DEVELOPMENT AND VALIDATION OF UV VISIBLE SPECTROPHOTOMETRIC, HPTLC AND RP-HPLC METHODS FOR SIMULTANEOUS ESTIMATION OF ALPRAZOLAM AND PROPRANOLOL HYDROCHLORIDE IN THEIR COMBINED DOSAGE FORM

A THESIS SUBMITTED TO

NIRMA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

BY

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UNDER THE GUIDANCE OF

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MAY 2011

CERTIFICATE

This is to certify that ***Ms. DEVAL C. PATEL*** *has prepared the thesis entitled "* **Development and Validation of UV Visible Spectrophotometric, HPTLC and RP-HPLC Methods For Simultaneous Estimation of Alprazolam And Propranolol Hydrochloride in their combined dosage form***", in partial fulfillment for the award of M. Pharm. degree of the Nirma University, under our guidance. She has carried out the work at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University.*

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I declare that the thesis " **Development and Validation of UV Visible Spectrophotometric, HPTLC and RP-HPLC Methods For Simultaneous Estimation of Alprazolam And Propranolol Hydrochloride in their combined dosage form***" has been prepared by me under the guidance of Dr. Priti J. Mehta, Sr. Associate Professor and Head and Mr. Nrupesh R. Patel, Assistant Professor, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.*

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ACKNOWLEDGEMENT

*First of all, I am thankful to **almighty (the supreme soul)** for always being with me and blessing me with good family, friends, teachers and well wishers and extend his helping hands to complete this project successfully.*

*I am highly thankful to **Dr. Manjunath Ghate** (I/C Director, Institute of Pharmacy, Nirma University, Ahmedabad) for providing all necessary help and facility for my work and also for his constant support and encouragement.*

*I am deeply grateful to Professor **Dr. Priti Mehta**, Ph.D., Head of the Department of Pharmacy, Institute of Pharmacy, Nirma University, for her detailed and constructive comments, and for her important support throughout this work. Her wide knowledge and her logical way of thinking have been of great value for me. Her understanding, encouraging and personal guidance have provided a good basis for the present thesis.*

*With a feeling of profound pleasure, I gratefully owe my sincere thanks to my co-guide, **Mr. Nrupesh Patel** (Asst. Professor, Dept of Pharmaceutics, Institute of Pharmacy, Nirma University) for his kind co-operation and continuous help in providing valuable suggestions for completing this project.*

*I am extremely grateful to **Dr. Charmy S. Kothari, Omkar Sir, Deepak Sir** Dept of Pharmaceutical analysis, Institute of Pharmacy, Nirma University for their continuous encouragement and everlasting support throughout the course of this dissertation work.*

*I express my special thanks to **Dr. Anuradha K. Gajjar**, Associate professor, Department of Medicinal Chemistry, Institute of Pharmacy, Nirma University, Dr. **Tejal A Mehta**, Associate Professor, Head, Dept. of Pharmaceutics and I/C Head, Dept of Pharmacology, Institute of Pharmacy, Nirma University **Dr. Vimal Kumar,** Professor, Dept. of Pharmacogonosy and Phytopharmaceuticals, Institute of Pharmacy, Nirma University, for their constant moral support and kind cooperation.*

*I would like to thank specially to Ph.D students **Deepak sir, Omkar sir, Jignesh sir, Prerakbhai, Mohitbhai and Som sir** for their selfless support, co-operation and*

valuable suggestion. I would like to thank my colleagues ***Divyam, Gargi, Hiral, Manish, Mikul, Pankti, Rakesh, Shalav and Kartik.*** *I would like to thank my seniors* ***Sagar, Deesha, Janki*** *and juniors* ***Ajay, Margi, Kartik, Shikha, Keval, Shweta, Jay, Barkat, Ankit, Jayesh, Divij.***

Our group ***cloud 9*** *has been a source of friendships as well as good advice and collaboration. I am especially grateful for the fun group of Cloud 9 group members who stuck it out with me****: Nishadh, Pankti, Gargi, Harsh, Shalav, Ravi, Tushar and Ujjval****.*

For the Infrared spectroscopic studies, ***Jigneshbhai*** *with the help of* ***Pankti and Gargi*** *provided us with the necessary FTIR studies of the final formulations which helped us in giving shape to our project. I would also like to acknowledge the Nirma University for providing us with such facilities. I also thank* ***Dr. Hardik Bhatt and Mr Kuntal Manna*** *for inspirational discussions with us regarding the interpretation of FTIR graphs.*

My time at ***Nirma University*** *was made enjoyable in large part due to the many friends and groups that became a part of my life. I am grateful for* ***Satejbhai's Shreyas bhai's and Bipin bhai's*** *hospitality as I finished up my degree, and for many other people and memories.*

I would like to thank ***my two family*** *for all their love and encouragement. For my parents who raised me with a love of science, supported me in all my pursuits and provided me with a vision and a goal to achieve in future. I am extremely thankful to my in-laws who always encourage me to pursue higher studies. And most of all for my loving, supportive, encouraging, and patient family including* ***my darling mom, my caring dad, my bhai and bhabhi*** *whose thorough support during the final stages of this M.Pharm. is very much appreciated. I am thankful to my brother in law* ***Nimeshbhai*** *who always brings a smile on my face with his good sense of humor and relieves my tension.*

Lastly my heartiest thanks to a special person in my life whom I dedicate my work, my loving husband ***Chirag.*** *His love, support and constant inspiration made things easy for me. Words are not enough to thank you but you were there with me like a shadow, showing me the way in my difficult times.*

Last, but not the least, I express my gratitude and apologize to anybody whose contributions, I could not mention in this page.

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LIST OF ABBREVIATIONS

IP.	: Indian Pharmacopoeia
BP.	: British Pharmacopoeia
USP.	: United States Pharmacopoeia
EU.	: European Pharmacopoeia
ICH	: International Conference on Harmonization
ALP	: Alprazolam
PNL	: Propranolol Hydrochloride
API	: Active Pharmaceutical Ingredient
Std.	: Standard
Conc.	: Concentration
Fig.	: Figure
Vol.	: Volume
Ref.	: Reference
U.V.	: Ultra Violet Spectroscopy
H.P.L.C.	: High Pressure Liquid Chromatography
H.P.T.L.C.	: High Pressure Thin Layer Chromatography
F.T.I.R.	: Fourier Transform Infrared Spectroscopy
S. F. C.	: Super Critical Fluid Chromatography
R.P.	: Reverse Phase
S.D.	: Standard Deviation
R.S.D.	: Relative Standard Deviation
L.O.D.	: Limit of Detection
L.O.Q.	: Limit of Quantitation
%	: Percentage
°C	: Degree Centigrade

MPa	: Mega Pascal
cm	: centimeter
µm	: micrometer
nm	: nanometer
g	: gram
mg	: milligram
µg	: microgram
ng	: nanogram
ml	: mililitre
µl	: microlitre
no.	: number
Rt	: Retention Time
Rf	: Retention Factor

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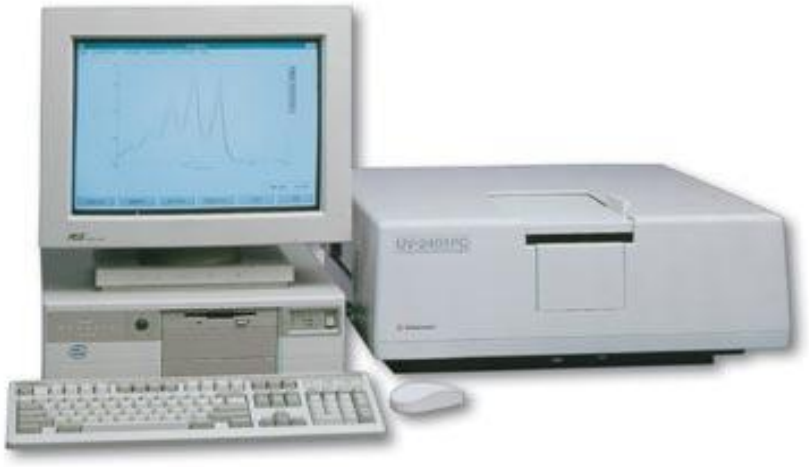
ABSTRACT

Three accurate, sensitive and reproducible methods are described for the quantitative determination of Alprazolam(ALP) and Propranolol hydrochloride(PNL) in their combined dosage form. The first method involves Dual wavelength spectrophotometric method. The method is based on determination of PNL at 319.4 nm using its absorptivity value and ALP at 258.2 nm after deduction of absorbance due to PNL. The two drugs follows the Beer- Lambert's law over the concentration range of 1-40 μg/ml for ALP and 80-200 μg/ml for PNL. The second method is based on separation of drugs by HPTLC followed by densitometric measurements of their spots at 248 nm. The separation was carried out on HPTLC aluminium sheets of silica gel 60 F_{254} using chloroform: methanol: ammonia (7:0.8:0.1 v/v/v) as mobile phase. The linear regression analysis was used for the regression line in the range of 100 - 600 ng/spot for ALP and 5-30 μg/spot for PNL , respectively. This system was found to give compact spots for ALP and PNL, after development. The third method is based on RP-HPLC separation of two drugs on the C_{18} column (150 mm length, 4.6 mm i.d, 5 μm particle size) at ambient temperature using a mobile phase consisting of acetonitrile: ammonium acetate buffer (25 mM, pH 4 with glacial acetic acid) (35:65 v/v) with 0.2 % triethylamine. Quantitation was achieved with photo diode array detection at 255 nm based on peak area with linear calibration curves at concentration ranges 0.5-50 μg/ml and 10-250 μg/ml for ALP and PNL, respectively. All the developed methods have been successively applied to marketed pharmaceutical formulation. No interference from the tablet excipients were found. All methods were validated according to ICH guidelines in terms of accuracy, precision, specificity, robustness, limits of detection and limit of quantitation.

DEVELOPMENT AND VALIDATION OF UV VISIBLE SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF ALPRAZOLAM AND PROPRANOLOL HYDROCHLORIDE IN THEIR COMBINED DOSAGE FORM



DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF ALPRAZOLAM AND PROPRANOLOL HYDROCHLORIDE IN THEIR COMBINED DOSAGE FORM.

DEVELOPMENT AND VALIDATION OF REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF ALPRAZOLAM AND PROPRANOLOL HYDROCHLORIDE IN THEIR COMBINED DOSAGE FORM.

Chapter 1
Introduction

1.1 INTRODUCTION TO MULTICOMPONENT FORMULATION[1]

Market is flooded with combination of drugs in various dosage forms. The multi-component formulations have gained a lot of importance nowadays due to greater patient acceptability, increased potency, multiple action, fewer side effects and quicker relief .

The multi-drug therapy is an ancient phenomenon to combat interrelated symptoms of diseased status of human beings. Since it ensure timely and complete medication for disorder and it has patient compliance, as it reduces the number of formulations to be taken at a time. Therefore, the pharmaceutical formulations with combinations of drugs have shown an increasing trend to counteract other symptoms specific to one drug n formulation, and hence analytical chemist will have to accept the challenge of developing reliable methods for analysis of drugs in such formulation.

Simultaneous analysis procedures are now being used more frequently for estimation of drugs in multi-component pharmaceutical formulations due to their inherent advantages viz. avoid time consuming extraction and separation, economical in the sense that use of expensive regents is minimized are equally accurate and precise. . For the estimation of multi-component formulation, the instrumental techniques, which are commonly employed, are spectrophotometery, GLC, high performance thin layer chromatography (HPTLC), HPLC etc.

- **Spectrophotometric multi-component analysis**

Absorption spectroscopy is one of the most useful and widely used tools available to the analyte for quantitative analysis. The relation between the concentration of analyte and the amount of light absorbed is the basis of most analytical applications of molecular spectroscopy. This method of analysis is gaining importance due to simple, rapid, precise, highly accurate and less time consuming. Spectrophotometric multi-component analysis can be applied where the spectra of drugs overlaps. In such cases of overlapping spectra,

simultaneous equation can be framed to obtain the concentration of individual component; otherwise multi-component analysis can be applied on any degree of spectral overlap provided that two or more spectra are not similar exactly.

- **High performance liquid chromatography (HPLC):**

This technique is based on the same method of separation as classical column chromatography. i.e. adsorption, partition, ion exchange and gel permeation but it differ from column chromatography, in that mobile phase is pumped through the packed column under high pressure. The technique is most widely used for all the analytical separation technique due to its sensitivity, its ready adaptability to accumulate quantitative determinations, its suitability for separating nonvolatile species or thermally fragile ones. In normal HPLC, polar solids such as silica gel; alumina (Al_2O_3) or porous glass beads and non-polar mobile phase such as heptane, octane or chloroform are used but if the opposite case holds, it is called as reversed phase HPLC.

- **High performance thin layer chromatography (HPTLC):**

The principle is based on plane chromatography. The mobile phase normally is driven by capillary action. The prominent advantages of this technique includes possibilities of separating of up to 70 samples and standard simultaneously on a single plate leading to high throughout, low cost analogs and the ability to construct calibration curves from standard chromatography under the same condition as the sample. Analyzing a sample by use of multiple separation steps and static post chromatographic detection procedures with various universal and specific visualization regents that are possible because all the sample components are stored on the layer without the chance of loss.

- **Gas chromatography (GC):**

GC is one of the most extensively used separation technique in which separation is accomplished by partitioning solute between a mobile gas phase and stationary phase, either liquid or solid. The chief requirement is same degrees of stability at the temperature necessary to maintain the substance in gas state.

1.2 RATIONALE FOR COMBINATION[2]

For panic attacks, the greatest benefit that medications can provide is to enhance the patient's motivation and accelerate progress toward facing panic and all of its repercussions. The most common benzodiazepines for panic attacks are alprazolam and clonazepam.

Those with social anxieties, medications can help to reduce the tensions associated with entering the fearful situation, to bring a racing heart and sweaty palms under control, and to reduce some shyness. The drugs with the longest history of use with social anxiety are the beta adrenergic blocking agents, also known as beta blockers. The most commonly used are propranolol. It is used for short-term relief of social phobia and reduces some peripheral symptoms of anxiety, such as tachycardia and sweating, and general tension, can help control symptoms of stage fright and public-speaking fears, has few side effects.

Hence, a high potency benzodiazepine like Alprazolam and beta blocker like propranolol in pharmaceutical formulation showed synergistic effect and proved to be the best for individuals with anxiety disorders.

