CHAPTER I

INTRODUCTION

Fluorescamine, 4-phenylspiro furan-2 (3H), 1'-phthalan-3, 3'-dione was first synthesized and utilized by Weigele *et al.*[1-2] It was introduced as a fluorometric reagent for the assay of primary amines and has been utilized for quantitative analysis of primary amino acids,[3-9] peptides,[9,10] and proteins.[9,11] Its usefulness has been demonstrated in many biochemical, chemical, and pharmaceutical applications.[12-28] Fluorescamine has also been used as a spray reagent for thin-layer chromatographic analysis for the detection of primary and secondary amines, such as, amino acids, peptides, alkaloids, drugs etc.[29-36]

Fluorescamine reacts with primary amines to form highly fluorescent pyrrolinones. These derivatives have an absorption maximum at about 380-410 nm. Fluorescamine also reacts directly with secondary amines to form nonfluorescent aminoenone chromophores, with an absorption maximum at 310-330 nm. Excess reagent is concomitantly hydrolyzed to yield nonfluorescent, water-soluble hydrolysis products.[3,6,15,16,25,37-40] Primary and secondary amines both react rapidly with fluorescamine at room temperature to give stable chromophores, but only those formed with primary amino compounds are fluorescent. Weigele *et al*[37] reported a method for analysis of secondary amino acids by transforming them into

fluorescamine sensitive primary amines via oxidative decarboxylation with N-chlorosuccinimide. It was used to determine many secondary amino compounds.[3,5,7,37,38] The aminoenone type chromophores formed with secondary amines have strong absorption which offers an idea to develope a spectrophotometric method for the determination of secondary amine drugs on the basis of their reaction with fluorescamine.

I. Chemistry

Amines are classified as primary, secondary, or tertiary according to the number of alkyl or aryl groups attached to the nitrogen atom. Primary amines have one alkyl or aryl group attached to the nitrogen (RNH_2), secondary have two (R_2NH) and tertiary have three (R_3N).[41] Secondary amine drugs are drugs, containing secondary amine group. These are for instance ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate, Metoprolol tartrate, propranolol hydrochloride and piperazine citrate.

II. General Organic Reaction of Secondary Amines

The reaction of secondary amines resulted to form such a useful organic substance. Some of them are alkaloids, some of them contains pharmacologic actions, some of them have been used as medicines. In order to understand the reaction, grouping of organic reaction of secondary amines can be put into six.

1. Aliphatic Nucleophilic Substitution[42]

In nucleophilic substitution the attacking reagent

(nucleophile) brings an electron pair to the substrate, using this pair to form the new bond, and the leaving group (nucleofuge) comes off with an electron pair.

$$R \curvearrowleft X + \bar{Y} \longrightarrow R - Y + \bar{X}$$

The S_N2 Mechanism - S_N2 stands for substitution nucleophilic bimolecular. In this mechanism there is back side attack : the nucleophilic approaches the substrate from a position 180° away from the leaving group. The reaction is one step process with no intermediate. The C-Y bond is formed as the C-X bond is broken :

$$\bar{Y} + \text{C}\curvearrowleft X \longrightarrow Y\cdots C\cdots X \longrightarrow Y\text{-C} + \bar{X}$$

The S_N1 Mechanism - The most ideal version of the S_N1 mechanism (substitution nucleophilic unimolecular) consists of two steps

step 1 $$R\text{-}X \underset{}{\overset{\text{slow}}{\rightleftharpoons}} R^{+} + X$$

step 2 $$R^{+} + \bar{Y} \xrightarrow{\text{fast}} R\text{-}Y$$

This reaction have been used for preparation of tertiary amine,[42] quaternary ammonium salt,[42] N, N-disubstituted amide,[41-46] N,N-disubstitued sulfonamide.[42] For example preparation of amide from acid chloride and secondary amine.[41-45]

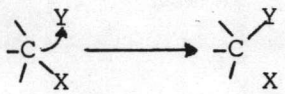
$$R\text{-}\overset{\overset{O}{\|}}{C}\text{-}Cl + R_2'\text{-}NH \longrightarrow R\text{-}\overset{\overset{O}{\|}}{C}\text{-}N\text{-}R_2' + HCl$$

2. <u>Aliphatic Electrophilic Substitution</u>[42]

