CHAPTER III

RESULTS AND DISCUSSION

Fluorescamine reacts efficiently with secondary amines to form nonfluorescent aminoenone chromophores which showed maximum absorption at 310-330 nm.[3] In this study, ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate, metoprolol tartrate, propranolol hydrochloride, and piperazine citrate were chosen to be a representative drugs containing secondary amino groups. These drugs were reacted with fluorescamine to form secondary amine drug-fluorescamine derivatives, whereas many other secondary amine drugs tested, eg. terbutaline sulfate, albuterol sulfate were not. It might be due to the steric effect of the bulky groups attached to the nitrogen atom which bonded to fluorescamine.

Determination of Maximum Absorption Wavelength

Secondary amine drugs reacted with fluorescamine to form secondary amine drug-fluorescamine derivatives. Therefore the quantity of secondary amine drugs can be determined by measuring the absorbance of their derivatives formed at the suitable wavelengths. The absorption spectra of fluorescamine derivatives of ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate, metoprolol tartrate, propranolol hydrochloride and piperazine citrate were determined spectrophotome-

trically by scanning in range from 300-450 nm. The absorption spectra of individual drug-fluorescamine derivatives were presented in Figure 1-7. The maximum absorption wavelength of the drug - fluorescamine derivatives showed at 317 nm for ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate, and metoprolol tartrate; 319 nm for propranolol hydrochloride and 325 nm for piperazine citrate, as summarized in Table 1.

Determination of pH Dependency

The effect of pH on the reaction of secondary amine drugs with fluorescamine was examined at various pHs by using phosphate buffer pH 2-12. The reaction was performed in the condition which was described in the determination of the maximum absorption wavelength. The measurement of absorbance of drug-fluorescamine derivatives also were observed at the maximum absorption wavelength of each secondary amine drugs. The fluorescamine derivatives of ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate and piperazine citrate exhibited high absorbance at pH 8-10, whereas the derivatives of metoprolol tartrate and propranolol hydrochloride exhibited at pH 9-10. There were no absorption can be observed at pH 2-6 for all drugs using in this study. The results indicated that the reaction of fluorescamine with secondary amine drugs was strongly pH dependent proceeding with increased rapidity as the pH increased up to the maximum at pH 8-10, as shown in Figure 8 and Table 2-8. It might be due to the protonation of the amine prior to reaction would retard the reaction. The absorbance at a particular pH was directly related to the extent of reaction at that pH. The pH 9 was selected as the optimal pH for

determination of all secondary amine drugs used in this study. For the determination of propranolol hydrochloride in the dosage form, pH 10 was selected as the optimum pH. At this pH drug-fluorescamine derivatives showed a maximum absorption which resulted in accurate data when compared with the official method.

Determination of The Effect of Time on Stability of Secondary Amine Drug-Fluorescamine Derivatives

The effect of time on stability of the secondary amine drug-fluorescamine derivatives at room temperature were investigated. The absorbance of the derivatives were measured at the specific wavelength, at selected interval of time within 3 hours and at 24 hours. The results are shown in Figure 9 and Tables 9-15. The absorbance of the fluorescamine derivatives of ephedrine hydrochloride and of pseudoephedrine hydrochloride were nearly constant within the first 30 minutes and decreased slightly in a period of 3 hours. The absorbance of phenylephrine-fluorescamine derivative remained nearly constant in the first 15 minutes then decreased slightly in 3 hours. The absorbance of the fluorescamine derivatives of epinephrine bitartrate and piperazine citrate decreased slightly in a period of 3 hours, but in the first hour the absorbance decreased more rapidly. The absorbance of metoprolol-fluorescamine derivatives decreased slightly in 3 hours. And the absorbance of propranolol-fluorescamine derivative was nearly constant over a period of 1 hour, then decreased slightly in 3 hours. At 24 hours the absorbance of all derivatives, in this study, decreased. From the present data, it was concluded that time was one factor that might effect the analysis.

It also showed that the measurement of absorbance could be determined between 2 and 20 minutes. After 20 minutes, the absorbance subsequently decreased due to instability of the reaction products. Therefore, in this study, the absorbance of all drug-fluorescamine derivatives were measured at 15 minuted after the reactions.