1.3 INTRODUCTION TO DRUG PROFILE[3]

1.3.1 ALPRAZOLAM

- **Molecular Formula:** $C_{17}H_{13}N_4Cl$
- **Molecular Weight:** 308.8
- **Structural Formula:**

- **IUPAC Name:** 8-Chloro–1–methyl–6–phenyl–4*H*-[1,2,4]triazolo[4,3-*a*][1,4]-benzodiazepine
- **CAS No:** 28981–97–7
- **Synonym:** Alpratsolaami; Alprazolám; Alprazolamas; Alprazolamum;
- **Proprietary Name:** Alpralid; Alpraz; Alprox; Alzam; Anxirid; Apo-Alpraz; Apox; Azor
- **Category:** Anxiolytic
- **Official Status:** Official in IP-96, BP, EU and USP[4,5,6,7]
- **Physicochemical Properties:**
    1. Appearance: A white crystalline powder.
    2. Solubility: Practically insoluble in water; sparingly soluble in alcohol and in acetone; freely soluble in chloroform and dichloromethane.
    3. Melting point: 228° - 228.5° (From ethyl acetate).
    4. Partition co-efficient: Log *P*, 2.12.

5. Dissociation Constant: pK_a : 2.4.
6. Storage: Store in cool dry place, protected from light

- **Pharmacological Actions and Clinical Pharmacology:**

1. Mechanism of action

Alprazolam binds to stereospecific benzodiazepine receptors on the postsynaptic GABA neuron at several sites within the central nervous system, including the limbic system, reticular formation. Enhancement of the inhibitory effect of GABA on neuronal excitability results by increased neuronal membrane permeability to chloride ions. This shift in chloride ions results in hyperpolarization (a less excitable state) and stabilization.

2. Pharmacokinetics

Alprazolam is well absorbed from the gastrointestinal tract after oral doses, peak plasma concentrations being achieved within 1 to 2 hours of a dose. The mean plasma half-life is 11 to 15 hours. Alprazolam is 70 to 80% bound to plasma proteins, mainly albumin. It is metabolized in the liver, primarily by the cytochrome P450 isoenzyme CYP3A4. Metabolites include α-hydroxyalprazolam, which is reported to be about half as active as the parent compound, 4-hydroxyalprazolam, and an inactive benzophenone. Plasma concentrations of metabolites are very low. Alprazolam is excreted in urine as unchanged drug and metabolites.

3. Interactions of Alprazolam:

Drugs	Interactions
Ethyl alcohol	Enhance the CNS depressant effect of Ethyl Alcohol
Aprepitant, Fosaprepitant	Increase the serum concentration of Benzodiazepines
Carbamazepine	Increase the metabolism of Benzodiazepines
CYP3A4 Inducers	Increase the metabolism of CYP3A4 Substrates
CYP3A4 Inhibitors	Decrease the metabolism of CYP3A4 Substrates.
Clozapine	Enhance the adverse/toxic effect of Clozapine
CNS Depressants	Enhance the adverse/toxic effect of other CNS Depressants, Exceptions: Olopatadine, Ophthalmic
Antifungal Agents (Azole Derivatives)	Decrease the metabolism of Benzodiazepines
Grapefruit Juice, Isoniazid, Calcium Channel Blockers (Nondihydropyridine), Macrolide Antibiotics, Cimetidine	Decrease the metabolism of Benzodiazepines

4. Indications for Alprazolam:

Panic disorder: Alprazolam is FDA-approved for the short-term treatment (up to 8 weeks) of panic disorder, with or without agoraphobia. Alprazolam is very effective in the short-term symptomatic relief of moderate to severe anxiety, essential tremor, and panic attacks. Alprazolam's effects may occur after 8 weeks and necessitate discontinuation or physician-directed dose escalation. The physician should periodically reassess the usefulness of the drug for the individual patient. Alprazolam is recommended for treatment resistant cases of panic disorder where there is no history of tolerance or dependence.

Anxiety disorders: Alprazolam is indicated for the management of anxiety disorders or the short-term relief of symptoms of anxiety. Alprazolam is sometimes prescribed for anxiety with associated depression. The antidepressant effects of Alprazolam may be due to its effects on beta-adrenergic receptors. Other benzodiazepines are not known to have antidepressant activity.

5. Contraindications:

Benzodiazepines require special precaution if used in children and in alcohol- or drug-dependent individuals. Use of Alprazolam should be carefully monitored by medical professionals in individuals with the conditions like Myasthenia gravis, acute narrow-angle glaucoma, severe liver deficiencies (e.g., cirrhosis), severe sleep apnea, pre-existing respiratory depression, marked neuromuscular respiratory weakness, acute pulmonary insufficiency, chronic psychosis, hypersensitivity or allergy to Alprazolam or other drugs in the benzodiazepine class, borderline personality disorder (may induce suicidality and dyscontrol).

6. Adverse Effects:

Central Nervous System	Abnormal coordination, Cognitive disorder, Depression, Drowsiness, Fatigue, Irritability, Memory impairment, Sedation, Agitation, Attention disturbance, Confusion, Depersonalization, Disorientation, Dizziness, Dream abnormalities, Hallucinations, Nightmares, Seizure, Talkativeness
Gastrointestinal System	Appetite increases/decreases, Constipation, Salivation decreases, Weight gain/loss, Xerostomia, Micturition difficulty
Neuromuscular & Skeletal System	Dysarthria (1% to 10%), Arthralgia, ataxia, Myalgia, Paresthesia
Cardiovascular System	Hypotension
Dermatologic System	Dermatitis, Pruritus, Rash
Endocrine & Metabolic System	Libido decreases/increases, Menstrual disorders
Genitourinary System	Incontinence
Hepatic System	Bilirubin increases, Jaundice, Liver enzymes increases
Respiratory System	Allergic rhinitis, Dyspnea <1% (Limited to important or life-threatening): Amnesia, falls
Postmarketing and/or Case Reports	Galactorrhea, Gynecomastia, Hepatic Failure, Hepatitis, Hhyperprolactinemia, Stevens-Johnson Syndrome

1.3.2 PROPRANOLOL

- **Molecular Formula:** $C_{16}H_{21}NO_2 \cdot HCl$
- **Molecular Weight:** 295.8
- **Structural Formula:**

- **IUPAC Name:** 1-[(1-Methylethyl) amino]-3-(1–naphthalenyloxy)-2–propanol
- **CAS No:** *318–98–9*; *3506–09–0* (±)
- **Synonym:** Propanolol-hidroklorid; Propanololi Hydrochloridum; Propranolol chlorhydrate de;
- **Proprietary Name:** Adrexan; Angilol; Apsolol; Avlocardyl; Bedranol; Berkolol; Betachron
- **Category:** Antihypertensive
- **Official Status:** Official in IP, BP and USP
- **Physicochemical Properties:**
    1. Appearance: A white powder
    2. Solubility: Soluble 1 in 20 of water and ethanol; slightly soluble in chloroform; practically insoluble in ether, benzene, and ethyl acetate.
    3. Melting point: 96° C (From cyclohexane).
    4. Partition co-efficient: Log *P* (octanol/pH 7.4) : 1.2
    5. Dissociation Constant: pK_a9.5 (24° C)
    6. Storage: Store in a cool dry place, protected from light

- **Pharmacological Actions and Clinical Pharmacology**

1. Mechanism of action

Propranolol is a non-selective beta blocker, that is, it blocks the action of epinephrine and norepinephrine on both β_1- and β_2-adrenergic receptors. It has little intrinsic sympathomimetic activity (ISA) but has strong membrane stabilizing activity (only at high blood concentrations, e.g. overdosage). Propranolol also has inhibitory effects on the norepinephrine transporter and/or stimulates norepinephrine release.

2. Pharmacokinetics

Propranolol is almost completely absorbed from the gastrointestinal tract, but is subject to considerable hepatic-tissue binding and first-pass metabolism. Peak plasma concentrations occur about 1 to 2 hours after an oral dose. Plasma concentrations vary greatly between individuals. Propranolol has high lipid solubility. It crosses the blood-brain barrier and the placenta, and is distributed into breast milk. Propranolol is about 90% bound to plasma proteins.

It is metabolised in the liver and at least one of its metabolites (4-hydroxypropranolol) is considered to be active, but the contribution of metabolites to its overall activity is uncertain.The metabolites and small amounts of unchanged drug are excreted in the urine. The plasma half-life of propranolol is about 3 to 6 hours. Propranolol is reported not to be significantly dialysable.

3. Interactions of Propranolol

Propranolol can mask the early warning symptoms of low blood sugar (hypoglycemia) and should be used with caution in patients receiving treatment for diabetes. Propranolol reduces the metabolism of thioridazine, increasing the concentration of thioridazine in the body and potentially causes abnormal heart beats.

4. Indications

Hypertension, Angina pectoris, Tachyarrhythmia, Myocardial infarction, Control of tachycardia/tremor associated with anxiety, hyperthyroidism or lithium therapy, Essential tremor, Migraine prophylaxis, Cluster headaches prophylaxis, Tension headache , Glaucoma, Primary exertional headache etc.

5. Contraindications

Propranolol should be used with caution in patients with Diabetes mellitus or hyperthyroidism, Peripheral vascular disease and Raynaud's syndrome, Phaeochromocytoma, Myasthenia gravis, drugs with bradycardic effects.

Propranolol is contraindicated in patients with Reversible airways disease, particularly asthma or chronic obstructive pulmonary disease (COPD), Bradycardia (<60 beats/minute), Sick sinus syndrome, Atrioventricular block (second or third degree), Shock, Severe hypotension, Cocaine toxicity.

6. Therapeutic Dosage

Disease	**Dosage**
Hypertension, Angina, Essential tremor	120–320 mg daily in divided doses
Migraine Prophylaxis	The initial dose is 80 mg daily in divided doses. The usual effective dose range is 160 mg to 240 mg per day.
Tachyarrhythmia, Anxiety (GAD), Hyperthyroidism	10–40 mg 3–4 times daily
Anxiety	5–10 mg 30min or 1.5hrs before and after performance, optionally 5–10 mg night before. Up to 40 mg if necessary, but side-effects may present.

7. Adverse Effects

Central Nervous System	Dizziness, lethargy, weakness, drowsiness, headache, insomnia, fatigue, anorexia, anxiety, mental depression, poor concentration, reversible amnesia and catatonia, vivid dreams with or without insomnia, hallucinations, paresthesia, incoordination
Gastrointestinal System	Anorexia, Nausea, Vomiting, Diarrhea, Abdominal pain, Cold extremities and Exacerbation of Raynaud's phenomenon, Congestive Heart Failure, Sleep disturbances, Vivid dreams, Dizziness, Fatigue and Bronchospasm
Cardiovascular System	Congestive Heart Failure and Bronchospasm, Syncope, Vertigo, Lightheadedness, Decreased Renal Perfusion, Postural Hypotension, Intensification of AV block and Hypotension, Severe Bradycardia, Claudication and Cold extremities, Raynaud's phenomenon, Dyspnea, Palpitations, Precordial Pain
Dermatologic System	A few cases of Erythmetous rashes and increases of facial acneiform lesions, Urticaria, Exfoliative Psoriasiform Eruption
Respiratory System	Bronchospasm, Laryngospasm and Respiratory distress
Others	Reduction or loss of libido, Reversible alopecia, Diminution, loss of hearing, Tinnitus, Visual disturbances, Diminished vision, Conjunctivitis, Thrombocytopenic purpura, Pharyngitis, Agranulocytosis, Fever combined with aching and sore throat, Flushing of the face

1.4 INTRODUCTION TO UV SPECTROPHOTOMETRY[8]

The spectrophotometric assay of drugs rarely involves the measurement of absorbance of sample containing only one absorbing component. The pharmaceutical analyst frequently encounters the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances, which potentially interfere in the assay.

The basis of all the spectrophotometric techniques for multi-component samples is the property that all wavelengths:

- The absorbance of a solution is the sum of absorbances of the individual components; or
- The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

In multi-component formulations the concentration of the absorbing substance is calculated from the measured absorbance using one of the following procedures:

1. Assay as a single-component sample
2. Assay using absorbance corrected for interference
3. Simultaneous equation method
4. Absorbance ratio method
5. Geometric correction method
6. Orthogonal polynomial method
7. Difference spectrophotometry
8. Derivative spectrophotometry
9. Least square approximation

1.5 INTRODUCTION TO HPTLC[9]

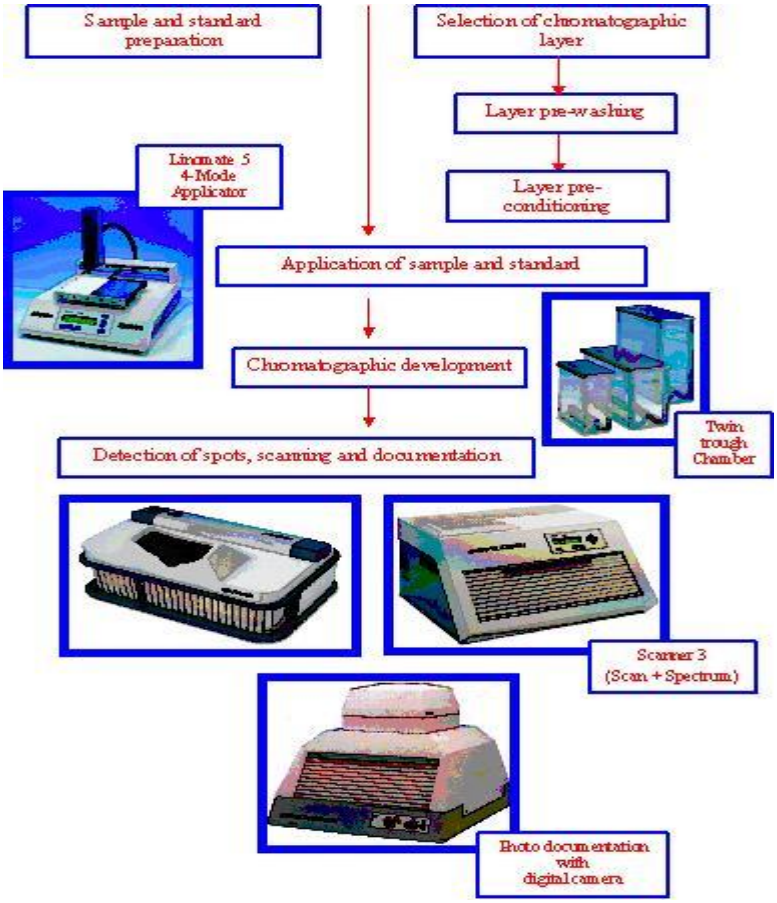


Figure 1.5 : Schematic Diagram of HPTLC

- **Features of HPTLC**

1. Simultaneous processing of sample and standard - better analytical precision and accuracy less need for Internal Standard
2. Several analysts work simultaneously
3. Lower analysis time and less cost per analysis
4. Low maintenance cost
5. Simple sample preparation - handle samples of divergent nature
6. No prior treatment for solvents like filtration and degassing
7. Low mobile phase consumption per sample

8. No interference from previous analysis - fresh stationary and mobile phases for each analysis - no contamination
9. Visual detection possible - open system
10. Non UV absorbing compounds detected by post-chromatographic derivatization.

