for ephedrine hydrochloride, 101.72% for codeine phosphate and 100.21% for promethazine hydrochloride. The results were summarized in Table 9.

4. Application

Results and chromatograms of the assay of five brands of cough syrup according to the proposed SPE and HPLC procedures were summarized in Table 10 and Figures 16-20, respectively.

The manufacturers' specification limits of the three active ingredients was 90.0-110.0% labeled amount. The mean percent labeled amounts of ephedrine hydrochloride and codeine phosphate from 5 commercial syrup samples were conformed to the specifications. The amount of promethazine hydrochloride in syrups No. 3-5 were quite low, especially for syrup No. 5. This could be explained by partial degradation of promethazine hydrochloride in these syrups as evidenced by the chromatograms in Figures 18-20.