Determination of the Effect of Temperature on Stability of Secondary Amine Drug-Fluorescamine Derivatives

The effect of temperature on the stability of secondary amine drug-fluorescamine derivatives were investigated. The derivatives formed were treated in constant temperature bath at the temperature of 40℃, 50℃, and compared to that treated at room temperature (30℃). The results are presented in Figure 10 and Table 16. The absorbance of the fluorescamine derivatives of ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate, metoprolol tartrate, propranolol hydrochloride and piperazine citrate at room temperature (30℃) were 1.055, 1.055, 0.969, 0.931, 1.054, 0.732 and 1.040; whereas that treated at 40℃ were 1.040, 0.951, 0.936, 0.864, 1.015, 0.702 and 0.985; and that treated at 50℃ were 0.973, 0.910, 0.869, 0.752, 0.972, 0.680, and 0.817, respectively. The results showed that the absorbance of the derivatives decreased slightly when the temperature increased. The decreased absorbance at high temperature might be due to the degradation of the derivatives. The measurement of absorbance at 30℃, showed a little variation when compared to the absorbance at 40℃ and 50℃, indicated that the drug-fluorescamine derivatives were stable at 30℃. The temperature below 30℃ was not performed because the

method of analysis would be convenient if the absorbance was measured at room temperature. Since the maximum absorption of all the drug-fluorescamine derivatives were obtained at room temperature (30℃), therefore the heating process were not necessary.

Determination of the Effect of Fluorescamine Concentration on Absorbance of Secondary Amine Drug-Fluorescamine Derivatives

The optimum fluorescamine concentration was investigated by using 1 ml of various concentrations of fluorescamine solutions reacted, with 0.2 ml of fixed concentration of solution of secondary amine drugs. The fluorescamine solution, 1×10^{-4}M to 2×10^{-3}M, were used to react with 1×10^{-3}M ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate and propranolol hydrochloride. A 1.22×10^{-3}M metoprolol tartrate (equivalent to 2.44×10^{-3}M metoprolol base) was used to react with different concentrations of fluorescamine solutions (2×10^{-4}M to 2.8×10^{-3}M). And 1.67×10^{-4}M piperazine citrate (equivalent to 5×10^{-4}M piperazine base) was used to react with different concentrations of fluorescamine solutions (0.5×10^{-4}M to 2×10^{-3}M). The experimental data are shown in Tables 17-23. The effect of concentration of fluorescamine and the mole-ratio of fluorescamine to secondary amine drugs on the absorbance of drug fluorescamine derivative were determined by plotting curve (Figure 11-12). The results indicated that the formation of drug-fluorescamine derivatives increased with increasing concentration of fluorescamine and reached a constant level at 8×10^{-4}M for ephedrine hydrochloride, 6×10^{-4}M for pseudoephedrine hydrochloride, 4×10^{-4}M

for phenylephrine hydrochloride and epinephrine bitartrate, 1.6 x 10^{-3}M for propranolol hydrochloride and 3 x 10^{-4}M for piperazine citrate. All of drug-fluorescamine derivatives, showed a suitable absorbance when the concentration of fluorescamine reach up to 2 x 10^{-3}M. The absorbance of metoprolol-fluorescamine derivative increased with increasing the fluorescamine concentration up to 1.6 x 10^{-3}M and then, increasing fluorescamine concentration, increased slightly in absorbance. The mole-ratio curve (Figure 12), also indicated that the secondary amine drugs, ephedrine, pseudoephedrine, phenylephrine, epinephrine, metoprolol propranolol and piperazine required about 4, 3, 2, 2, 4, 8 and 3, respectively, equivalents of fluorescamine for the reaction to go to completion. Competing with the reaction of fluorescamine and secondary amine drugs that yields the secondary amine drug-fluorescamine derivatives, was the hydrolysis of excess fluorescamine to a water-soluble product.[11,18] In order to ensure an excess fluorescamine used and to achieve a maximum absorption, the fluorescamine concentration of 2 x 10^{-3}M was chosen for quantitative determination of all secondary amine drugs used in this study.

Determination of the Linearity of Absorbance with Concentration of Secondary Amine Drugs

For quantitative analysis, the linearity of absorbance with concentration is one of the requirements. Since the absorbance is proportional to the amount of drugs, then the relationship between absorbance and concentration of drugs should be adherence to Beer's law. Under the suitable conditions, the linearity of absorbance with

concentration of secondary amine drugs; ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate metoprolol tartrate, propranolol hydrochloride and piperazine citrate were found. The calibration curves were prepared by plotting absorbance against concentration of the secondary amine drugs, mcg per ml of the final solution, as shown in Tables 24-30 and Figures 13-19, respectively. Table 31 summarized the linear concentration range, slope and the % of coefficient of variation range of the secondary amine drugs used in this study. The precision of the study was shown by the results of four experiments.

The linear absorbance concentration relationship were a concentration range of 4.04 - 20.20 mcg/ml for ephedrine hydrochloride and pseudoephedrine hydrochloride, 4.08 - 24.48 mcg/ml for phenylephrine hydrochloride, 6.66 - 46.62 mcg/ml for epinephrine bitartrate, 8.34 - 41.70 mcg/ml for metoprolol tartrate, 5.92 - 29.60 mcg/ml for propranolol hydrochloride and 2.14 - 14.98 mcg/ml for piperazine citrate.