- **Steps involved in HPTLC**

1. Selection of chromatographic layer
2. Sample and standard preparation
3. Layer pre-washing
4. Layer pre-conditioning
5. Application of sample and standard
6. Chromatographic development
7. Detection of spots
8. Scanning
9. Documentation of chromatic plate

1.6 INTRODUCTION TO HPLC[10]

HPLC was derived from classical column chromatography and has found important place in analytical techniques.This technique is based on the separation of components due to the difference in the migration rate of solute through a stationary phase by a liquid mobile phase.

The parameters involved in chromatographc separation are as follows :

- Capacity factor
- Resolution
- Column Efficiency
- Column Selectivity
- Distribution or Partition Coefficient

The main components of HPLC system are high pressure pump, a column, an injector system and detector. The system works as follows: the eluent is filtered and pumped through a chromatographic column, the sample is loaded and injected onto the column, and the effluent is monitored using a detector and recorded as peaks. In HPLC quantification of drugs is based on the peak area of the chromatogram.

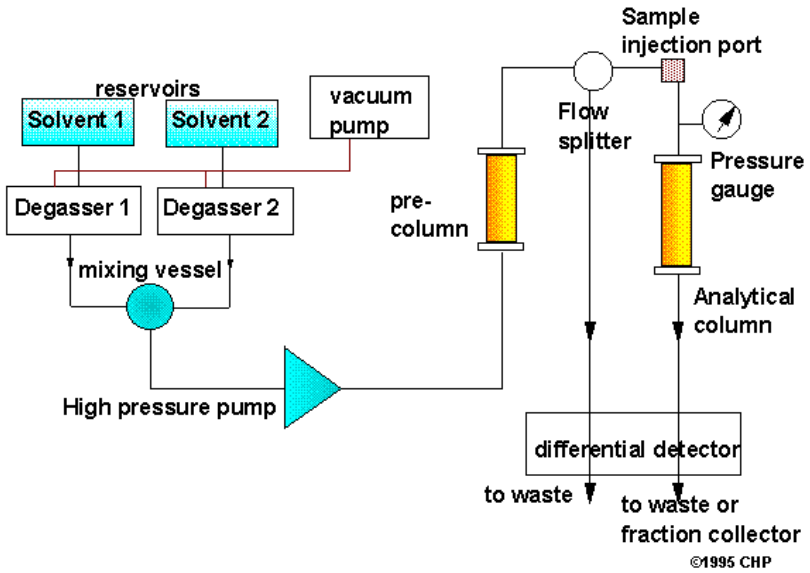


Figure 1.6 : Schematic diagram of HPLC system

1.7 ANALYTICAL METHOD VALIDATION[11]

1.7.1. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

1.7.2. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

1.7.3. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.This is sometimes termed trueness.

1.7.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

- **Repeatability**

  Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision .

- **Intermediate precision**

  Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

- **Reproducibility**

  Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

1.7.5. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

1.7.6. Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

1.7.7. Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

1.7.8. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

2.1. LITERATURE REVIEW OF ALPRAZOLAM

2.1.1. Reported HPLC methods of Alprazolam

NO	MATRIX	OTHER ANALYTES	COLUMN	MOBILE PHASE	CONDITION	REMARK	REF NO
1	Tablets	Propranolol	C_{18} column (250 mm length **,** 4.6 mm, i.d. 5 µm particle size)	Acetonitrile: water (adjusted to pH 2.3 with ortho phosphoric acid**)** 60:40 v/v	**Flow rate :**1.0 ml/min **Detector** : UV detection at 214 nm.	**Linear range :** 4 -30 µg/ml for Propranolol hydrochloride and 0.05 – 0.375 mg/ml for alprazolam	12
2	Tablets	Sertraline	Nucleosil C_{18} column (150 mm length , 4.6 mm i.d; and 5 µm particle size)	Acetonitrile and phosphate buffer pH 5.5 (50: 50, v/v),	**Flow rate** : 1.0 ml/min, **Detector** : UV detection at 230 nm	**Linear range**: 3-18 µg/ml for both drugs.	13
3	Tablets	Fluoxetine hydrochloride	Nucleosil C_8 column (150 mm length, 4.6 mm id, 5 µm particle size)	Acetonitril-phosphate buffer pH 5.5(45:55, v/v)	**Flow rate** :1.0 ml/min **Detector** : UV detection at 230 nm	**Linear range** : 4-14 µg/ml for both drugs.	14

4	Tablets	Sertraline	Reversed-phase C18 column	75 mM potassium dihydrogen phosphate buffer (pH 4.3)–acetonitrile–methanol (50:45:5, v/v/v)	**Flow rate**: 0.9 ml/min **Detector** : UV detection at 227 nm	**Linear Range** : Alprazolam : 1–80 µg/ml Sertraline : 5–200 µg/ml	15
5	Human Plasma,	α-Hydroxy alprazolam	-	-	Cyanopropyl bonded-phase extraction . **Detector:** UV detection at 230nm	Estazolam as an internal standard.	16
6	Dog Serum,	Triazolam	-	Acetonitrile: isopropanol: water (94:5:1)	**Flow rate** : 0.75 ml/min, **Detector:** UV detection at 221 nm	**Internal standards:** Triazolobenzodiazepines	17
7	Human Plasma,	Bromazepam, Diazepam, Lorazepam, Lormetazepam, Tetrazepam	-	-	**Detector:** Diode-array(HPLC-DAD) at detection wavelength 230 nm.	**Linear range** of benzodiazepine of 20-4000 ng/ml	18

2.1.2. Reported HPTLC method of Alprazolam

NO	MATRIX	OTHER ANALYTES	COLUMN	MOBILE PHASE	CONDITION	REMARK	REF NO
1	Tablets	Fluoxetine Hydrochloride	Aluminum-backed layer of silica gel $60F_{254}$	Acetone-toluene-ammonia (6.0: 3.5: 0.5, v/v/v)	**Detector:** UV detection at 230 nm	**Linear range** :400-1400 ng/spot for both drugs	19
2	Tablets	Sertraline	Silica gel plates with fluorescent indicators	Carbon tetrachloride, methanol, acetone, and ammonia (12:3:5:0.1, v/v/v/v)	**Detector:** 254 nm by using UV absorption densitometry	**R_f value:** Alprazolam 0.52 Sertraline 0.70**,** **LOD:** Alprazolam 0.05 µg/ml Sertraline 2.5 µg/ml, **LOQ:** Alprazoalm 0.2 µg/ml Sertraline 10 µg/ml	20
3	Tablets	Sertraline	Aluminum-backed layer of silica gel 60 F_{254}	Acetone, toluene, ammonia (6.0:3.0:1.0, v/v/v)	**Detector:** UV detection at 230 nm	**Linear range**: 400-1400 ng/spot for both drugs	21

2.1.3. Reported UV Visible Spectrophotometric methods of Alprazolam

NO	MATRIX	OTHER ANALYTES	METHOD	CONDITION / REMARKS	REF NO
1	Tablets	Fluoxetine Hydrochloride	First Derivative Spectroscopy Method	Dual wavelength selected for the estimation where λ1 (235nm) & λ2 (246.7nm) for Fluoxetine and λ3 (222.2nm) & λ4 (230nm) for Alprazolam where one had the same absorbance while others had marked difference of absorbance	22
			Second-Derivative Spectro photo- metry Method,	Quantitative determination of the drugs was performed at **232.14 nm** and at **225.25 nm** for Alprazolam and Fluoxetine, respectively **LOQ**: 4-14 µg/ml for both drugs	
2	Tablets	Fluoxetine	Simultaneous Estimation Method	Fluoxetine and Alprazolam were estimated at 226.4 nm and 263.2 nm, respectively	23

2.1.4. Reported GC-MS / LC-MS methods of Alprazolam

NO	MATRIX	OTHER ANALYTES	COLUMN	METHOD	CONDITION / REMARKS	REF NO
1	Human Plasma,	alpha-hydroxy alprazolam	Restek-200 capillary column,	GC-negative ion chemical ionization MS	Deuterium-labeled internal standards, **Reagent gas**: methane	24
2	Urine	*a*-hydroxy alprazolam &3-hydroxy methyl-5-methyltriazolyl chloro-benzo phenone	Dual capillary column and dual nitrogen detector of GC	GC	*a*-hydroxyalprazolam was found in the highest concentration and separated best from endogenous urine substances	25
3	Rabbit Plasma	-	DB-5MS analytical column	GC-MS Determined by a Quadrupole Mass Detector operated under selected ion monitoring mode (SIM),	**Internal standard**: Medazepam **LOD** :15 ng/ml, **LOQ** : 50 ng/ml,	26
4	Tablets	-	-	GC-FID and GC-MS method,	**Internal standard:** Tetracosane **linear range** : GC-FID: 0.25-5 µg/ml GC MS: 50-1000 ng/ml **LOD** GC-FID:200 ng/ml GC-MS :40 ng/ml	27

5	Rat hair and plasma,	Estazolam, Midazolam and their metabolites	Semi-micro HPLC column (100 mm length, 2mm i.d,3 µm particle size	RP-LC with electrospray ionization (ESI-MS),	-	28
6	Human Plasma	4- and α-hydroxy alprazolam	-	LC-MS	The extraction recovery was more than 82% for alprazolam and its metabolites. The within- and between-assay coefficients of variation were in the range of 1.9–17.9%	29

2.1.5. Reported Miscellaneous methods of Alprazolam

NO	MATRIX	OTHER ANALYTES	METHOD	CONDITION / REMARKS	REF NO
1	Human Serum	-	Hanging Mercury Drop Electrode, Adsorptive Stripping Voltammetry	**LOD:** 0.07 ng/ml for accumulation in water (accumulation time 240 s) and 0.3 ng/ml for accumulation in serum extract (accumulation time 30 s)	30
2	Blood	Nordiazepam, Triazolam, Lorazepam	Immunoassay Detection	When extracted with an organic solvent (butyl chloride) for precipitation with methanol or zinc sulfate, benzodiazepines can be detected at low concentrations	31

3	Tablets	-	Photostability- studies at accelerated pH	The photochemical degradation of alprazolam was performed in buffered solutions at pH 2.0 and 3.6. The higher rate of reaction was observed at pH = 2.0, the main photodegradation products were isolated	32
4	Tablets		Spectrofluorimetric assay for the photodegradation products	The photostability of alprazolam was evaluated at pH 2.0, 3.6 and 5.0, The drug was exposed to UV radiations, the photodegradation of alprazolam was followed by HPLC & Spectrofluorimetric assay.	33
5	Tablets	Amitriptyline HCl, Trifluoperazine HCl, Risperidone	Aluminum-backed layer of silica gel 60 F_{254} Carbon tetrachloride : acetone: triethylamine (8:2:0.3, *v/v/v*)	**Detector**: Densitometric measurements at 250 nm **Linear range :** Amitriptyline HCl (50–1,200 ng/spot), Trifluoperazine HCl (50–1,200 ng/spot), Risperidone (100–2,400 ng/spot) and Alprazolam (25–600 ng/spot), **LOD :** Amitriptyline HCl (20 ng/spot), Trifluoperazine HCl (20 ng/spot), Risperidone (40 ng/spot) Alprazolam (5 ng/spot)	34

2.2. LITERATURE REVIEW OF PROPRANOLOL

2.2.1. Reported HPLC methods of Propranolol

NO	MATRIX / OTHER ANALYTES	COLUMN/ MOBILE PHASE	CONDITION / REMARK	REF NO
1	Human serum/ plasma	Hypersil Cyano column Acetonitrile-aqueous acetic acid (1%) containing 0.2% triethylamine (35:65, v/v) (pH 3.6) **Detector** :Fluorescence detector set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm	**Flow rate** : 1.5 ml/ min, **Internal standard** : pronethalol **Linear range :** 5-200 ng/ ml **Retention times** : pronethalol 7.5 min propranolol 9.5 min,.	35
2	Human plasma Metoprolol	10-micron particle, 8 mm X 10 cm CN cartridge is used in conjunction with a radial compression separation system Monobasic sodium phosphate (pH 3) solution/methanol/acetonitrile (760:84:156 v/v/v), **Detector :** Fluorescence detection	**Flow rate :** 6 ml/min. **Retention time:** Propranolol 3.13 min metoprolol 1.42 min,	36
3	Human plasma Atenolol	Nucleosil RP-18 column Acetonitrile, water, triethylamine and phosphoric acid, pH 3	**Linear range** 3.13–100 ng/ml for Propranolol	37

NO	MATRIX / OTHER ANALYTES	COLUMN/ MOBILE PHASE	CONDITION / REMARK	REF NO
4	Rat hepatic microsomes	ODS column Phosphate buffer (ph 3.5, 0.067 mol/l) and methanol (55:45, v/v) as mobile phase. **Detector** :UV detection at 220 nm	**Internal standard** : p-nitrobenzoic acid **Linear range**: 0.50 -20.0 µmol/l. **LOD** : 0.15 µmol/l **LOQ** : 0.5 pmol/l	38
5	Rabbit plasma Furosemide	Nucleosil C_{18} column **Mode** : Isocratic 0.02 M potassium dihydrogen phosphate and acetonitrile (80:20, v/v) adjusted to ph 4.5 , **Detector** : UV detection 235 nm	**Linear range :** Furosemide 0.1–200 Propranolol **:**0.5–200 µg/ml	39
6	Rat plasma Buparva- quone, Atenolol, PropranololQuinidine Verapamil.	C_4 column Ammonium acetate buffer (0.02 M, pH 3.5) and acetonitrile in the ratio of 30:70 (v/v) **Detector :** UV detection at 251 nm	**Flow rate :**1.0 ml/min. **Retention time:** Atenolol, 4.30 Quinidine 5.96, Propranolol5.96, verapamil , 6.55, BPQ 7.98 min, **LOQ** :Atenolol 15 µg/ml, Quinidine, 0.8 µg/ml, Propranolol 5 µg/ml, Verapamil 10 µg/ml, BPQ. 200 ng/ml	40
7	Human plasma		**LOD:**Propranolol 0.5ng/ml NLA 2 ng/ml Propranolol HCl (20-100 ng/ml) and NLA (0.2-2 µg/ml)	41

