CHAPTER II

MATERIALS AND METHODS

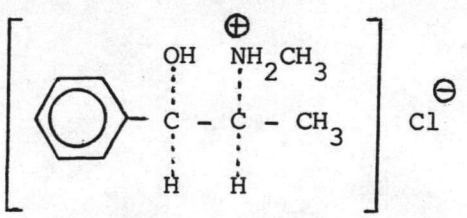
Instruments

1. Spectrophotometer - Shimadsu UV-180
2. Recorder - Shimadsu U-135
3. pH meter - Radiometer Copenhagen, PHM 62 STANDARD pH METER
4. Temperature controlled - water bath - Memmert

Chemicals

1. Ephedrine hydrochloride BP

The chemical name of ephedrine hydrochloride is benzenemethanol, α-[1-(methylamino) ethyl]-, hydrochloride, [R-(R*, S*)] and its structure is[55]



$C_{10}H_{15}NO.HCl$

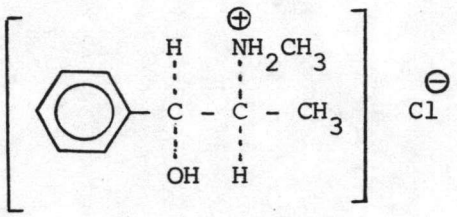
MW. 201.70

Description[62] : Fine, white, odorless crystals or powder, affected by light; melting range 217-220°; specific rotation -33° to -35.5°.
Solubility[62] : 1 g in about 3 ml water and about 14 ml alcohol; insoluble in ether.

2. Pseudoephedrine hydrochloride BP

The chemical name of pseudoephedrine hydrochloride is

benzenemethanol, α-[1-(methylamino) ethyl]-, [S-(R*, R*)]-hydrochloride and its structure is[62]



$C_{10}H_{15}NO.HCl$

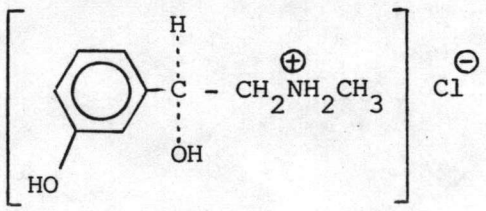
MW. 201.70

Description[52,62] : White, crystalline powder; almost odorless; melts between 182° and 185°; solution are neutral to litmus.

Solubility[52] : Soluble in 1.6 part of water, 1 in 4 parts of ethanol (96%), and in 60 parts of chloroform.

3. Phenylephrine hydrochloride USP

The chemical name of phenylephrine hydrochloride is benzenemethanol, 3-hydroxy-α-[(methylamino) methyl]-, hydrochloride (S) and its structure is[62]



$C_9H_{13}NO_2.HCl$

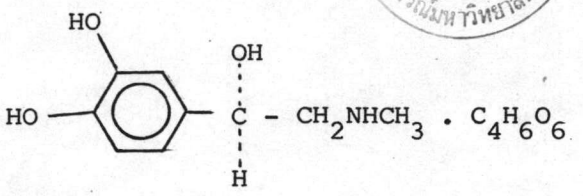
MW. 203.67

Description[62] : White or nearly white, odorless, bitter crystals; its solution are acid to litmus; specific rotation -42° to -47.5°; melts between 140° and 145°.

Solubility[62] : Freely soluble in water and alcohol.

4. Epinephrine bitartrate BP

The chemical name of epinephrine bitartrate is (-)-3, 4-dihydroxy-α-[(methylamino) methyl] benzyl alcohol tartrate (1:1) salt and its structure is[62]



$C_9H_{13}NO_3 \cdot C_4H_6O_6$

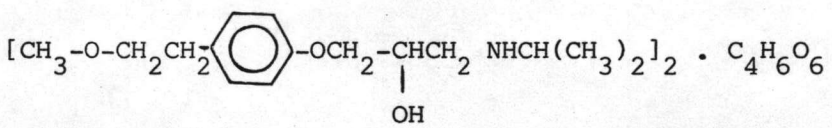
MW. 333.29

Description[62] : White, grayish white, or light brownish gray crystalline powder; odorless; slowly darkens on exposure to air and light; melting range 147° to 152°, with decomposition; pH (1% solution) 3.5.

