STUDIES ON SYNTHESIS OF BIOACTIVE COMPOUNDS AND HPLC PROFILE OF SOME PHARMACEUTICAL FORMULATION

A THESIS
SUBMITTED TO THE
SAURASHTRA UNIVERSITY
FOR THE DEGREE OF

Doctor of Philosophy

IN

THE FACULTY OF SCIENCE (CHEMISTRY)

BY

Pankaj K. Kachhadia

UNDER THE GUIDANCE

OF

Dr. H. S. Joshi

DEPARTMENT OF CHEMISTRY
(DST-FUNDED, UGC-SAP SPONSORED),
SAURASHTRA UNIVERSITY
(**** BY NAAC),
RAJKOT - 360 005.
INDIA

March-2008

Gram: UNIVERSITY
Fax: 0281-2577633

Phone: (R) 0281-2584221
(O) 0281-2578512

SAURASHTRA UNIVERSITY

University Road
Rajkot - 360 005

Dr. H. S. Joshi
M.Sc., Ph.D., F.I.C.S.
Associate Professor,
Department of Chemistry

Residence:
B-1, Amidhara Appartment,
2- Jalaram Plot,
University Road,
Rajkot - 360 005
GUJARAT (INDIA)

No.

Date: 11-03-2008

Statement under o. Ph. D. 7 of Saurashtra University

The work included in the thesis is my own work under the supervision of ***Dr. H. S. Joshi*** and leads to some contribution in chemistry subsidized by a number of references.

Date: 11-03-2008
Place: Rajkot

(Pankaj K. Kachhadia)

This is to certify that the present work submitted for the Ph.D. Degree of Saurashtra University by ***Pankaj K. Kachhadia*** his own work and leads to advancement in the knowledge of chemistry. The thesis has been prepared under my supervision.

Date: 11-03-2008
Place : Rajkot

Dr. H. S. Joshi
Associate Professor
Department of Chemistry
Saurashtra University
Rajkot-360 005

Dedicated
to my
beloved Parents

ACKNOWLEDGEMENTS

First and foremost I pay all my homage and devote my emotions to "My Parents" without whose blessing this task would not have been accomplished. I bow my head in utter humility and complete dedication from within my heart. Hats off to the Omnipresent, Omniscient and Almighty God, The glorious fountain and continuous source of inspirations! I offer salutation to the Omnipotent Lord "Krishna".

I would like to express my sincere gratitude to my supervisor Associate Professor Dr. H. S. Joshi for accepting me as his student and who made this research a success. It is with Dr. Joshi's enthusiasm and integral view on research combined with his willingness to provide quality chemistry and not less that kept me going and I wish to say thank you for showing me this way of research. Besides being a wonderful Supervisor, Dr. Joshi is as close as family and a very good friend and I am deeply honored to have known him in my life. I wish to say thank you so much again Dr. Joshi for all the help you offered over the years both in and out of my academic life. It is with no doubt that without your help I would not be where I am now.

I also owe to, from the deepest corner of heart, deepest sense of gratitude and indebtedness to Professor P. H. Parshania, Head and also Professor Anamik Shah, Department of Chemistry, as I have been constantly benefited with them lofty research methodology and the motivation as well as them highly punctual, affectionate. I will never forget Dr. A. R. Parikh, Dr. N. A Chauhan and Dr.(Mrs.) H. H. Parekh ex-professors of Department, for their constant inspiration with keen interest and ever vigilant guidance without which this task could not have been achieved.

An endeavor such as a Ph.D. is impossible to accomplish without the generous help and support of my family, friends and colleagues. I would like to take this opportunity to thank those whom I was fortunate to know, work and form friendship with over the past three years.

Who in this world can entirely and adequately thank the parents who have given me everything that I possess in my life? I bow my head with utter respect to my beloved mother Smt. Kusumben for her continuous source of inspiration, motivation and devotion to me, and my father Shri Kantibhai for the uncompromising principles that guided my life. Also I can never forget my younger brother Hitesh and my younger sister Snehal, I assure them to be worthy of whatever they have done for me.

How could I ever forget to my instructor Mr. Ashish Doshi, for constant encouragement and providing his valuable guidance through out of my research work.

I would like to reserve a special thank for Jignesh Akbari for his most willing co-operation and comprehensive exchange of ideas during the course of my research work.

As with the completion of this task, I find myself in difficult position on attempting to express my deep indebtedness to Satish Tada and Manoj Dhaduk.