NO	MATRIX / OTHER ANALYTES	COLUMN/ MOBILE PHASE	CONDITION / REMARK	
8	Rat bile 3 metabolites	**Mode** : Gradient elution **Detector** : Fluorescence detection	**LOQ** : 1.25 µg/ml of compound using 50 µl to 100 µl of bile sample.	42
9	Human plasma R and S Hyoscyamine	Oasis MCX cartridges **Mode** : Gradient elution methanol:acetic acid:triethylamine which was varied from 100:0.05:0.04 to 100:0.05:0.1 (v/v/v) over 30 min	**Flow rate** : 1 ml/min	43
10	Human plasma 4-hydroxy Propranolol	Homochiral derivatizing agent 2,3,4,6-tetra-*O*-acetyl-β-d-glucopyranosyl isothiocyanate (TAGIT) **Detector :** Fluorescence detection	**Linear range :** Propranolol: 2.0–200 ng/ml 4-hydroxyPropranolol :5.0 to 200 ng/ml	44
11	Human plasma 4 hydroxy Propranolol	Solid phase extraction and detected in positive ion mode by tandem mass spectrometry with a turbo ion spray interface	**Internal standards** : Deuterium-labeled Propranolol and 4-hydroxy Propranolol, Propranolol-d7 and 4-hydroxy Propranolol-d7 **Linear range** : Free Propranolol : 0.20-135.00 ng/mL Free 4-hydroxy Propranolol :0.20-25.00 ng/mL **LOQ :** 0.20 ng/mL for both analytes **LOD :** Propranolol:50pg/mL 4-hydroxy Propranolol :100 pg/mL	45

NO	MATRIX / OTHER ANALYTES	COLUMN/ MOBILE PHASE	CONDITION / REMARK	REF NO
12	Serum extracts Procainamide Disopyramid Quinidine,	C_8 reversed phase column An initial mobile phase of 80% phosphate (25 mmol/L, pH 3.5), 20% organic acetonitrile:methanol,(2:3)was maintained for 2 min at which time a linear gradient was used to change the mobile phase to 30% phosphate, 70% organic at 20 min after injection. **Detector** : Absorbance at 212 nm	**Internal standard** :N-propionyl Procainamide	46
13	Human plasma	Reversed-phase cyanopropylsilane column 70% acetonitrile and 30% 0.02 *M* acetate buffer, pH 7.0 **Detector** : Fluorescence detection	The reproducibility and precision of the method are shown from the analyses of samples containing 10-150 ng/ml of plasma.	47
14	Human plasma Metoprolol PHenol red	55% methanol, 45% of 0.05 M KH_2PO_4 aqueous solution (adjusted to pH 6) and 0.2 % (v/v) triethylamine. **Detector** : UV detection at 227 nm	**Flow rate** : 1 ml/min with a 9 min run time **Linear range** : 7.5-125 ng/ml **LOD** : 4.24, 2.18 and 8.57 ng/ml **LOQ** : 14, 7.2 and 28.3 ng/ml for Metoprolol, Propranolol and PHenol red respectively.	48
15	Alprazolam	Waters C_{18} column (250 × 4.6 mm, i.d. 5 µ) **Mobile phase :** acetonitrile: water (adjusted to ph 2.3 with ortho phosphoric acid**)** 60:40 v/v **Detector** : UV detection at 214 nm.	**Flow rate** :1.0 mL/min **Linear range** : 4 -30 µg/ml for Propranolol hydrochloride and 0.05 – 0.375 mg/ml for alprazolam	12

2.2.2. Reported HPTLC methods of Propranolol

NO	MATRIX/ OTHER ANALYTES	METHOD	CONDITION/REMARKS	REF NO
1	Atenolol, Acebutolol, and Bisoprolol	HPTLC Chromatographic Densitometry, **Detector :** UV densitometric Measurements	Silica gel 60 F_{254} HPTLC plates chloroform: methanol: ammonia, (15: 7 :0.2 v/v/v),	49
2	Bulk Drug Tablet	HPTLC Chromatographic Densitometry,	Detection wavelength : 290 nm **Linear range** : 200-2000 ng/spot	50

2.2.3. Reported Miscellaneous methods of Propranolol

NO	MATRIX/ OTHER ANALYTES	METHOD	CONDITION/REMARKS	REF NO
1	Bulk Drug	Novel ion selective PVC membrane electrode Method,	Silicotungstic acid is used as the counter ion and di-isononyl phthalate (DNP) used as the plastizer.,	51
2	Atenolol, Dipyridamole and Amiloride	Non-linear variable-angle synchronous fluorescence spectrometry	**Linear range** : 10–400, 6–200, 5.6–280 and 5-100ng/ml, respectively. As a result, the analyses were performed in an ethanol–water (70%(v/v)) medium at a ph 7.5, adjusted by using trishydroxymethyl amino methane (0.08 M) as a buffer solution	52
3	Bulk Drug	Calorimetric Method	The method involves nitration of the drug with a mixture of potassium nitrate and sulphuric acid. The coloured nitro-derivative has an absorption maxima at 360 nm. **Linear range** : 10–50 μg/ml.	53
4	Tablets	Flow injection-chemiluminescence (CL) Method for Propranolol HCl	**Linear range** :1.0-17.5mg/l **LOD**: 70ng/ml	54

NO	MATRIX/ OTHER ANALYTES	METHOD	CONDITION/REMARKS	REF NO
5	Tablets	Colorimetric by Derivatization	chemical derivatizatio by using diazotized 4-amino-3,5-dinitrobenzoic acid (ADBA) as the chromogenic derivatizing reagent and resultant formation of azo dyes, **Linear range** : 1–8 μg/mL **LOD** : 0.76 μg/mL and is reproducible	55
6	Terbutaline, and ketamtine	On-line capillary electrophoresis- (CE ESI-MS),	Separation of the different chiral forms has been achieved by introducing cyclodextrins (CDs), which act as chiral selectors, into the CE operating electrolytes..	56
7	Serum or Plasma	Fluoroimmuno assay	Propranolol coupled to magnetizable solid-phase particles and fluorescein-labeled Propranolol as tracer	57
8	Human plasma	Displacement electrophoresis in a two-layer polyacrylamide gel using fluorimetric detection	Drug is extracted from human plasma into a chloroform + heptane mixture in the presence of ammonia	58
9	Racemic mixtures	Capillary Electrophoresis method for Enantiomeric purity determination	Cyclodextrins as chiral additives and uncoated fused-silica capillaries thermostated at 15^{o}C.	59

NO	MATRIX/ OTHER ANALYTES	METHOD	CONDITION/REMARKS	REF NO
10	Biological fluids	Solid-phase microextraction SPME	**Linear range :** (0.5-100 μg/mL) **LOD** : 0.32 μg/mL by UV detection.	60
11	Saliva	Ion-Transfer Voltammetric Determination	Silicon Membrane-Based Liquid|Liquid Microinterface Arrays . **Linear range** : 0.05−1 μM **LOD** :0.02 μM	61
12	Tablet Metoprolol, Acebutolol	Sequential Injection Analysis (SIA) technique	**Linear range** : Metoprolol (40.52 - 250 mg/l), Acebutolol (32.85 - 140 mg/l) and Propranolol (16.58 - 120 mg/l)	62
13	Tablets	Spectro photometric determination	Oxidative coupling with 3-methyl benzothiazoline-2-one hydrazone. A mixture of an acidic solution of the chromogenic agent and the drug upon treatment with ceric ammonium sulfate produces an orange color peaking at 496 nm.	63
14	Human plasma 4-hydroxy Propranolol	Solid phase extraction and liquid chromatography/electrospray tandem mass spectrometry	**Internal standards:** Deuterium-labeled Propranolol, 4-hydroxy Propranolol, Propranolol-d7 and 4-hydroxy Propranolol-d7, **LOQ** : 0.20 ng/mL for both analytes with **LOD** : 50 and 100 pg/mL for Propranolol and 4-hydroxy Propranolol, respectively **Linear range** 1.00–500.00 ng/mL for total Propranolol and 1.00–360.00 ng/mL for total 4-hydroxy Propranolol.	64

NO	MATRIX/ OTHER ANALYTES	METHOD	CONDITION/REMARKS	REF NO
15	Metoprolol tartrate	AAS and spectro-photometric determination	**Linear range** : 40 and 60 µg /ml Drug acts as a secondary amine: (a) with carbon disulphide, the formed complex extracted into iso-butyl methyl ketone (IBMK) after chelation with Cu(II) ions at ph 7.5, followed by measuring the absorbance at 435.4 nm or indirectly for the drug by flame atomic absorption spectrophotometry (AAS).	65
16	Tablets Piroxicam	Indirect Spectrophotometric Determination	**Linear ranges** : 0.5 - 12.5 and 0.3 - 16.0 µg/ml for Propranolol, and 0.4 - 7.5 and 0.2 - 10 µg/ml for Piroxicam	66
17	Bulk drug	Liquid PHase Chemiluminescence Development	Sensitive flow injection-chemiluminescence detection (FI-CL) method based on pyrogallol (Pg) chemiluminescent reagent (CL-reagent) oxidized by periodate	67
18	Tablet	Spectrophotometric method	Bromopyrogallol Red(BPR)-molybdenum(VI) for complex formation **Linear range** : 1.5 - 30 µg/10 ml; the apparent molor absorptivity is 7.8 * 104 l mol^{-1} cm^{-1} at 618 nm.	68

NO	MATRIX/ OTHER ANALYTES	METHOD	CONDITION/REMARKS	REF NO
19	Tablets	Calorimetric method	Nitration of the drug with a mixture of potassium nitrate and sulphuric acid. The coloured nitro-derivative has an absorption maxima at 360 nm. **Linear range** : 10–50 μg/ml.	69
20	Tablet Hydro chlorothiazide	Simultaneous estimation by derivative, multi-component and two-wavelength Spectroscopy	Propranolol hydrochloride shows absorbance maxima at 289 nm and hydrochlorothiazide shows absorbance maxima at 271.2 nm in 0.1 N HCI.	70
21	Urine Levodopa	Simultaneous spectro-fluorimetric determination	Measurement of natural fluorescence of these drugs in the micellar media of sodium dodecyl sulfate (SDS) **Linear ranges :** Levodopa : 2.0×10^{-8} to 1.0×10^{-5} mol/L Propranolol : 3.6×10^{-9} to 1.8×10^{-6} mol/L	71
22	Tablets	Polarographic determination	Propranolol was reacted with nitric acid to give nitroPropranolol and was then measured in Britton–Robinson solutions in the ph range 2.0–12.0 by differential-pulse polarography. **Linear range** : 5.0×10^{-7} –5.0×10^{-5} M **LOD** : 5 nM	72

No	MATRIX/ OTHER ANALYTES	METHOD	CONDITION/REMARKS	REF NO
23	Brain tissue Propranolol glycol and N-desisopropyl-Propranolol	Electron capture gas chromatography	Tissues are homogenized in perchloric acid-acetonitrile. Propranolol and its metabolites are isolated from the supernantant by solvent extraction and separated and detected as their trifluoroacetyl derivatives by electron capture gas chromatography.. **Linear Range** :$0.7–2.0 \times 10^{-16}$ moles/sec.	73
24	Tablets	Phosphori-metric method	Potassium iodide as heavy atom salt and sodium sulphite as oxygen scavenger, were used to obtain phosphorescent signal of Propranolol in solution. The phosphorescence intensity was measured at 492 nm. **LOD** : 14.4 ng/ml,	74
25	Tablets	Spectro-photometric method	Nitration of the drug with uranyl nitrate or thorium nitrate in sulphuric acid medium. The yellow coloured nitro derivative has an absorption maximum at 377 nm. The nitro derivative obeys Beer's Law in concentration range of 2-32 µg/ml and 1-30 µg/ml for uranyl nitrate and thorium nitrate respectively.	75

Chapter 3
Aim of Present Work

3.1 AIM OF PRESENT WORK

Pharmaceutical analytical procedures may be used for identification and quantitative analysis of the active moiety in the sample of drug substances or products. In the past few decades, number of new chemical entities and newer formulation of the known entities have been introduced into the market. Multicomponent dosage forms are also introduced as they are known to be beneficial. The development of an assay procedure for such dosage forms poses considerable challenges to the analytical chemist owing to complexity of these dosage forms as it contain multiple drug entities.

Several methods were reported for the individual estimation of ALP and PNL and their combination with other drugs but no single spectrophotometric and HPTLC method has been reported for simultaneous estimation of these drugs in combined dosage forms. A single HPLC method is reported for this combination. The reported HPLC method uses more amount of organic phase and the optimised mobile phase is not compatible with LC-MS or GC-MS.Hence it cannot be extended for further study of drug analytes in biological fluid, characterization of degradation products and impurity profiling. Therefore, it was endeavored to develop an accurate, precise and sensitive UV Visible Spectrophotometric, HPTLC and alternative RP-HPLC method to estimate both the drugs in their combined dosage form.

3.2 OBJECTIVES OF PRESENT WORK

- To develop a suitable UV Visible Spectrophotometric, HPTLC and RP-HPLC method for simultaneous estimation of Alprazolam and Propranolol hydrochloride in their combined dosage form.
- To validate the developed method in terms of Linearity, Range, Accuracy, Precision, Limit of Detection, Limit of Quantification, Robustness, Specificity as per ICH guidelines.

Chapter 4
Identification of Drugs

4.1 IDENTIFICATION OF DRUGS

Identification of drugs was carried out by Melting point, UV Visible Spectroscopy, Raman spectroscopy and IR spectroscopy.

- **Instrumentation**
    1. Melting point apparatus (Model T0603160; manufactured by EIE Instruments Pvt Ltd.
    2. UV Visible Spectrophotometer (Model UV-2450 PC Series; manufactured by Shimadzu Inc; Japan)
    3. Raman Spectrophotometer (Model R-3000 series; manufactured by Raman Systems, Inc.USA)
    4. FT-IR Spectrophotometer (Model FT-IR 6100; manufactured by Jasco,Inc,Japan)

4.1.1 MELTING POINT DETERMINATION

Melting point of Alprazolam(ALP) and Propranolol HCl(PNL) has been determined using melting point apparatus. The melting point of the pure drugs was taken by open capillary method.

Table: 4.1 Melting Point of Alprazolam and Propranolol HCl

Drug	Reported Melting Point(°C)[76]	Observed Melting Point(°C)
Alprazolam	228 -228.5 **°C**	226-228 **°C**
Propranolol HCl	163-164 **°C**	162-165 **°C**

4.1.2 UV SPECTRA OF DRUG

10 µg/ml solution of standard ALP and PNL each was prepared in methanol and scanned in UV Visible Spectrophotometer in range of 200-400 nm to determine the absorption maxima of both the drugs.