Solubility[62] : 1 g in about 3 ml water and about 550 ml alcohol; almost insoluble in chloroform and ether.

5. Metoprolol tartrate

The chemical name of metoprolol tartrate is (±) -(Isopropylamino)-3-p-(β-methoxyethyl) phenoxy-2-propranolol tartrate and its structure is[61,63]



$(C_{15}H_{25}NO_3)_2 \cdot C_4H_6O_6$

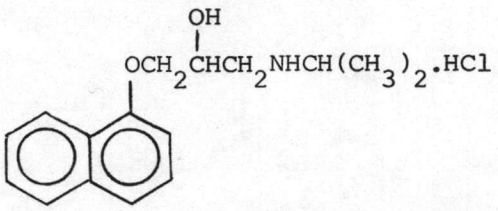
MW. 684.85

Description[61] : White, crystalline powder, odorless; bitter taste; melts at 121° to 122°.

Solubility[61] : Very soluble in water; soluble in alcohol and chloroform; practically insoluble in ether.

6. Propranolol hydrochloride BP

The chemical name of propranolol hydrochloride is 2-propranol, 1-[(1-methylethyl) amino]-3-(1-naphthalenyloxy), hydrochloride and its structure is[55]



$C_{16}H_{21}NO_2.HCl$

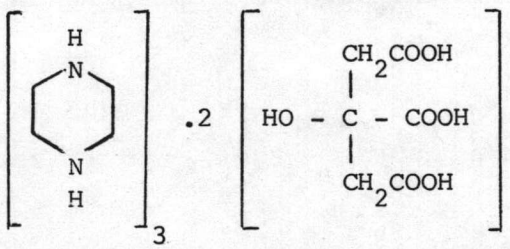
MW. 295.81

Description[52] : A white or almost white powder; odorless; pH (1% - solution) 5.0-6.0.

Solubility[52] : Soluble in 20 parts of water and in 20 parts of ethanol (96%); slightly soluble in chloroform.

7. Piperazine citrate BP

The chemical name of piperazine citrate (3:2) is diethylenediamine, citrate and its structure is[52,]



$(C_4H_{10}N_2)_3.2C_6H_8O_7$

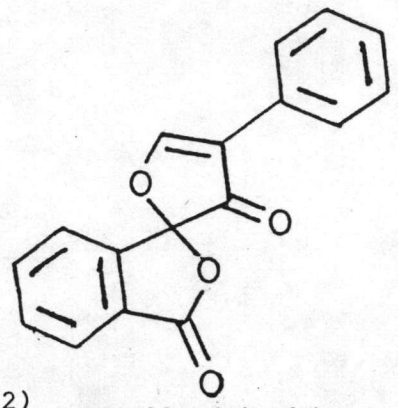
anhydrous MW. 642.66

Description[62] : White, crystalline powder, having not more than a slight odor; a 1 in 10 solution is acid to litmus, having a pH of 5 to 6.

Solubility[62] : Soluble in water; insoluble in alcohol and ether.

8. Fluorescamine - Sigma

The chemical name of fluorescamine is 4-phenylspiro [furan-2-(3H), 1'-phthalan]-3,3'-dione and its structure is[4]



$C_{17}H_{10}O_4$

MW. 278.30

Description[2] : A yellowish white powder; melting range 154° to 155°; store below 30℃

Solubility[11] : Insoluble in water; soluble in acetone and dioxane.

9. Acetone - BDH Chemical
10. Potassium hydrogen phosphate - Merck
11. Potassium dihydrogen phosphate - Merck
12. Sodium hydroxide - Merck
13. Hydrochloric acid - Merck
14. Heptane - Carlo Erba

All of chemicals used were analytical grade and secondary amine drugs used were pharmaceutical grade.

Reagents

Preparation of Fluorescamine solutions

Dissolved fluorescamine in acetone to make concentrations of 2.8×10^{-3}M, 2.4×10^{-3}M, 2.0×10^{-3}M, 1.8×10^{-3}M, 1.6×10^{-3}M, 1.4×10^{-3}M, 1.2×10^{-3}M, 1.0×10^{-3}M, 8.0×10^{-4}M, 6.0×10^{-4}M, 4.0×10^{-4}M, 2.0×10^{-4}M, and 1.0×10^{-4}M, aged for 24 hours (by standing at room temperature). Fluorescamine solution in acetone showed effect of aging wh ch affected reproducibility. This was overcome by "aging" the solution for at least 24 hours prior to use.