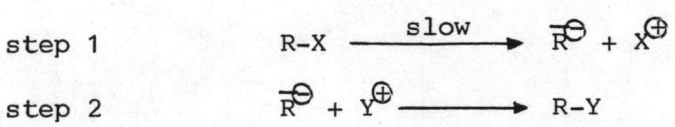
For aromatic system the most common leaving is the proton. The proton is also the leaving group in aliphatic systems,

but the reactivity depends on the acidity. Protons in saturated alkanes are very unreactive, but electrophilic substitution are often easily carried out at more acidic positions, for example, α to a carbonyl group, or at an alkynyl position (RC≡CH)

The S_E2 Mechanism - The bimolecular mechanism for electrophilic aliphatic substitution are analogous to the S_N2 mechanism in that the new bond forms as the the old one breaks.



The S_E1 mechanism is analogous to the S_N1. It involves two steps : a slow ionization and a fast combination.



This reaction have been used to prepare N-nitroso derivative or nitrosamine,[42-45,47] N-halo secondary amine,[42,44,47] N,N-disubstituted formamide.[42]

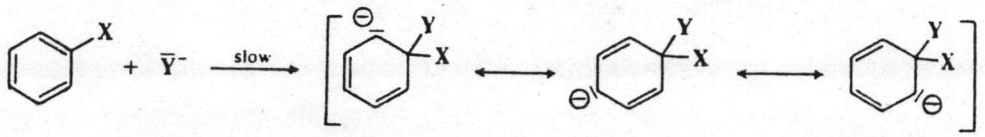
3. Aromatic Nucleophilic Substitution[42]

Nucleophilic substitutions proceed so slowly at an aromatic carbon. However, there are exceptions to previous statement. Reaction which are successful at an aromatic substrate are largely of three kinds : (1) reaction activated by electron-withdrawing groups ortho and para to the leaving; (2) reaction catalyzed by very strong base and proceeding through aryne intermediates; and (3) reaction in which the nitrogen of a diazonium salt is replaced by a

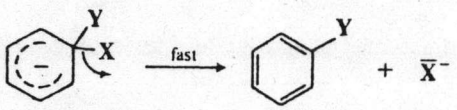
nucleophile.

The S_NAr Mechanism - Consisting of two steps, the first step is usually rate determining. This mechanism is somethimes called the S_N2 mechanism.

Step 1

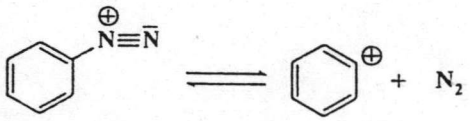


Step 2

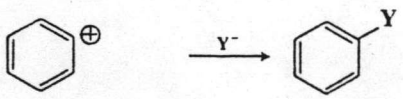


The S_N1 Mechanism - For diazonium salt, this mechanism is important.

Step 1

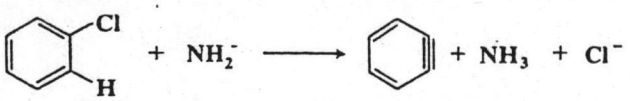


Step 2

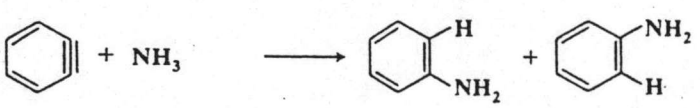


The Benzyne Mechanism - These substitutions occur on aryl halides which have no activating groups; bases are required stronger than normally used.

Step 1



Step 2



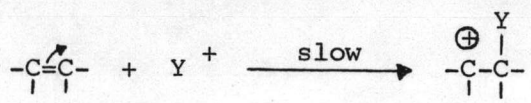
Activated aryl halides react quite well with secondary amines.(42)

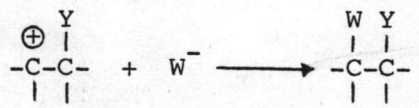
$$ArBr + R_2NH \longrightarrow ArNR_2 + HBr$$

4. Addition of Carbon-Carbon Multiple Bonds(42)

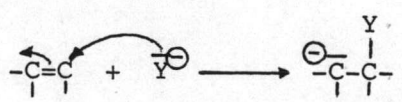
There are four ways in which addition to double bond or triple bond can take place. Three of these are two-step process and the fourth of mechanism is simultaneous.