Table: 4.2 UV Spectra of Alprazolam(10 µg/ml) and Propranolol HCl(10 µg/ml) in MeOH

Drug	Reported Peak(nm) [77]	Observed Peak(nm)
Alprazolam	222.00 nm	221.0 nm
Propranolol HCl	290.00, 306.00, 319.00 nm	289.5, 306.4, 319.40 nm

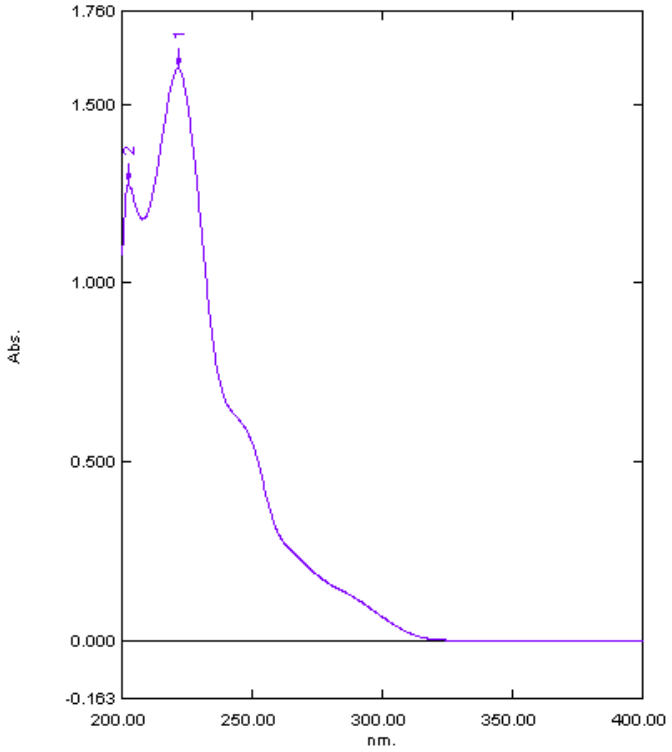


Figure 4.1 : UV Spectra of ALP (10 µg/ml) in MeOH

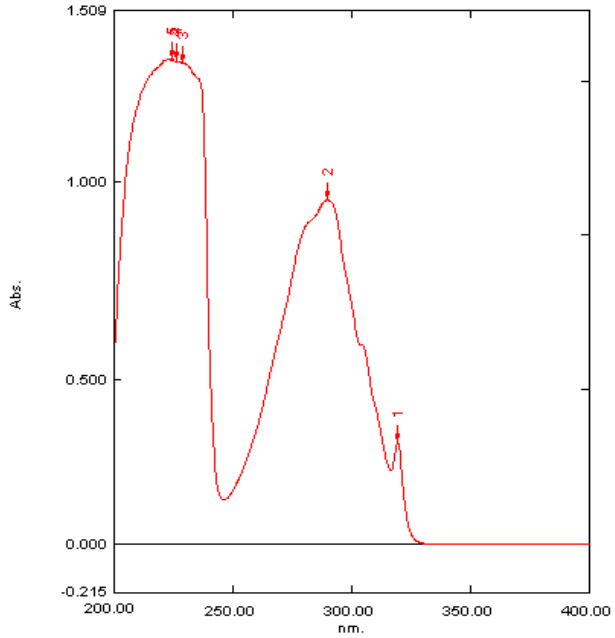


Figure 4.2 : UV Spectra of PNL (10 µg/ml) in MeOH

4.1.3 RAMAN SPECTRA OF DRUGS

Raman Spectra of pure drugs of ALP and PNL was taken by Raman spectrophotometer. The Raman spectra obtained were verified with the reported Raman spectra available in literature. [78]

4.1.3.1 Raman Spectra of Alprazolam

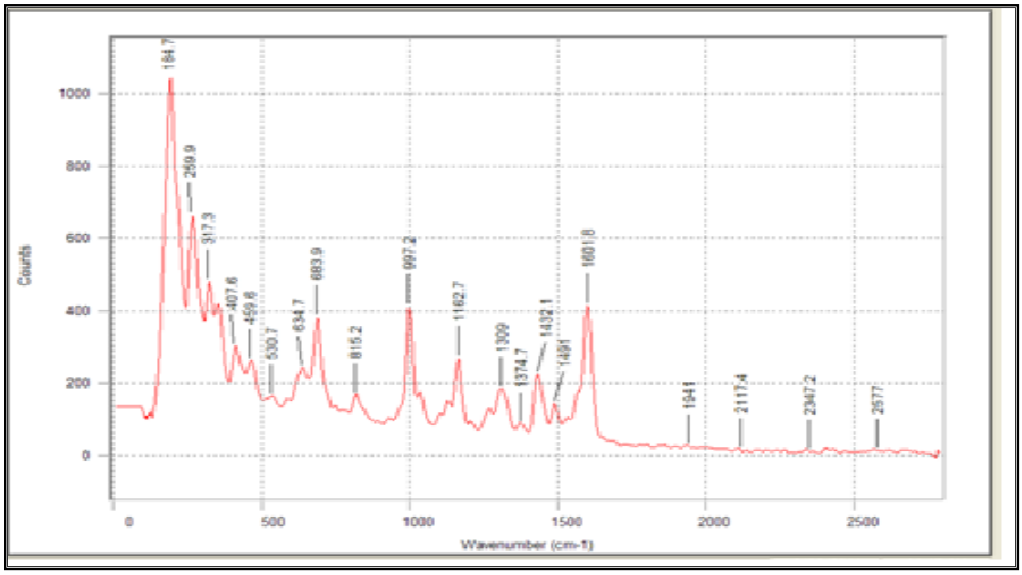


Figure 4.3 : Recorded Raman Spectra of ALP

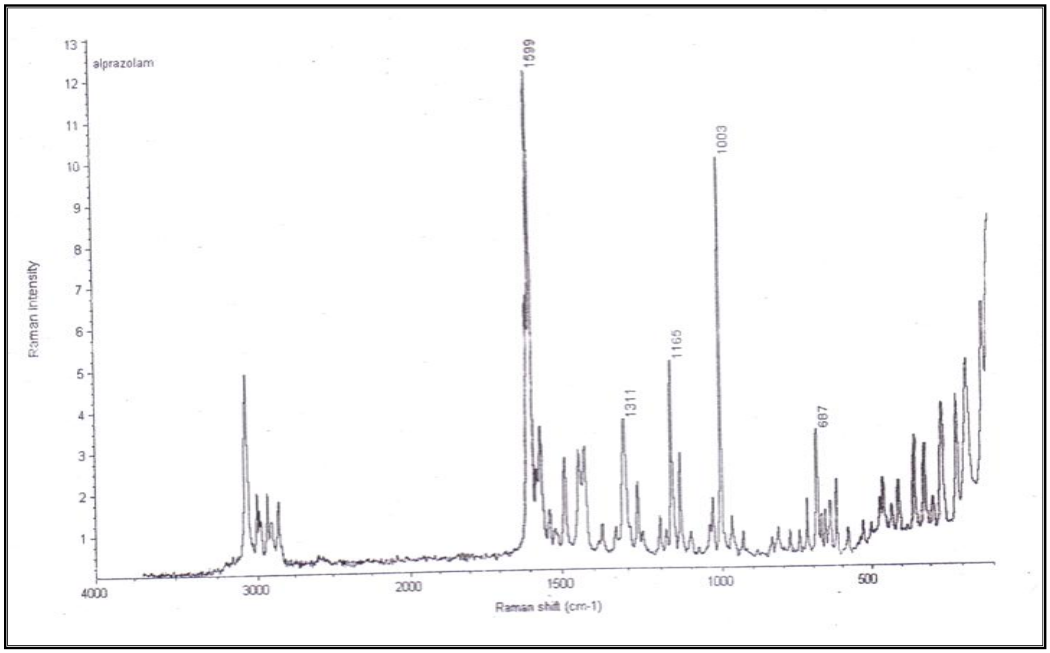


Figure 4.4 : Reported Raman Spectra of ALP

Table 4.3 : Reported and Recorded Raman peaks of Alprazolam

Functional Group	Reported Raman Peaks (cm^{-1})	Recorded Raman Peaks (cm^{-1})
Ring stretches(benzene derivative)	1599 cm^{-1}	1601.76 cm^{-1}
In plane C-H deformation	1311 cm^{-1}	1309.04 cm^{-1}
C-C stretches	1165 cm^{-1}	1162.68 cm^{-1}
Trigonal ring breathing	1003 cm^{-1}	997.17 cm^{-1}
C-Cl stretch	687 cm^{-1}	683.93 cm^{-1}

4.1.3.2 Raman Spectra of Propranolol Hydrochloride

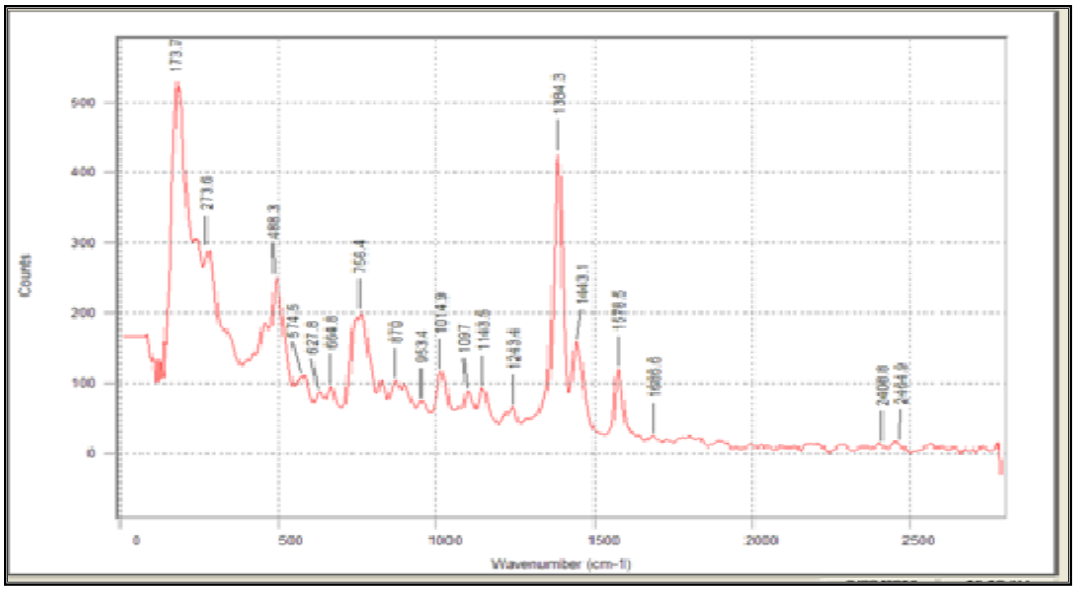


Figure 4.5 : Recorded Raman Spectra of Propranolol HCL

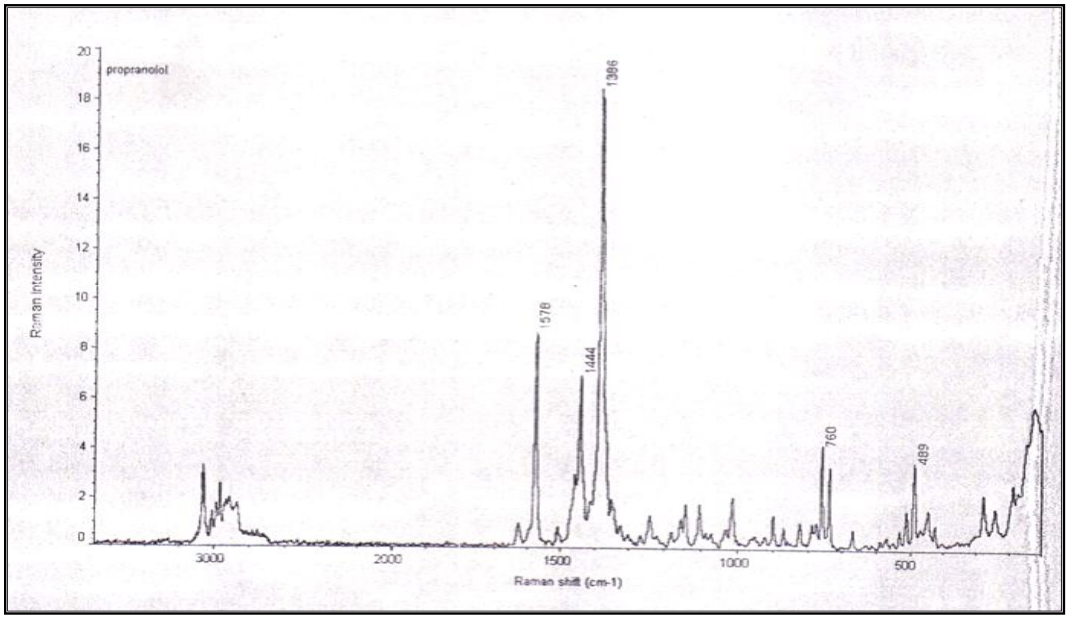


Figure 4.6 : Reported Raman Spectra of Propranolol HCl

Table 4.4 : Reported and Recorded Raman peaks of Propranolol HCl

Functional Group	Reported Raman Peaks (cm^{-1})	Recorded Raman Peaks (cm^{-1})
Ring stretches(benzene derivative)	1578 cm^{-1}	1578.51 cm^{-1}
CH_3-CH_2 deformation	1444 cm^{-1}	1443.09 cm^{-1}
Ring stretches (anthracenes)	1386 cm^{-1}	1384.27 cm^{-1}
Symmetric C-N-C stretch(secondary amines)	-	869.96 cm^{-1}
Skeletal stretch(isopropyl group)	760 cm^{-1}	756.42 cm^{-1}

4.1.4 IR SPECTRA OF DRUGS

IR Spectra of pure drugs was taken using FT-IR spectrophotometer.IR spectra obtained was verified with the reported IR spectra available in literature. [79]