Solution must be refrigerated when not in use and has an effective shelf-life of two week.[17]

Preparation of Buffer solutions

0.05 M Phosphate buffer pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0. A 0.05 M potassium dihydrogen phosphate (6.80 gm KH_2PO_4 per litre) and 0.05 M potassium hydrogen phosphate (8.71 gm K_2HPO_4 per litre) were mixed to give the desired pH range using a pH meter adjusted with dilute hydrochloric acid or 0.5 N sodium hydroxide solution.

0.2 M Phosphate buffer pH 10.0. A 0.2 M potassium hydrogen phosphate (34.84 gm K_2HPO_4 per litre) was adjusted with 0.5 N sodium hydroxide solution to make pH 10.0 by using a pH meter.

Standard solutions

1×10^{-3}M Ephedrine hydrochloride solution or 0.202 mg/ml. Dissolved ephedrine hydrochloride 20.2 mg in distilled water to make 100 ml.

1×10^{-3}M Pseudoephedrine hydrochloride solution or 0.202 mg/ml. Dissolved pseudoephedrine hydrochloride 20.2 mg in distilled water to make 100 ml.

1×10^{-3}M Phenylephrine hydrochloride solution or 0.204 mg/ml. Dissolved phenylephrine hydrochloride 20.4 mg in distilled water to make 100 ml.

1×10^{-3}M Epinephrine bitartrate solution or 0.333 mg/ml Dissolved epinephrine bitartrate 33.3 mg in distilled water to make 100 ml.

6.09×10^{-4}M Metoprolol tartrate solution equivalent to 1.22×10^{-3}M metoprolol base or 0.417 mg/ml. Dissolved metoprolol tartrate 41.7 mg in distilled water to make 100 ml.

1.22×10^{-3}M Metoprolol tartrate solution equivalent to 2.44×10^{-3}M metoprolol base or 0.834 mg/ml. Dissolved metoprolol tartrate 83.4 mg in distilled water to make 100 ml.

1×10^{-3}M Propranolol hydrochloride solution or 0.296 mg/ml. Dissolved propranolol hydrochloride 29.6 mg in distilled water to make 100 ml.

2×10^{-4}M Piperazine citrate solution equivalent to 6×10^{-4}M piperazine base or 0.129 mg/ml. Dissolved piperazine citrate 12.9 mg in distilled water to make 100 ml.

1.67×10^{-4}M Piperazine citrate solution equivalent to 5×10^{-4}M piperazine base or 0.107 mg/ml. Dissolved piperazine citrate 26.8 mg in distilled water to make 250 ml.

Methods

1. Determination of Maximum Absorption Wavelength

Each of 0.4 ml 1×10^{-3}M ephedrine hydrochloride solution, 1×10^{-3}M pseudoephedrine hydrochloride solution, 1×10^{-3}M phenylephrine hydrochloride solution, 1×10^{-3}M epinephrine bitartrate solution, 1×10^{-3}M propranolol hydrochloride solution, and 0.3 ml 1.22×10^{-3}M metoprolol tartrate solution, 1.67×10^{-4}M piperazine citrate solution were pipetted into 15 ml tubes. A 0.05 M phosphate buffer pH 9.0 was added into the tubes to make 4 ml, mixed, added 1 ml of 2×10^{-3}M fluorescamine solution and then mixed vigorously

for at least 10 seconds. After standing for 15 minutes, the solution were scanned in 1 cm cells against a reagent blank by the UV spectrophotometer using wavelength from 300-450 nm. The maximum absorption wavelength of the derivatives were determined.

The procedure was repeated four times. The absorption spectra were shown in Figure 1-7 and the maximum absorption wavelength of the derivatives were shown in Table 1.