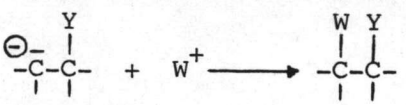
Electrophilic Addition - A positive species approaches the double or triple bond and in the first step form a bond by converting the ¶ pair of electron into a σ pair :

step 1 

step 2 

Nucleophilic Addition - In the first step a nucleophilic brings its pair of electrons to one carbon atom of the double or triple bond, Forcing the ¶ electrons to become centered on the other carbon, creating a carbanion. The second step is combination of this carbanion with a positive species.

step 1 

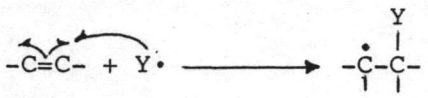
step 2 

Free Radical Addition - A radical is generated by :

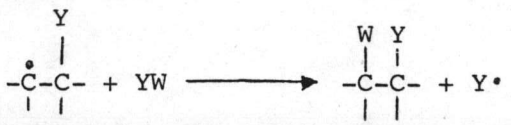
$$YW \xrightarrow[\text{spontaneous dissociation}]{h\nu \text{ or}} Y\cdot + W\cdot$$

or R•(from some other source) + YW ⟶ RW + Y•

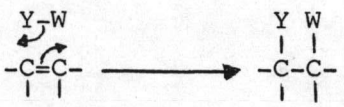
step 1



step 2



Cyclic Mechanism - The initial attack is not one carbon of the double bond, but both carbons are attacked simultaneously.



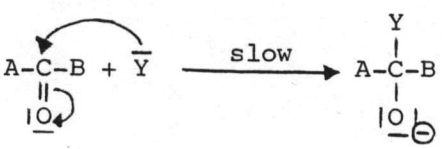
This reaction have been used to prepare enamine, tertiary amine, N, N-disubstituted amide,amidine,vic-diamine.[42]

5. Addition to Carbon-Hetero Multiple Bonds[42]

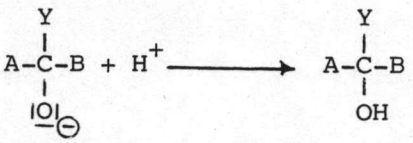
The reactions involve addition to carbon-oxygen, carbon-nitrogen, and carbon-sulfur double bonds and to the carbon-nitrogen triple bond. The C=O, C=N, and C≡N bonds are strongly polar, with the carbon always the positive end. Nucleophilic attacking species always go to the carbon and electrophilic ones to the oxygen or nitrogen.

Nucleophilic addition - The nucleophile attacks first and the electrophile attacks in step 2

step 1

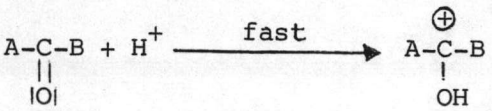


step 2

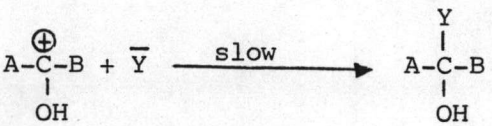


Electrophilic addition - The electrophile attacks first and the nucleophile attacks in step 2.

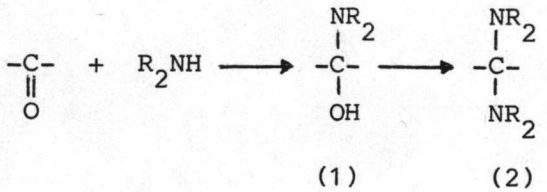
step 1



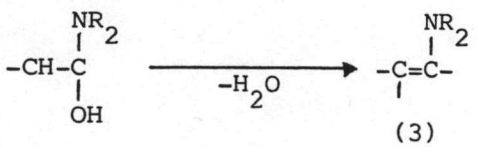
step 2



When secondary amines are added to aldehydes or ketones, the initially formed N, N-disubstituted hemiaminals (1) cannot loss water in the same way, and it is posible to isolate them. However, they are generally unstable, and under the reaction conditions usually react further. If no α hydrogen is present, (1) is converted to the more stable aminal (2). However, if an α hydrogen is present, water from (1) can be lost in that direction to give enamines (3).[42,47]



(1) (2)