4.1.4.1 IR Spectra of Alprazolam

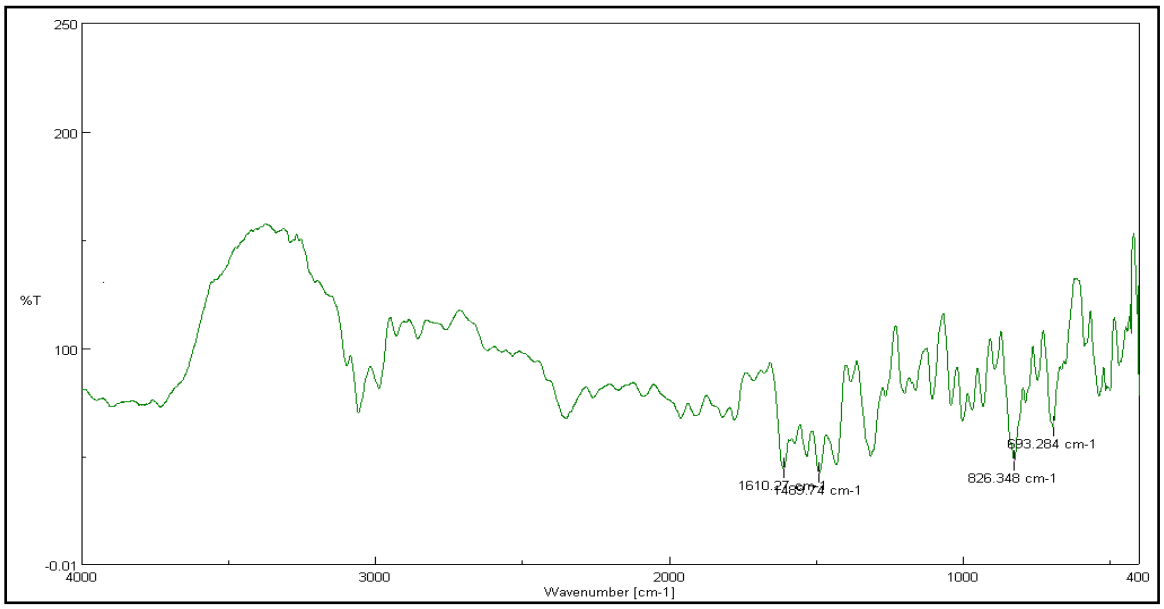


Figure 4.7 : Recorded IR Spectra of Alprazolam

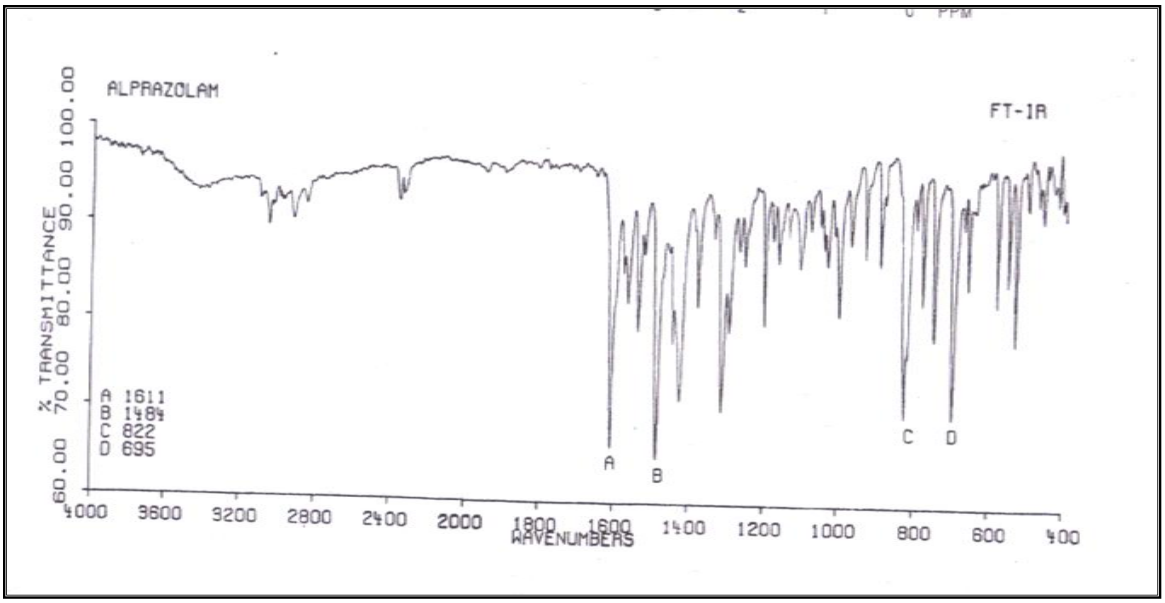


Figure 4.8 : Reported IR Spectra of Alprazolam

Table 4.5 : Reported and Recorded IR peaks of Alprazolam

Functional Group	**Reported IR Peaks (cm^{-1})**	**Recorded IR Peaks (cm^{-1})**
C=C Aromatic	1611 cm^{-1}	1610.27 cm^{-1}
-CH_2 bending vibration	1484 cm^{-1}	1489.74 cm^{-1}
C-H Aromatic(out of plane, bending vibration	822 cm^{-1}	826.348 cm^{-1}
C-Cl (chloride)	695 cm^{-1}	693.284 cm^{-1}

4.1.4.2 IR Spectra of Propranolol hydrochloride

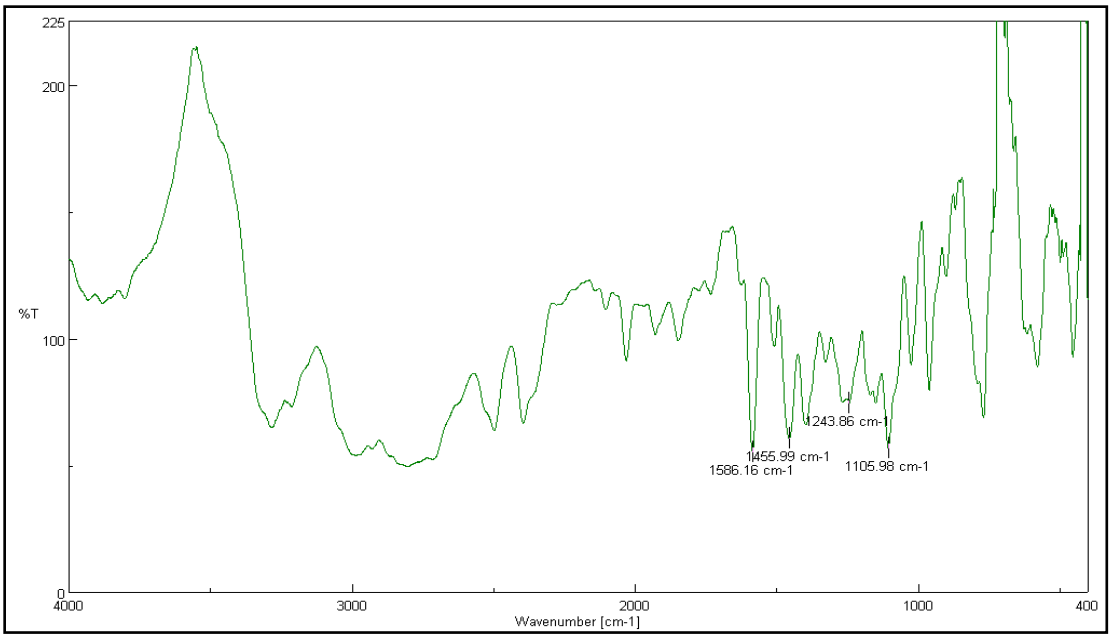


Figure 4.9 : Recorded IR Spectra of Propranolol HCl

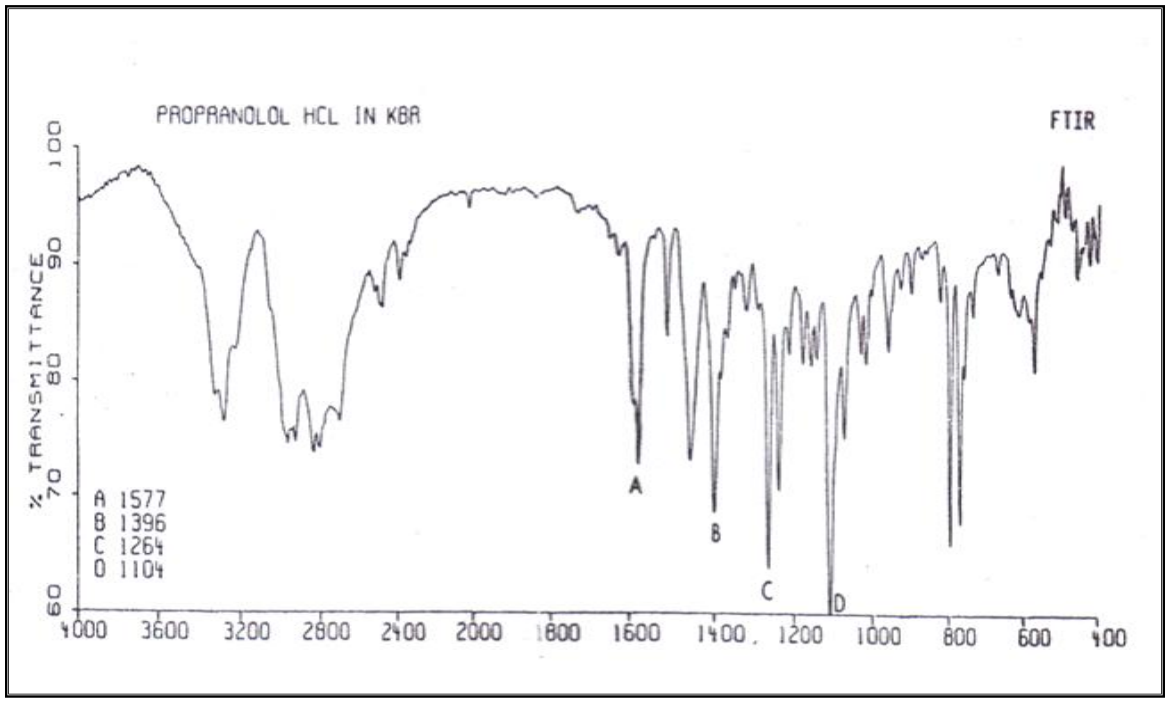


Figure 4.10 : Reported IR Spectra of Propranolol HCl

Table 4.6 : Reported and Recorded IR peaks of Propranolol HCl

Functional Group	Reported IR Peaks (cm^{-1})	Recorded IR Peaks (cm^{-1})
N-H bending vibration secondary amine	1577 cm^{-1}	1586.1 6cm^{-1}
C-H bending vibraton	1396 cm^{-1}	1455.99 cm^{-1}
C-N (amines)	1264 cm^{-1}	1243.86 cm^{-1}
C-O (alcohols)	1104 cm^{-1}	1105.98 cm^{-1}

Chapter 5
UV-Visible Spectrophotometric Method

5.1 EXPERIMENTAL WORK

5.1.1 Instrumentation

- **UV Visible Spectrophotometer**

  Model 2450 UV Visible Spectrophotometer(Shimadzu, JAPAN) having slit width of …and wavelength accuracy of … was used.

- **Analytical Balance**

  Model CX 220 analytical balance (CITIZEN, India) having capacity of 10 mg to 220 mg was used.

- **Sonicator**

  Model Trans-O-Sonic, D compact having capacity of 2 liter was used.

5.1.2 Materials And Methods

5.1.2.1 Reagents And Chemicals

- Market Formulation (Tablet) – Lam Plus (Tes Med India Pvt Ltd)
- API Alprazolam - Gift sample from Astron Research Centre
- API Propranolol Hydrochloride - Gift sample from Torrent Research Centre
- Methanol - AR Grade, S.D Fine chemicals Ltd., Mumbai, India

5.1.3 Preparation of Standard Stock Solution of Alprazolam (100 µg/ml) and Propranolol (1000 µg/ml)

ALP(25 mg) and PNL(25 mg) was weighed accurately and transferred to individual 25 ml amber colored volumetric flasks and dissolved in methanol. The solution was sonicated for 10 minutes. The flasks were shaken and volume was made up to the mark with methanol to give solution containing 1000 µg/ml of ALP and PNL. Aliquot of 1 ml was pipetted out from stock solution of ALP(1000 µg/ml) and transferred to 10 ml amber colored volumetric flask. The volume was made with methanol to obtain final concentration of 100 µg/ml of ALP.

5.1.4 Preparation of Binary mixtures of ALP and PNL

Appropriate volume of aliquots 1.0, 1.25, 1.5, 1.75 and 2.0 ml was pipetted out from 10 µg/ml standard ALP solution and 0.8, 1.0, 1.2, 1.4 and 1.6 ml was pipetted out from 1000 µg/ml standard PNL solutions and transferred to10 ml amber colored volumetric flask. The volume was made upto the mark with methanol to obtain final concentration of 1.0, 1.25, 1.5, 1.75 and 2.0 µg/ml of ALP and 80, 100, 120, 140 and 160 µg/ml of PNL in binary mixture. The binary mixture was made in the ratio of 1(ALP):80(PNL).

5.1.5 Analysis of Tablet Sample

A total of twenty tablets were weighed accurately and powdered . An amount of tablet powder equivalent to 10 mg of PNL (0.125 mg of ALP) was transferred to 10 ml amber colored volumetric flask. 5 ml of methanol was added to flask and sonicated for 10 minutes. The solution was shaken and volume was made up to the mark with methanol to give 1000 µg/ml PNL(12.5 µg/ml of ALP). The above solution was filtered through Whatmann filter paper (No.41). Aliquot 1 ml was pipetted out and transferred to 10 ml amber colored volumetric flask and volume was made up to the mark with methanol to get the solution containing 100 µg/ml of PNL (1.25 µg/ml of ALP).

5.1.6 PREPARATION OF SOLUTION FOR METHOD VALIDATION

5.1 Preparation of Linearity Curve

Appropriate volume of aliquot 0.1,0.5,1.0,2.0,3.0 and 4.0 ml was pipetted out from 100 µg/ml standard solution of ALP and 0.8,1.0,1.2,1.4,1.6,1.8 and 2.0 ml was pipetted out from 1000 µg/ml standard solution of PNL, transferred to individual 10 ml amber colored volumetric flask. The flasks were shaken and volume was made upto the mark with methanol to obtain final concentration of 1,5,10,20,30 an 40 µg/ml of ALP and 80,100,120,140,160,180 and 200 µg/ml of PNL.

5.2 Accuracy

The accuracy of the method was determined by performing the recovery studies from previously analyzed tablet sample by standard addition method at three different levels (80,100,120 %).

The concentration of tablet sample selected for recovery studies was 80 µg/ml PNL (1 µg/ml of ALP).For this 0.8 ml aliquot was taken from 1000 µg/ml stock solution of tablet and transferred to each 10 ml amber colored volumetric flask marked as A(for 80% recovery), B(for 100 % recovery) and C(for 120 % recovery).

Suitable aliquot of 0.8, 1.0 and 1.2 ml was pipetted out from 10 µg/ml standard solution of ALP and transferred to flask A,B and C respectively. Suitable aliquot of 0.64, 0.8 and 0.96 ml was pipetted out from 1000 µg/ml standard solution of PNL and transferred to flask A,B and C respectively. The flasks were shaken thoroughly and volume was made upto the mark with methanol to obtain mixture of solution of tablet and standard drug with following concentration.