The slope of the calibration curves of ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate, metoprolol tartrate, propranolol hydrochloride and piperazine citrate were to be 0.0628, 0.0668, 0.0602, 0.0358, 0.0160, 0.0331 and 0.1266 ml/mcg, respectively.

The % of coefficient of variation range of four experiments in calibration curves were 0.13 - 2.77 for ephedrine hydrochloride, 0.27 - 3.27 for pseudoephedrine hydrochloride, 0.27 - 1.69 for phenylephrine hydrochloride, 0.49 - 4.84 for epinephrine bitartrate,

0.42 - 3.90 for metoprolol tartrate, 0.61 - 7.24 for propranolol hydrochloride and 0.26 - 3.56 for piperazine citrate. From the data obtained indicated that phenylephrine hydrochloride showed best reproducibility, followed by ephedrine hydrochloride, pseudoephedrine hydrochloride, piperazine citrate, metoprolol tartrate, epinephrine bitartrate and propranolol hydrochloride, respectively.

The calibration curves of ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, piperzine citrate showed x intercept, which the calibration curves of epinephrine bitartrate, metoprolol tartrate, propranolol hydrochloride showed y intercept.

Determination of Percent Labelled Amount of Propranolol Hydrochloride in Propranolol Hydrochloride Tablets Using Fluorescamine Method and USP Method

Propranolol hydrochloride tablet, represented for drug containing secondary amine groups, was selected as a preparation for analysis. Because this preparation is official in USP and therefore the comparision of two methods of analyses can be made. The content of propranolol hydrochloride in propranol hydrochloride tablets was determined by both fluorescamine method and the official USP method.[55] Only minor modification of fluorescamine method using 0.2 M phosphate buffer pH 10.0 instead of 0.05 M phosphate buffer pH 9.0, was required for determination of propranolol hydrochloride in the formulations. The results obtained were compared in Table 32. The mean percentage value for five determinations was 99.68% with 0.65% of coefficient of variation by fluorescamine method and 99.50%

with 0.21% of coefficient of variation by official USP method. The data presented showed a good precision and a close relationship between the two methods. It was indicated that fluorescamine method gave reproducibility results compared well with official USP method.

The results obtained were within the USP limit of content in tablet (90.0 - 110.0%) thus this preparation was subsequently used for testing the accuracy of the method.

Determination of the Percent Recovery of Propranolol Hydrochloride in Propranolol Hydrochloride Tablets by Fluorescamine Method and USP Method

The accuracy of the proposed method was checked by determining the percent recovery. Since other inactive excipients in the preparation might interfere with the determination of sample. Therefore, these interferences could be tested by adding accurately varying amount of standard propranolol hydrochloride, 5, 10 and 15 mg, to propranolol hydrochloride tablet which the exact amount of propranolol hydrochloride was known and determined by using the proposed method and official USP method. The mean percent recoveries were calculated from five determinations, as shown in Table 33. For the weight of propranolol hydrochloride added : 5, 10 and 15 mg, fluorescamine method gave the percent recoveries of 100.18, 100.93 and 100.13 with 1.37, 1.16 and 0.84% of coefficient of variation; respectively. In official USP method the percent recoveries were 99.08, 100.31 and 101.01 with 0.75, 1.40 and 0.43% coefficient of

variation for the weight of propranolol hydrochloride added 5, 10 and 15 mg; respectively. The results showed that both fluorescamine and official USP methods produced good recoveries with high reproducibility. Therefore, the presence of other excipients produced no effect on the propranolol hydrochloride determination.

Comparative Analysis of Preparation Containing Propranolol Hydrochloride

To test the validity of the proposed method, three commercial formulations with different dosage form, 1 mg per ml injection, 10 mg tablets and 40 mg tablets, were analyzed by fluorescamine method compared with official USP method. The results obtained were the mean value of five replicated determinations of each sample expressed in percentage of amount labelled as shown in Tables 34-36. The mean percent labelled amount and % of coefficient of variation values of all preparations containing propranolol hydrochloride were compared in Table 37. The percent labelled amount of propranolol hydrochloride injection, 1 mg per ml, was 98.32 and 98.44 with 0.42 and 0.78% of coefficient of variation determined by fluorescamine method and official USP method, respectively. For propranolol hydrochloride 10 mg tablets, the percent labelled amount were 97.58 and 97.51 with 0.38 and 0.30% of coefficient of variation dertermined by fluorescamine method and official USP method, respectively. And the mean value of percentage of propranolol hydrochloride 40 mg tablets was 98.59 with 1.01% of coefficient of variation for

fluorescamine method and 99.25 with 0.26% of coefficient of variation for official USP method. The results obtained indicated that the proposed method compared well with the USP method could be used for the determination of propranolol hydrochloride in pharmaceutical preparations.