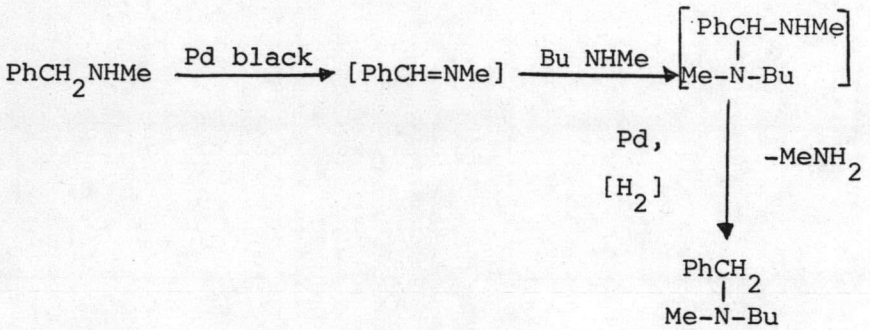


(3)

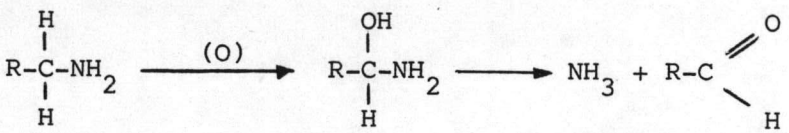
The most well known reaction in this group is Manich reaction.[42,44]

6. Oxidation[42]

Secondary amines can be dehydrogenated to imines with palladium back. The imine initially formed by the dehydrogenation reacts with another molecule of the same or a different amine to give an aminal, which losses RNH_2 to give tertiary amine. An example is the reaction between N-methylbenzylamine and N-methyl-butylamine, which produce 95% N-methyl N-butylbenzylamine.



The oxidation of amines with strong oxidizing agents; such as permanganate, results in the rupture of the C-N valence bond. In alkaline solutions the oxidation proceeds rapidly.



Secondary aliphatic amines have been cleaved to give aldehydes,ketones, or carboxylic acids with aqueous bromine and with neutral permanganate. The other product of this reaction is the amine with one less alkyl group.

III Analytical Methods for Secondary Amine Drugs

Various methods have been employed for quantitative analysis of secondary amine drugs. These methods are described below.

1. Titrimetric Methods

1.1 Aqueous Acid-Base Titrations

Aliphatic amines include secondary amines, are basic enough to titrate with a strong acid in aqueous solution. Although the feasibility of the titration depends on concentration of the base as well as upon its strength, the rough generalization can be given that accurate titration is possible with visual end point detection if the pKa is 8 or greater (pKb is 6 or smaller). This limitation can be extended by another pK unit by means of potentiometric detection of the end point. The addition of alcohol to the titration medium is helpful in sharpening titration end point for titrating aromatic and many other weakly basic amines than is obtained in water alone.[48-50]

General titration method has been used to determine ephedrine and other alkaloids. This was done by extracting with dichloromethane, (CH_2Cl_2) (for ephedrine), evaporated, dissolved residue in methanol and titrated with 0.02 N sulfuric acid using methyl red as an indicator.[51] The official methods[51-52] and the analytical methods committee of the S.A.C.[53], for determination of ephedrine in ephedrine hydrochloride tablet and nasal spray were performed by steam distillation. The collected distillate in excess standard acid was titrated with sodium hydroxide solution using methyl red as an indicator (residual titration of excess acid.) For piperazine, titration could be performed with standard acid in aqueous solution using methyl orange as an indicator, but the titration curve was not sharp and this method was not recommended.[53]

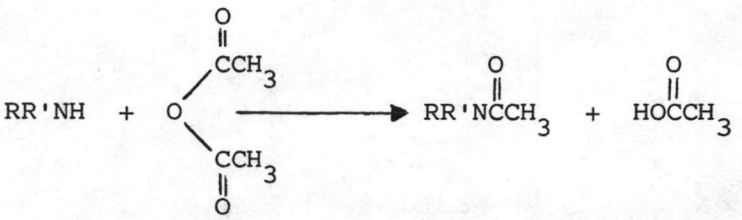
1.2 Non Aqueous Acid-Base Titrations

The titration of secondary aliphatic and aromatic amines in non-aqueous systems is very favourable method of analysis. More experience has been gained with glacial acetic acid as a non aqueous titration medium than with any other solvent. Amine salt can be titrated in non aqueous media but it cannot be accomplished by direct titration with acid, because the amine is already protonated. To liberate a free amine for the titration, mercuric acetate was added to react with amine salt. Crystal violet and cresol red have been suggested as suitable indicators, but potentiometric detection of the end point, using glass and calomel electrodes, appears to be superior.[49-50]

The non aqueous titratic procedure was used for determination of secondary amine drugs such as ephedrine hydrochloride,[53-55] pseudoephedrine hydrochloride,[52,56] epinephrine bitartrate,[53,54,57] propranolol hydrochloride,[52,55] and piperazine citrate[53,57] by dissolving the drugs in glacial acetic acid, titrated with perchloric acid to end point.