Flask A (80 % recovery) :

Tablet sample conc : 80 µg/ml PNL (1 µg/ml of ALP)

Standard conc : 64 µg/ml PNL (0.8 µg/ml of ALP)

Flask B (100 % recovery) :

Tablet sample conc : 80 µg/ml PNL (1 µg/ml of ALP)

Standard conc : 80 µg/ml PNL (1 µg/ml of ALP)

Flask C (120 % recovery) :

Tablet sample conc : 80 µg/ml PNL (1 µg/ml of ALP)

Standard conc : 96 µg/ml PNL (1.2 µg/ml of ALP)

5.3 Precision

- **Intraday and Interday Precision**

The concentration selected for intraday and interday precision was 1, 10, 30 µg/ml for ALP and 100, 140, 180 µg/ml for PNL. For this aliquot of 0.1, 1.0 and 3.0 ml was pipetted out from 100 µg/ml standard solution of ALP and aliquot of 1.0,1.4 and 1.8 ml was pipetted out from 1000 µg/ml standard solution of PNL, transferred to individual six 10 ml amber colored volumetric flask. The volume of all the flasks was made upto the mark with methanol to obtain solution with following concentration.

Flask 1 : 1 µg/ml ALP
Flask 2 : 10 µg/ml ALP
Flask 3 : 30 µg/ml ALP
Flask 4 : 100 µg/ml PNL
Flask 5 : 140 µg/ml PNL
Flask 6 : 180 µg/ml PNL

Absorbance of each solution was measured three times in a day for intraday precision and at three different days for interday precision at their estimating wavelength.

- **Repeatability**

Repeatability was determined by measuring the absorbance of one concentration six times. at three different concentration for both the drugs. The concentration selected was 10 µg/ml of ALP and 100 µg/ml of PNL.

For this aliquot of 1.0 ml was pipetted out from 100 µg/ml standard solution of ALP and aliquot of 1.0 ml was pipetted out from 1000 µg/ml standard solution of PNL and transferred to individual two 10 ml amber colored volumetric flask. The volume of both the flasks was made upto the mark with methanol to give solution with following concentration.

Flask 1 : 10 µg/ml ALP

Flask 2 : 100 µg/ml PNL

Absorbance of each solution was measured six times at their estimating wavelength.

5.4 Limit of Detection

The calibration curve was repeated six times and the standard deviation of intercepts was calculated.LOD was calculated using following equation.

$LOD = 3.3\ \sigma/s$

Where, σ = standard deviation of intercepts

s = slope of straight line.

5.5 Limit of Quantification

The calibration curve was repeated six times and the standard deviation of intercepts was calculated.LOD was calculated using following equation.

$LOD = 10\ \sigma/s$

Where, σ = standard deviation of intercepts

s = slope of straight line.

Chapter 6
HPTLC Method

6.1 EXPERIMETNAL WORK

6.1.1 Instrumentation

- **HPTLC**

Camag Applicator Linomat 5 Semiautomatic application by spray on technique (2-500µl) was used. Camag 100 µl Applicator Syringe (Hamilton, Bonaduz, Schweiz) was used for application of spots. Twin trough glass chamber (Camag) having dimensions 20 x 10 cm was used for plate development.TLC Scanner 3 with scanning speed upto 10 mm/s and spectral range 190-800 nm was used for scanning developed plates. UV cabinet with dual wavelength (254 and 366 nm) UV lamp was used for detection of spots. Stationary phase used was precoated silica gel on aluminium sheet G60 F254.

- **Analytical Balance**

Model CX 220 analytical balance (CITIZEN, India) having capacity of 10 mg to 220 mg was used.

- **Sonicator**

Model Trans-O-Sonic, D compact () having capacity of 2 liter was used.

6.1.2 Materials And Methods

6.1.2.1 Reagents and Chemicals

- Market Formlation (Tablet) – Lam Plus (Tes Med India Pvt Ltd)
- API Alprazolam - Gift sample from Astron Research Centre
- API Propranolol Hydrochloride - Gift sample from Torrent Research Centre
- Methanol - AR Grade, S.D Fine chemicals Ltd., Mumbai, India
- Chloroform - AR Grade, S.D Fine chemicals Ltd., Mumbai, India
- Ammonia - AR Grade, S.D Fine chemicals Ltd., Mumbai, India

6.1.3 Preparation of Standard Stock Solution of Alprazolam (100µg/ml) and Propranolol (1000 µg/ml) as per subsection 5.1.2.2(page 47)

6.1.4 Analysis of Tablet Samples

A total of twenty tablets were weighed accurately and powdered . An amount of tablet powder equivalent to 100 mg of PNL (1.25 mg of ALP) was transferred to 10 ml amber colored volumetric flask. 5 ml of methanol was added to flask and sonicated for 10 minutes. The solution was shaken and volume was made up to the mark with methanol to obtain solution containing 10,000 µg/ml PNL (12.5 µg/ml of ALP). The above solution was filtered through Whatmann filter paper (No.41). Aliquot 1.6 ml was pipetted out from this solution and transferred to 10 ml amber colored volumetric flask and volume was made up to the mark with methanol to obtain the solution containing 1600 µg/ml of PNL (20 µg/ml of ALP).

6.1.5 PREPARATION OF SOLUTION FOR METHOD VALIDATION

6.1.7.1 Preparation of Linearity Curve

- **Alprazolam**

Linearity of Alprazolam was taken by increasing concentration of ALP while keeping the concentration of PNL constant. For this, appropriate volume of aliquot 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 ml was pipetted out from 100 µg/ml standard solution of ALP. It was transferred to individual six 10 ml amber colored volumetric flask. Aliquot 1.6 ml was pipetted out from 10,000 µg/ml standard solution of PNL and added to each above flask containing different concentration of ALP. The flasks were thoroughly shaken and volume was made upto the mark with methanol to obtain mixture of solution with following concentration.

Flask 1 : 10 µg/ml ALP, 1600 µg/ml PNL
Flask 2 : 20 µg/ml ALP, 1600 µg/ml PNL
Flask 3 : 30 µg/ml ALP, 1600 µg/ml PNL
Flask 4 : 40 µg/ml ALP, 1600 µg/ml PNL
Flask 5 : 50 µg/ml ALP, 1600 µg/ml PNL
Flask 6 : 60 µg/ml ALP, 1600 µg/ml PNL

10µl of each solution was applied to the activated TLC plate to obtain spots of following concentration on plate.

Spot 1 : 100ng/spot ALP, 16 µg/spot PNL
Spot 2 : 200ng/spot ALP, 16 µg/spot PNL
Spot 3 : 300ng/spot ALP, 16 µg/spot PNL
Spot 4 : 400ng/spot ALP, 16 µg/spot PNL
Spot 5 : 500ng/spot ALP, 16 µg/spot PNL
Spot 6 : 600ng/spot ALP, 16 µg/spot PNL

Spot 1 and Spot 6 was repeated two times since the first and the last spot might not run properly due to solvent run and frowning might occur in those spots. The spotted plate was analysed as per the optimized HPTLC conditions mentioned in subsection 6.1.3.

- **Propranolol**

Linearity of Propranolol was taken by increasing concentration of PNL while keeping the concentration of ALP constant. For this, appropriate volume of aliquot 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml was pipetted out from 10,000 µg/ml standard solution of PNL. It was transferred to individual six 10 ml amber colored volumetric flask. Aliquot 2.0 ml was pipetted out from 100 µg/ml standard solution of ALP and added to each above flask containing different concentration of PNL. The flasks were thoroughly shaken and volume was made upto the mark with methanol to obtain mixture of solution with following concentration

Flask 1 : 500 µg/ml PNL, 20 µg/ml ALP
Flask 2 : 1000 µg/ml PNL, 20 µg/ml ALP
Flask 3 : 1500 µg/ml PNL, 20 µg/ml ALP
Flask 4 : 2000 µg/ml PNL, 20 µg/ml ALP
Flask 5 : 2500 µg/ml PNL, 20 µg/ml ALP
Flask 6 : 3000 µg/ml PNL, 20 µg/ml ALP

10µl of each solution was applied to the activated TLC plate to obtain spots of following concentration on plate.

Spot 1 : 5µg/spot PNL, 200 ng/spot ALP
Spot 2 : 10µg/spot PNL, 200 ng/spot ALP
Spot 3 : 15µg/spot PNL, 200 ng/spot ALP
Spot 4 : 20µg/spot PNL, 200 ng/spot ALP
Spot 5 : 25µg/spot PNL, 200 ng/spot ALP
Spot 6 : 30µg/spot PNL, 200 ng/spot ALP

Spot 1 and Spot 6 was repeated two times since the first and the last spot might not run properly due to solvent run and frowning might occur in those spots. The spotted plate was analysed as per the optimized HPTLC conditions mentioned in subsection 6.1.3.

Each solution of calibration curve for ALP and PNL was analysed six times as per the optimised HPTLC conditions mentioned in subsection 6.1.3.

6.1.7.2 Accuracy

The accuracy of the method was determined by performing the recovery studies from previously analyzed tablet sample by standard addition method at three different levels (80,100,120 %).

The concentration of tablet sample selected for recovery studies was 1200 µg/ml PNL (15 µg/ml of ALP).For this 1.2 ml aliquot was pipetted out from 10,000 µg/ml stock solution of tablet and transferred to each 10 ml amber colored volumetric flask marked as A(for 80% recovery), B(for 100 % recovery) and C(for 120 % recovery).

Suitable aliquot of 1.2, 1.5 and 1.8 ml was pipetted out from 100 µg/ml standard solution of ALP and transferred to flask A,B and C respectively. Suitable aliquot of 0.96, 1.2 and 1.44 ml was pipetted out from 10,000 µg/ml standard solution of PNL and transferred to flask A,B and C respectively. The flasks were shaken thoroughly and volume was made upto the mark with

methanol to obtain mixture of solution of tablet and standard drug with following concentration.

Flask A (80 % recovery) :

Tablet sample conc : 1200 µg/ml PNL (15 µg/ml of ALP)

Standard conc : 960 µg/ml PNL (12 µg/ml of ALP)

Flask B (100 % recovery) :

Tablet sample conc : 1200 µg/ml PNL (15 µg/ml of ALP)

Standard conc : 1200 µg/ml PNL (15 µg/ml of ALP)

Flask C (120 % recovery) :

Tablet sample conc : 1200 µg/ml PNL (15 µg/ml of ALP)

Standard conc : 1440 µg/ml PNL (18 µg/ml of ALP)

10 µl of each solution of Flask A,B and C was applied to the activated TLC plate three times to obtain nine spots with following concentration.

Spot 1
Spot 2 → 270 ng/spot (21600 ng /spot PNL)
Spot 3

Spot 4
Spot 5 → 300 ng/spot (24000 ng /spot PNL)
Spot 6

Spot 7
Spot 8 → 330 ng/spot (26400 ng /spot PNL)
Spot 9

The spots were analysed as per the optimised HPTLC conditions mentioned in subsection 6.1.3.

6.1.7.3 Precision

- **Intraday and Interday Precision**

The concentration selected for intraday and interday precision was 10, 30, 50 µg/ml for ALP and 1000, 2000, 3000 µg/ml for PNL. For this aliquot of 1.0, 3.0 and 5.0 ml was pipetted out from 100 µg/ml standard solution of ALP and aliquot of 1.0, 2.0 and 3.0 ml was pipetted out from 10,000 µg/ml standard solution of PNL, transferred to individual six 10 ml amber colored volumetric flask. The volume of all the flasks was made upto the mark with methanol to obtain solution with following concentration.

Flask 1 : 10 µg/ml ALP
Flask 2 : 30 µg/ml ALP
Flask 3 : 50 µg/ml ALP
Flask 4 : 1000 µg/ml PNL
Flask 5 : 2000 µg/ml PNL
Flask 6 : 3000 µg/ml PNL

10µl of each solution was applied to the activated TLC plate to obtain spots of following concentration on plate.

Spot 1 : 100 ng/spot ALP
Spot 2 : 300 ng/spot ALP
Spot 3 : 500 ng/spot ALP
Spot 4 : 10µg/spot PNL
Spot 5 : 20µg/spot PNL
Spot 6 : 30µg/spot PNL

Spot 1 and Spot 6 was repeated two times since the first and the last spot might not run properly due to solvent run and frowning might occur in those spots. The spotted plate was analysed as per the optimized HPTLC conditions mentioned in subsection 6.1.3.

Each solution was analysed three times in a day for intraday precision and at three different days for interday precision as per the optimised HPTLC conditions mentioned in subsection 6.1.3.

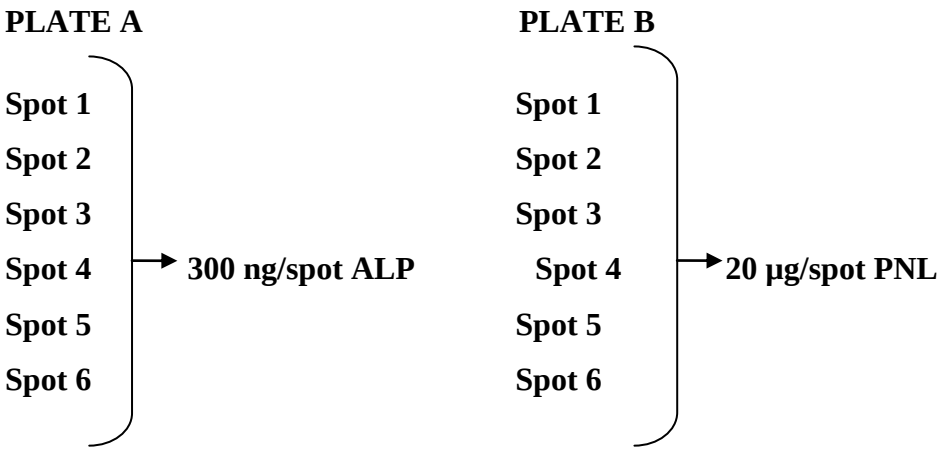
- **Repeatability**

Repeatability was determined by analysing one concentration six times for both the drugs. The concentration selected was 5 µg/ml of ALP and 100 µg/ml of PNL.

For this aliquot of 3.0 ml was pipetted out from 100 µg/ml standard solution of ALP and aliquot of 2.0 ml was pipetted out from 10,000 µg/ml standard solution of PNL, transferred to individual two 10 ml amber colored volumetric flask. The volume of both the flasks was made upto the mark with methanol to obtain solution with following concentration.

Flask 1 : 30 µg/ml ALP **Flask 2** : 2000 µg/ml PNL

10µl of each solution was applied six times to two activated TLC plate to obtain spots of following concentration on plate.



Spot 1 and Spot 6 was repeated two times since the first and the last spot might not run properly due to solvent run and frowning might occur in those spots. The spotted plate was analysed as per the optimized HPTLC conditions mentioned in subsection 6.1.3.

6.1.7.4 Limit of Detection

The calibration curve was repeated six times and the standard deviation of intercepts was calculated.LOD was calculated using following equation.

LOD = 3.3 σ/s

Where, σ = standard deviation of intercepts

s = slope of straight line.