2. Determination of pH Dependency

Each of 0.4 ml of 1×10^{-3}M ephedrine hydrochloride solution 1×10^{-3}M pseudoephedrine hydrochloride solution, 1×10^{-3}M phenylephrine hydrochloride solution, 1×10^{-3}M epinephrine bitartrate solution, 1×10^{-3}M propranolol hydrochloride solution, and 0.3 ml of 1.22×10^{-3}M metoprolol tartrate solution, 2×10^{-4}M piperazine citrate solution were pipetted into the 15 ml tubes. A 0.05 M phosphate buffer pH 2.0 was added into the tubes to make 4 ml, mixed, added 1 ml of 2×10^{-3}M fluorescamine solution, and then mixed vigorously for at least 10 seconds. After standing for 15 minutes, the absorbances were measured using UV spectrophotometer at the maximum absorption wavelength against a reagent blank in 1 cm cells.

Each sample was repeated using 0.05 M phosphate buffer pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 instead of buffer pH 2.0.

The procedure was repeated four times. The results obtained were shown in Table 2-8 and Figure 8

3. Determination of the Effect of Time on Stability of Secondary Amine Drug-Fluorescamine Derivatives.

The 2 ml each of 1 x 10^{-3}M ephedrine hydrochloride solution, 1 x 10^{-3}M pseudoephedrine hydrochloride solution, 1 x 10^{-3}M phenylephrine hydrochloride solution, 1 x 10^{-3}M epinephrine bitartrate solution, 1 x 10^{-3}M propranolol hydrochloride solution, 1.5 ml of 1.22 x 10^{-3}M metoprolol tartrate solution, and 1.2 ml of 1.67 x 10^{-4}M piperazine citrate solution was pipetted into the separated 25 ml volumetric flasks. About 12 ml of 0.05 M phosphate buffer pH 9.0 was added into each flask, mixed. Pipetted 2 x 10^{-3}M fluorescamine solution 5 ml into each volumetric flasks, mixed vigorously at least 10 seconds. Adjusted to volume with 0.05 M phosphate buffer pH 9.0. The absorbances of the derivatives were measured at maximum absorption wavelength at 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 90, 120, 150, 180 minutes and 24 hours against a reagent blank in 1 cm cells.

The procedure was repeated four times. The results were shown in Table 9-15 and Figure 9.

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

The 0.4 ml each of 1 x 10^{-3}M ephedrine hydrochloride solution, 1 x 10^{-3}M pseudoephedrine hydrochloride solution, 1 x 10^{-3}M phenylephrine hydrochloride solution, 1 x 10^{-3}M epinephrine bitartrate soluiton, 1.22 x 10^{-3}M metoprolol tartrate solution, 1 x 10^{-3}M propranolol hydrochloride solution, and 1.67 x 10^{-4}M piperazine citrate solution was pipetted into the separated 15 ml tubes. Pipetted 3.6 ml of 0.05 M phosphate buffer pH 9.0 into the tubes mixed. Then

added 1 ml 2 x 10^{-3}M fluorescamine solution, mixed vigorously for at least 10 seconds. Left at room temperature (30℃) for 15 minutes. The absorbances of each solution were measured using UV spectrophotometer at maximum absorption wavelength against a reagent blank in 1 cm cells.

Each sample was repeatedly determined the absorbances by heating the solution at 40℃ and 50℃.

The procedure was repeated four times. The results obtained were shown in Table 16 and Figure 10.

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

The 0.2 ml each of 1 x 10^{-3}M ephedrine hydrochloride solution, 1 x 10^{-3}M pseudoephedrine hydrochloride solution, 1 x 10^{-3}M phenylephrine hydrochloride solution, 1 x 10^{-3}M epinephrine bitartrate solution, 1 x 10^{-3}M propranolol hydrochloride solution was pipetted into the separate 15 ml tubes. Pipetted 3.8 ml of 0.05 M phosphate buffer pH 9.0 into the tubes, mixed, and added 1 ml of 1 x 10^{-4}M fluorescamine solution, mixed vigorously for at least 10 seconds. After standing for 15 minutes, the absorbances were measured using UV spectrophotometer at maximum absorption wavelength against a reagent blank in 1 cm cells.

Each sample was repeatedly determined the absorbances by using 1 ml each of 2 x 10^{-4}, 4 x 10^{-4}, 6 x 10^{-4}, 8 x 10^{-4}, 1 x 10^{-3}, 1.2 x 10^{-3}, 1.4 x 10^{-3}, 1.6 x 10^{-3}, 1.8 x 10^{-3} and 2 x 10^{-3}M fluorescamine solution replaced of 1 x 10^{-4}M fluorescamine solution.