1.3 Titration following an Acylation Reaction

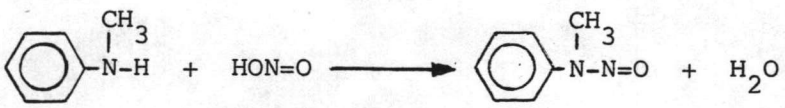
The reaction between acetic anhydride or acetyl chloride with primary, secondary aliphatic or aromatic amine provides an excellent method for analysis when a direct acidimetric titration is not possible, e.g., in the presence of a tertiary amine. Both primary and secondary amines react quantitatively.[49,50,58,59] Ephedrine could be acetylated quantitatively in aqueous solution to yield a neutral N-acetyl derivative.[53,58]



The quantity of amine (primary or secondary) after acetylation, may be determined by hydrolyzing the excess anhydride and titrated the acetic acid formed with standard alkali. The hydrolizing process can be done by adding a known amount of water and the excess water was determined by using Karl Fisher reagent. This method was more complex because Karl Fisher reagent was difficult to prepare, it must be standardized each day with standard water solution and all solutions used must be free of water. The amount of water in the samples also has to be known.[48,50]

1.4 Titration of the Nitrosation Reaction

Secondary amines (aromatic and aliphatic) react with nitrous acid in acid medium to form N-nitrosamines.[48] This reaction might be employed for the direct nitrite titration of some secondary amines.[50]



$$R_2NH + HON{=}O \longrightarrow R_2N{-}N{=}O + H_2O \quad (R \text{ is alkyl})$$

As commonly used, a solution of sodium nitrite was added to an ice cold solution of amine in hydrochloric acid at a slow rate, and the solution was tested with starch-iodide paper for the excess of unreacted nitrite after standing a few minutes.

This procedure was designed to overcome the volatilization and decomposition of nitrous acid. The starch-iodide paper test might be replaced by potentiometric determination using platinum and calomel electrodes at 10 to 15℃.[49]

1.5 Oxidation-Reduction Titrations

Ephedrine was determined by the reaction with alkaline iodine solution at 50℃ to form iodoform and titration of excess iodine in acid solution.[53] For epinephrine, the oxidation was performed at pH 5.4 to 5.5 with iodine and the excess iodide being removed with thiosulphate for quantitative measurement.[53]

Adrenaline and noradrenaline can be oxidized with manganese dioxide or potassium ferricyanide at pH 5 to 6 to form adrenochrome and noradrenochrome,respectively. But at pH 3 to 4 only adrenaline was oxidized. Phenylephrine hydrochloride could be determined by addition of excess of 0.1 N bromine in the presence of hydrochloric acid, addition of potassium iodide solution, and back titration of liberated iodine with 0.1 N thiosulfate.[53]

2. Gravimetric Method

Precipitation of the picrate is the method generally restricted to aliphatic amines and is particularly useful for determination of sympathomimatic amines of ephedrine class. Variation of this method have been used for the determination of secondary amines,[60] such as piperazine. The precipitation of piperazine as dipicrate precipitate can be determined quantitatively by gravimetric method.[52,53,57]

Ephedrine could be acetylated quantitatively (acylation) in aqueous solution to yield a neutral N-acetyl derivative which could be extracted with chloroform, and determined the weight of the residue from the chloroform layer.[61] AOAC method was used to determine ephedrine in ephedrine elixir by extracting with chloroform and proceeded for the gravimetric assay.[53]

3. Chromatographic Methods

Chromatographic methods are used in the purification and isolation as well as in the analysis of drugs. Amines could be acylated with trifluoroacetic anhydride $(CF_3CO)_2O$, and the resulting trifluoroacetamides was separated by gas chromatography.[50] The mixed triacetyl derivatives of adrenaline and noradrenaline which usually present in adrenaline obtained from natural sources might be separated by partition chromatography using water supported on Celite as the stationary phase and benzene as the mobile phase.[53] Ion-pair column partition method combined with spectrophotometric method were used to determine phenylephrine hydrochloride in tablets and capsules.[51] Acetylation of piperazine with acetic anhydride to form diacetyl piperazine which was separated by chromatographic method. The eluate, obtained by using chloroform as mobile phase, was determined by gravimetric method.[51]