6.1.7.5 Limit of Quantification

The calibration curve was repeated six times and the standard deviation of intercepts was calculated.LOD was calculated using following equation.

LOD = 10 σ/s

Where, σ = standard deviation of intercepts

s = slope of straight line.

Chapter 7
HPLC Method

7.1 EXPERIMENTAL WORK

7.1.1 Instrumentation

- **HPLC**

JASCO 200 Series HPLC model (JASCO, Inc JAPAN) with PU-2080 plus pump, MX-2080-31 mixer, Rheodyne model 7125 with 20µl fixed loop injector and Photo Diode Array detector was used. The software used was Borwin version 1.50

- **pH Meter**

pH meter 111E/101E (Analabs Scientific Instruments Ltd,) having resolution of ±0.01 pH and accuracy of ±0.01pH was used.

- **Analytical Balance**

Model CX 220 analytical balance (CITIZEN, India) having capacity of 10 mg to 220 mg was used.

- **Sonicator**

Model Trans-O-Sonic, D compact () having capacity of 2 liter was used.

7.1.2 Materials And Methods

7.1.2.1 Reagents and Chemicals

- Market Formulation (Tablet) – Lam Plus (Tes Med India Pvt Ltd)
- API Alprazolam - Gift sample from Astron Research Centre
- API Propranolol Hydrochloride - Gift sample from Torrent Research Centre
- Acetonitrile - HPLC Grade, S.D Fine chemicals Ltd., Mumbai, India
- Ammonium phosphate crystals - AR Grade, S.D Fine chemicals Ltd , Mumbai, India
- Glacial Acetic acid - S.D Fine chemicals Ltd , Mumbai, India
- Triethyl amine - S.D Fine chemicals Ltd , Mumbai, India

7.1.3 Preparation of Standard Stock Solution of Alprazolam (100µg/ml) and Propranolol(1000 µg/ml) as per subsection 5.1.2.2

7.1.4 Analysis of Tablet Samples

A total of twenty tablets were weighed accurately and powdered . An amount 0f tablet powder equivalent to 10 mg of PNL (0.125 mg of ALP) was transferred to 10 ml amber colored volumetric flask. 5 ml of methanol was added to flask and sonicated for 10 minutes. The solution was shaken and volume was made up to the mark with methanol to obtain solution containing 1000 µg/ml PNL (12.5 µg/ml of ALP). The above solution was filtered through Whatmann filter paper (No.41). Aliquot 1.6 ml was pipetted out from this solution and transferred to 10 ml amber colored volumetric flask and volume was made up to the mark with methanol to obtain the solution containing 160 µg/ml of PNL (2 µg/ml of ALP).

7.1.5 PREPARATION OF SOLUTION FOR METHOD VALIDATION

7.1.7.1 Preparation of Linearity Curve

- **Alprazolam**

Linearity of Alprazolam was taken by increasing concentration of ALP while keeping the concentration of PNL constant. For this, appropriate volume of aliquot 0.5 and 1.0 ml was pipetted out from 10 µg/ml standard solution of ALP and aliquot 1.0, 2.0, 4.0 and 5.0 ml was pipetted out from 100 µg/ml standard solution of ALP. It was transferred to individual six 10 ml amber colored volumetric flask. Aliquot 0.2 ml was pipetted out from 1000 µg/ml standard solution of PNL and added to each above flask containing different concentration of ALP. The flasks were thoroughly shaken and volume was made upto the mark with diluent to obtain mixture of solution with following concentration

Flask 1 : 0.5 µg/ml ALP, 20 µg/ml PNL
Flask 2 : 1 µg/ml ALP, 20 µg/ml PNL
Flask 3 : 10 µg/ml ALP, 20 µg/ml PNL
Flask 4 : 20 µg/ml ALP, 20 µg/ml PNL
Flask 5 : 40 µg/ml ALP, 20 µg/ml PNL
Flask 6 : 50 µg/ml ALP, 20 µg/ml PNL

- **Propranolol**

Linearity of Propranolol was taken by increasing concentration of PNL while keeping the concentration of ALP constant. For this, appropriate volume of aliquot 1.0 ml was pipetted out from 100 µg/ml standard solution of PNL and aliquot 0.5,1.0,1.5, 2.0 and 2.5 ml was pipetted out from 1000 µg/ml standard solution of PNL. It was transferred to individual six 10 ml amber colored volumetric flask. Aliquot 0.2 ml was pipetted out from 1000 µg/ml standard solution of ALP and added to each above flask containing different concentration of PNL. The flasks were thoroughly shaken and volume was made upto the mark with diluent to obtain mixture of solution with following concentration

Flask 1 : 10 µg/ml PNL, 20 µg/ml ALP
Flask 2 : 50 µg/ml PNL, 20 µg/ml ALP
Flask 3 : 100 µg/ml PNL, 20 µg/ml ALP
Flask 4 : 150 µg/ml PNL, 20 µg/ml ALP
Flask 5 : 200 µg/ml PNL, 20 µg/ml ALP
Flask 6 : 250 µg/ml PNL, 20 µg/ml ALP

Each solution of calibration curve for ALP and PNL was analysed six times as per the optimised HPLC conditions mentioned in subsection 7.1.4.

7.1.7.2 Accuracy

The accuracy of the method was determined by performing the recovery studies from previously analyzed tablet sample by standard addition method at three different levels (80,100,120 %).

The concentration of tablet sample selected for recovery studies was 100 µg/ml PNL (1.25 µg/ml of ALP).For this 1.0 ml aliquot was pipetted out from 1000 µg/ml stock solution of tablet and transferred to each 10 ml amber colored volumetric flask marked as A(for 80% recovery), B(for 100 % recovery) and C(for 120 % recovery).

Suitable aliquot of 1.0, 1.25 and 1.5 ml was pipetted out from 10 µg/ml standard solution of ALP and transferred to flask A,B and C respectively. Suitable aliquot of 0.8, 1.0 and 1.2 ml was pipetted out from 1000 µg/ml standard solution of PNL and transferred to flask A,B and C respectively. The flasks were shaken thoroughly and volume was made upto the mark with diluent to obtain mixture of solution of tablet and standard drug with following concentration.

Flask A (80 % recovery) :

Tablet sample conc : 100 µg/ml PNL (1.25 µg/ml of ALP)

Standard conc : 80 µg/ml PNL (1.0 µg/ml of ALP)

Flask B (100 % recovery) :

Tablet sample conc : 100 µg/ml PNL (1.25 µg/ml of ALP)

Standard conc : 100 µg/ml PNL (1.25 µg/ml of ALP)

Flask C (120 % recovery) :

Tablet sample conc : 100 µg/ml PNL (1.25 µg/ml of ALP)

Standard conc : 120 µg/ml PNL (1.5 µg/ml of ALP)

Each solution was analysed three times as per the optimised HPLC conditions mentioned in subsection 7.1.4.

7.1.7.3 Precision

- **Intraday and Interday Precision**

The concentration selected for intraday and interday precision was 1, 10, 50 µg/ml for ALP and 10, 100, 200 µg/ml for PNL. For this aliquot of 0.1, 1.0 and 5.0 ml was pipetted out from 100 µg/ml standard solution of ALP and aliquot of 0.1, 1.0 and 2.0 ml was pipetted out from 1000 µg/ml standard solution of PNL, transferred to individual six 10 ml amber colored volumetric flask. The volume of all the flasks was made upto the mark with diluent to obtain solution with following concentration.

Flask 1 : 1 µg/ml ALP **Flask 4** : 10 µg/ml PNL

Flask 2 : 10 µg/ml ALP
Flask 3 : 50 µg/ml ALP
Flask 5 : 100 µg/ml PNL
Flask 6 : 200 µg/ml PNL

Each solution was analysed three times in a day for intraday precision and at three different days for interday precision as per the optimised HPLC conditions mentioned in subsection 7.1.4.

- **Repeatability**

Repeatability was determined by analysing one concentration six times for both the drugs. The concentration selected was 5 µg/ml of ALP and 100 µg/ml of PNL.

For this aliquot of 0.5 ml was pipetted out from 100 µg/ml standard solution of ALP and aliquot of 1.0 ml was pipetted out from 1000 µg/ml standard solution of PNL, transferred to individual two 10 ml amber colored volumetric flask. The volume of both the flasks was made upto the mark with diluent to obtain solution with following concentration.

Flask 1 : 5 µg/ml ALP
Flask 2 : 100 µg/ml PNL

Each solution was analysed six times as per the optimised HPLC conditions mentioned in subsection 7.1.4.

7.1.7.4 Limit of Detection

The calibration curve was repeated six times and the standard deviation of intercepts was calculated.LOD was calculated using following equation.

$LOD = 3.3\ \sigma/s$

Where, σ = standard deviation of intercepts
s = slope of straight line.

7.1.7.5 Limit of Quantification

The calibration curve was repeated six times and the standard deviation of intercepts was calculated.LOD was calculated using following equation.

$LOD = 10\ \sigma/s$

Where, σ = standard deviation of intercepts

s = slope of straight line.

Chapter 8
Comparison of Developed Methods

8.1 Comparison of Developed Methods

Comparison of the developed UV Spectrophotometric, HPTLC and RP-HPLC methods was carried out by applying ANOVA test to the assay results of both drugs by developed methods.

Table 8.2 : Assay Results of ALP and PNL by developed methods

DRUG	% ASSAY RESULT BY DIFFERENT METHODS		
	UV	**HPTLC**	**RP-HPLC**
ALP	98.835	98.972	99.559
	99.424	100.017	99.278
	99.052	99.746	100.709
PNL	99.143	98.538	99.690
	98.276	100.734	101.801
	99.038	98.711	98.431

Table 8.2 : Result of ANOVA test of ALP

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	3	297.311	99.103	0.088		
Column 2	3	298.735	99.578	0.294		
Column 3	3	299.546	99.848	0.574		
ANOVA						
Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.853	2	0.426	**1.337**	0.331	**5.143**
Within Groups	1.915	6	0.319			
Total	2.768	8				

Table 8.3 : Result of ANOVA test of PNL

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	3	296.457	98.819	0.224		
Column 2	3	297.983	99.328	1.491		
Column 3	3	299.922	99.974	2.900		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.011	2	1.005	**0.654**	0.554	**5.143**
Within Groups	9.229	6	1.538			
Total	11.239	8				

8.2 CONCLUSION

The developed UV Spectrophotmetric, HPTLC and RP-HPLC methods were compared by applying ANOVA test and it was found that F calculated value was less than F critical value. Hence there was no significant difference between the developed methods and all the developed methods can be successfully applied to pharmaceutical formulation.

Chapter 9
Summary & Future Scope

9.1 SUMMARY

The simultaneous estimation of Alprazolam and Propranolol hydrochloride in their combined dosage form was performed using simple, accurate, precise and sensitive UV-Visible Spectrophotometric, HPTLC and RP-HPLC method.

In Dual wavelength UV Visible Spectrophotometric method,quantification was done by absorbance. The estimating wavelength for ALP and PNL was done at 258.2 nm and 319.4 nm respectively. The linear concentration range of ALP was 1-40 µg/ml while for PNL it was 80-200 µg/ml .The regression equation for ALP was $y=0.028x + 0.009(r^2 = 0.999)$ and for PNL it was $y=0.005x + 0.015(r^2 = 0.999)$.% Recovery was found to be 99.675-100.595 for ALP and 98.981-100.042 for PNL. LOD was found to be 0.125 µg/ml for ALP and 1.104 µg/ml for PNL while LOQ value was found to be 0.370 µg/ml for ALP and 3.345 µg/ml for PNL.% Assay was found to be 98.88 for ALP and 98.95 for PNL.

In HPTLC method, quantification of ALP and PNL was done by peak area. The separation of drugs was carried out using Chloroform : Methanol : Ammonia (7 : 0.8 : 0.1 v/v/v) as mobile phase on a 10 x 10 cm activated TLC silica gel G60 F_{254} plate in a 20 x 10 cm TLC chamber which was saturated for 30 mins. The distance run was 80 mm. The densitometric detection of the spots was done at 248 nm having slit dimension of 5 x 0.45 mm. The linear concentration range of ALP was 100-600 ng/spot while for PNL it was 5-30 µg/spot. The regression equation for ALP was $y=9.063x + 363.3(r^2 = 0.997)$ and for PNL it was $y=958.7x + 5795(r^2 = 0.996)$.% Recovery was found to be 99.084-100.589 for ALP and 98.737-99.504 for PNL. LOD was found to be 12.56 ng/spot for ALP and 0.80 µg/spot for PNL while LOQ value was found to be 38.08 ng/spot for ALP and 2.43 µg/spot for PNL.% Assay was found to be 99.183 for ALP and 99.028 for PNL.

In HPLC method, quantification was done by peak area. The separation of drugs was carried out on C_{18} Phenomenex column (150 mm length, 4.6 i.d, 5

um particle size) with Acetonitrile : 25 mM Ammonium acetate buffer pH 4 with glacial acetic acid (35 : 65) with 0.2 % Triethyl amine as mobile phase. The flow rate was 1 ml/min with runtime 12 min. Detection was carried out at 255 nm with PDA detector. The linear concentration range of ALP was 0.5-50 µg/ml while for PNL it was 10-250 µg/ml .The regression equation for ALP was y=67718x - 8420 (r^2 = 0.999) and for PNL it was y=28934x – 19358 (r^2 = 0.998).% Recovery was found to be 99.278-99.846 for ALP and 98.608-100.228 for PNL. LOD was found to be 0.126 µg/ml for ALP and 0.143 µg/ml for PNL while LOQ value was found to be 0.382 µg/ml for ALP and 0.436 µg/ml for PNL.% Assay was found to be 100.226 for ALP and 99.558 for PNL.

The developed methods were validated according to ICH guidelines. The assay results of the developed method was compared by applying ANOVA test and it was found that F calculated value is less than F critical value. Hence there is no significant difference between the developed method and all the methods can be successfully applied for the routine quality control laboratories for analysis of pharmaceutical formulation containing ALP and PNL in combined dosage form.

9.2 FUTURE SCOPE

Literature review reveals that not a single LC-MS or GC-MS analytical technique has been reported for the analysis of ALP and PNL in combination. Hence it can be endeavour to develop such methods..The developed RP-HPLC method can be extended to mass spectrometry for analysis of drug analytes in biological fluid, characterization of degradation product and impurity profiling. Spectrofluorimetric method for estimation of ALP and PNL individually has been reported but not in combination so both drugs can be estimated simultaneously by inducing fluorescence through fluorescent inducers like metal ions, complexing agents etc. Both ALP and PNL are photosensitive drugs. A single photostability studies has been reported for ALP individually but not reported for PNL. Hence photostability studies for combination can be developed..

Chapter 10
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