A 0.2 ml of 1.22×10^{-3}M metoprolol tartrate solution was added into 15 ml tube. Pipetted 3.8 ml of 0.05 M phosphate buffer pH 9.0 into the tube, mixed, and added 1 ml of 2×10^{-4}M fluorescamine solution, mixed vigorously for at least 10 seconds. After standing for 15 minutes, the absorbance was measured using UV spectrophotometer at 317 nm against a reagent blank in 1 cm cell.

The sample was repeatedly determined the absorbance by using 1 ml each of 4×10^{-4}, 8×10^{-4}, 1.2×10^{-3}, 1.6×10^{-3}, 2×10^{-3}, 2.4×10^{-3} and 2.8×10^{-3}M fluorescamine solution replaced of 2×10^{-4}M fluorescamine solution.

A 0.2 ml of 1.67×10^{-4}M piperazine citrate solution was added into a 15 ml tube. Pipetted 3.8 ml of 0.05 M phosphate buffer pH 9.0 into the tube, mixed, and added 1 ml of 0.5×10^{-4}M fluorescamine solution, mixed vigorously for at least 10 seconds. After standing for 15 minutes, the absorbance was measured at 325 nm against a reagent blank in 1 cm cells.

The sample was repeatedly determined the absorbance by using 1×10^{-4}, 2×10^{-4}, 3×10^{-4}, 4×10^{-4}, 5×10^{-4}, 6×10^{-4}, 7×10^{-4}, 8×10^{-4}, 9×10^{-4}, 1×10^{-3}, 1.5×10^{-3} and 2×10^{-3}M fluorescamine solution instead of 0.5×10^{-4}M fluorescamine solution.

The procedure was repeated four times. The results obtained were shown in Table 17-23 and Figure 11.

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

Each of 1×10^{-3}M ephedrine hydrochloride solution, 1×10^{-3}M pseudoephedrine hydrochloride solution, 1×10^{-3}M phenylephrine hydrochloride solution, 1×10^{-3}M epinephrine bitartrate solution, 6.09×10^{-4}M metoprolol tartrate solution, 1×10^{-3}M propranolol hydrochloride solution and 1.67×10^{-4}M piperazine citrate solution was used ot determine. A 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 ml of each solution were mixed with 0.05 M Phosphate buffer pH 9.0 to make 4 ml into 15 ml tube. Added 1 ml of 2×10^{-3}M fluorescamine solution, mixed vigorously for at least 10 seconds. After standing for 15 minutes, the absorbances were measured at maximum absorption wavelength against a reagent blank in 1 cm cells.

The procedure was repeated four times. The results were shown in Table 24-31 and Figure 12-18.

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

Standard preparation-Dissolved an accurately weighed quantity of propranolol hydrochloride reference standard about 20 mg in 100 ml volumetric flask, added 0.01 N hydrochloric acid to volume, mixed.

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* = USP XX

Assay preparation-Weighed and finely powdered not less than 20 propranolol hydrochloride tablets. Weighed accurately a portion of the powder, equivalent to about 20 mg of propranolol hydrochloride and transferred with the aid of about 70 ml. 0.01 N hydrochloric acid to a 100 ml volumetric flask. Swirled by mechanical means for 30 minutes, diluted with 0.01 N hydrochloric acid to volume, mixed and filtered.

Each of 0.5 ml of standard preparation and assay preparation was pipetted to each of two tubes. Pipetted 0.2 M phosphate buffer pH 10.0 3.5 ml into the tubes, mixed. Added 2×10^{-3}M fluorescamine solution 1 ml, mixed vigorously at least 10 seconds. After standing for 15 minutes, the absorbances were measured at 319 nm against a reagent blank in 1-cm cells. The amount of propranolol hydrochloride in tabelts was calculated from the following formula.

$$\% \text{ Labelled Amount} = \frac{0.1 \times C \times Au \times Wt \times 100}{As \times W \times P}$$

Au = Absorbance of sample

As = Absorbance of standard

C = Concentration of standard preparation in mcg per ml.

P = Label amount

W = Weight of sample powder

Wt = Average weight per tablet

USP Method

Standard preparation-Dissolved an accurately weighed quantity of propranolol hydrochloride reference standard about 20 mg in 100 ml volumetric flask, added distilled water to volume, mixed.