4. Optical Methods

4.1 Colorimetric Methods

Colorimetric methods are generally resorted to when only small samples are available. When the constituent being analyzed presence in a very low concentration, or when a

specific color reaction, permits the determination of one component in the presence of the others is available, then the colorimetric method is selected.[60]

Many amines and quaternary ammonium compounds, for example ephedrine, could be determined in aqueous solution by forming a salt or ion pair between the positive charge nitrogen and a negative charge of dye or indicator molecule. The complex formation can be extracted with organic solvent and determined by using spectrophotometric method.[60] Typical dyes, have been used, were bromcresol purple, bromthymol blue, bromcresol green and methyl orange.[50] The identity test of ephedrine of the BP had been modified for the anlysis of ephedrine tablets and injection to give quantitative results. The color formation was stable and can be quantitatively extracted with cyclohexane. The intensity of color was measured at 515 nm.[53] A colorimetric method for the determination of adrenaline in pharmaceutical products containing bisulphite was developed, the color formation reached maximum intensity quickly and was constant for some hours.[53,56] Adrenaline was also determined by using persulfate reaction, and spectrophotometric method.[53] Phenylephrine hydrochloride in various preparations, such as nasal sprays might be determined by a modification of the 4-aminophenazone method and measured at 500 nm.[53] Colorimetric method for determination of phenylephrine was performed by oxidizing with ferricyanide, forming color complex with 4-aminoantipyrine in borate solution and measuring the absorbance. The automated method using automatic analyzer was also used in the determination of phenylephrine.[51]

4.2 UV Spectrophotometric Methods

The UV spectrophotometric method is widely used in quantitative analysis. It is extremely advantageous when only small amounts of material are available for assay. Two official methods for determining propranolol in propranolol tablets and prapranolol injection are BP method[52] and USP method.[55] The BP method, propranolol hydrochloride was extracted by methanol and measured the absorbance at 290 nm. The USP method, propranolol hydrochloride was treated with sodium hydroxide to form propranolol base, extracted with heptane and measured the absorbance at 293 nm. Ephedrine in ephedrine hydrochloride tablets[53] and phenylephrine in phenylephrine injection[52] were determined by dissolving in sulphuric acid and measuring the absorbances at 256.5 and 273 nm., respectively. Pseudoephedrine hydrochloride tablets were also determined by UV spectrophotometry.[56]

4.3 Infrared Spectrophotometric Methods

Infrared analysis is one of the the most powerful tools available for the determination of the structures of organic compounds. Several important functional groups are infrared active and their absorbances at particular wavelengths are indication of their presence. This absorption has been used in qualitative and quantitative analysis of some drugs.[60] Piperazine in piperazine powder and syrup was determined using near-infrared spectrophotometer.[51]

4.4 Spectrofluorometric Methods

Fluorescence arises through the return of an

optically excited molecule to its lowest electronic state by emission of radiation. The intensity of light emitted by a fluorescent substance in solution is directly proportional to its concentration only in a small range at low concentrations.

The method for the simultaneous determination of epinephrine and norepinephrine in admixture, performed by oxidation with manganese dioxide at pH 6.5. Both epinephrine and norepinephrine were oxidized to adrenochrome and noradrenochrome, respectively. At pH 3.0, only epinephrine was oxidized. Therefore the determination of both compounds can be done by difference. Fluorescence in each case was produced by the addition of alkali to convert the adrenochrome and noradrenochrome to adrenolutine and noradrenolutine respectively.[60] A mixture of adrenaline and noradrenaline might be determined simultaneously. The method was based on the observations that when adrenaline and noradrenaline were condensed with ethylenediamine, the fluorescence due to the adrenaline derivative was about five times that of the noradrenaline derivative at 550 nm. However the fluorescence spectra of the two compounds was similar.[53]

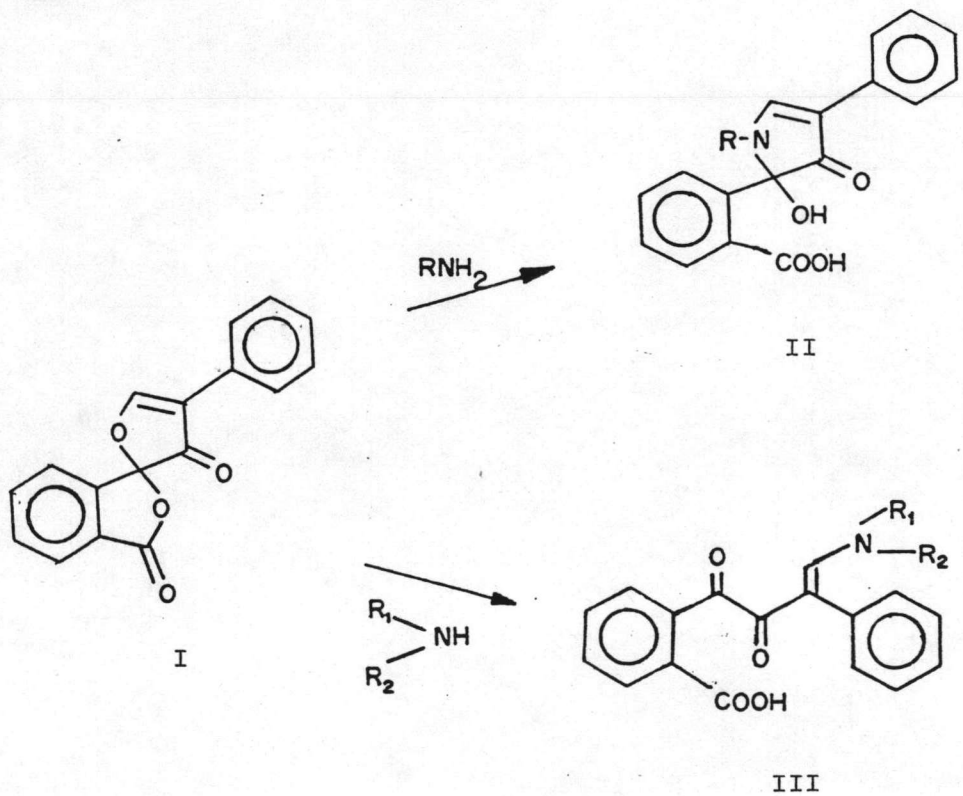
4.5 Polarimetric Methods

Polarimetry, the measurement of the degree of rotation of plane polarized light by a solution of an optically active substance, finds its main use in the characterization and establishment of purity of isolated and synthetic materials. Under specific conditions of solvent, concentration range, temperature, light source. etc., it is possible to determine the concentration of solutions of optically active substances in the absence of

interfering optically active substances. Epinephrine might be determined quantitatively by polarimetry.[53,60] This method had been adopted by the USP for epinephrine nasal solution, epinephrine inhalation, epinephrine injection, sterile epinephrine oil suspension, and epinephrine bitartrate ophthalmic solution.[57]

5. The Proposed Method

Fluorescamine (I) is known to react almost instantaneously with primary and secondary amines to give fluorescent pyrrolinones (II) and nonfluorescent aminoenones (III), respectively.[38]



The aminoenone type chromophores have strong UV absorption at 310-330 nm.[3] Based on these observations, a UV spectrophotometric method was developed for determination of secondary amine drugs. Ephedrine hydrochloride, pseudoephedrine

hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate, metoprolol tartrate, propranolol hydrochloride, and piperazine citrate, were all secondary amine drugs selected for this trial. The effect of various experimental conditions, such as pH, time, temperature, concentration of fluorescamine used, and linearity of absorbance-concentration relationship were examined. The optimum values for the variables were used to develop the method for determining secondary amine drugs. This method was used to determine propranolol hydrochloride in the formulations and the results obtained were compared with official USP method.[55]

The outline of this thesis was based on the following statements :

1. Secondary amine drugs used in this study were ephedrine hydrochloride, pseudoephedrine hydrochloride, phenylephrine hydrochloride, epinephrine bitartrate, metoprolol tartrate, propranolol hydrochloride, and piperazine citrate.

2. Secondary amine drugs were determined by the reaction with fluorescamine. The various reaction conditions, such as maximum absorption wavelenght, pH, time, temperature, concentration of fluorescamine used, and linearity of concentration range were studied and the optimum conditions for the assay were selected.

3. The accuracy and precision of the proposed method were measured by determining the percent recoveries compared to those obtained by the USP method.

4. The proposed method was applied to determine the content uniformity of propranolol hydrochloride in commercial pharmaceutical preparations. The results obtained were compared to those obtained by the USP method.