Assay preparation-Weighed and finely powdered not less than 20 propranolol hydrochloride tablets. Weighed accurately a portion of the powder, equivalent to about 100 mg of propranolol hydrochloride and transferred with the aid of about 350 ml of 0.1 N hydrochloric acid to 500 ml volumetric flask. Swirled by mechanical means for 30 minutes, diluted with 0.1 N hydrochloric acid to volume, mixed and filtered.

Transferred 5.0 ml each of standard preparation and the assay preparation to separate separators, and treated each as follows. Added 10 ml of distilled water, 1 ml of sodium hydroxide solution (1 in 5), and 25 ml of heptane. Shaked for 5 minutes, and allowed the layers to separate. Concomitantly determined the absorbances of both heptane solution in 1-cm cells at 293 nm, using heptane as the blank. Calculated the amount of propranolol hydrochloride in tablet from the following formula.

$$\% \text{ Labelled Amount} = \frac{0.5 \times C \times Au \times Wt \times 100}{As \times W \times P}$$

Au = Absorbance of sample

As = Absorbance of standard

C = Concentration of standard preparation in mcg per ml

Wt = Average weight per tablet

W = Weight of the sample powder

P = Label Amount

The procedure was repeated five times for each method. The results were shown in Table 32

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

Fluorescamine Method

Standard preparation-Prepared the same as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-fluorescamine method.

Assay preparation-Weighed and finely powdered not less than 20 propranolol hydrochloride tablets. Weighed accurately several portions of the powder equivalent to about 10 mg of propranolol hydrochloride, and transferred with the aid of about 60 ml 0.01 N hydrochloric acid to separate 100 ml volumetric flasks. Then added 5, 10, 15 ml of propranolol hydrochloride standard solution (1 mg per ml in 0.01 N HCl) respectively. Swirled by mechanical means for 30 minutes, diluted with 0.01 N hydrochloric acid to volume, mixed and filtered.

The procedure proceeded as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-fluorescamine method.

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* = USP XX

USP Method

Standard preparation-Prepared the same as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-USP method.

Assay preparation-Weighed and finely powdered not less than 20 propranolol hydrochloride tablets. Weighed accurately several portions of the powder equivalent to about 10 mg of propranolol hydrochloride, and transferred with the aid of about 60 ml 0.1 N hydrochloric acid to separate 100 ml volumetric flasks, Then added 5, 10, 15 ml of propranolol hydrochloride standard solution (1 mg per ml in 0.1 N HCl) respectively, Swirled by mechanical means for 30 minutes, diluted with 0.1 N hydrochloric acid to volume, mixed and filtered.

The procedure proceeded as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-USP method.

Calculated percent recovery both fluorescamine method and USP method from the following formula.

$$\% \text{ Recovery} = \frac{(Wf - Ws) \times 100}{Wa}$$

Wf = Weight of propranolol hydrochloride found

Ws = Weight of propranolol hydrochloride from tablets

Wa = Weight of propranolol hydrochloride added

The procedure was repeated five times for each method. The results were shown in Tablet 33

9. Comparative Analysis of Preparation Containing Propranolol Hydrochloride

Three commercial formulations with different dosage form (10 mg tablets 40 mg tablets 1 mg per ml injection) were analyzed by fluorescamine method compared with USP method.

Fluorescamine Method

Standard preparation-Prepared the same as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-fluorescamine method.

Assay preparation (tablets)-Prepared the same as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-fluorescamine method.

Assay preparation (injection)-Transferred and accurately measured volume of propranolol hydrochloride injection, equivalent to about 5 mg of propranolol hydrochloride to a 25 ml volumetric flask, diluted with 0.01 N hydrochloric acid to volume, mixed.

The procedure proceeded as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-fluorescamine method.

USP Method

Standard preparation-Prepared the same as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-USP method.

Assay preparation (tablets)-Prepared the same as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-USP method.

Assay preparation (injection)-Transferred an accurately measured volume of propranolol hydrochloride injection, equivalent to about 5 mg of propranolol hydrochloride, to a 25 ml volumetric flask, diluted with distilled water to volume, mixed.

The procedure proceeded as directed in determination of percent labelled amount of propranolol hydrochloride in propranolol hydrochloride tablets-USP method.

The procedure was repeated five times for each method. The results were shown in Tablet 34-36.