Thank to my best friends in the Dr. H. S. Joshi research group like Hiteshbhai, Ram, Kaushik, Dr. Virendra, Dr. Dipen, Dr. Paresh, Dr. Praful, Dr. Dinesh, Dr. Dushyant, Dr. Sunil, Dr. Mayur, Dr. Mahesh, and my research colleagues Atul, Rupesh, Thanki, Sekhada, Meera, Nainesh, Jagdish, Sukla, Arti, Vishal, Nimisha, Mathukiya, Janak, Nikunj, Ashif, Vrajesh, , Nikhil, Dr. Madi, Jitendra, Samir, Viren, Vashu, for their help and friendship which lighten my day and did not make me feel alone in my research work.

I cherish the affection and help rendered by my best friends like Atul, Hetal and Jagdish who ever stood beside me with their helping hands and moral support.

Big thanks to the staff at the Department of Chemistry and also to Mr. Harshadbhai Joshi and Mrs. Namrataben for their kind support and providing chemicals and glass wares on time.

I also remember well wishers and all those persons who helped me directly or indirectly during my Ph.D.

I gratefully acknowledge the most willing help and co-operation shown by SAIF, CIL, Chandigarh for spectral studies. Finally, I express my grateful acknowledgment to Department of Chemistry, Saurashtra University for providing me the excellent laboratory facilities, and kind furtherance for accomplishing this work.

Pankaj Kachhadia

CONTENTS

P A R T - [B]

STUDIES ON SYNTHESIS OF BIOACTIVE COMPOUNDS

SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL SCREENING OF 2-THIOXOTETRAHYDROPYRIMIDINES AND THEIR DERIVATIVES

Section-I

Synthesis, characterization and antimicrobial screening of 2-thioxotetrahydropyrimidines

Section-II:

Synthesis, characterization and antimicrobial screening of thiazolo[3,2-*a*]pyrimidines

Synopsis

A comprehensive summary of the work to be incorporated in the thesis entitled "**Studies on synthesis of bioactive compounds and HPLC profile of some pharmaceutical formulation**" has been describe as under.

PART-[A]: HPLC METHOD DEVELOPMENT AND VALIDATION OF SOME PHARMACEUTICAL FORMULATION.

PART-[B]: STUDIES ON SYNTHESIS OF BIOACTIVE COMPOUNDS.

PART-[A]: HPLC METHOD DEVELOPMENT AND VALIDATION OF SOME PHARMACEUTICAL FORMULATION.

The research work undertaken in these studies mainly addresses analysis and validation protocol, development of stability indicating HPLC methods according to ICH guidelines. Our strategy for active pharmaceutical ingredient and formulation.

Aim of work

To develop stability indicating high performance liquid chromatographic methods for the estimation of some active pharmaceutical ingredient from their single/combine pharmaceutical dosage forms by HPLC and to perform the validation procedure for same

Experimental Work

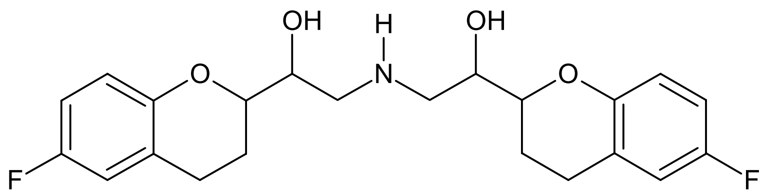
- **Development of analytical method**
  - Selection of mobile phase
  - Selection of stationary phase
  - Choice of flow-mode and flow rate
  - Selection of wave-length
  - Injection volume / Concentration
  - Selection of diluent

- **Validation of analytical method**
  - Specificity study
  - Linearity and range study
  - Precision and Intermediate precision study
  - Accuracy study
  - Robustness study
  - Solution stability study
  - System suitability
  - Limit of detection and limit of quantification

We have undertaken the work on **nebivolol**, **aspirin and clopidogrel** and **aceclofenac and tramadol hydrochloride** which are described as under

Section-I : HPLC Method Development and Validation of Nebivolol

Nebivolol is an antihypertensive compound. It is chemically 1-(6-fluorochroman-2-yl)-2-[(2-(6- fluorochroman-2-yl)-2-hydroxy-ethyl] amino] ethanol (Figure 1). Its molecular formula is $C_{22}H_{25}F_{2}NO_{4}$ having molecular weight 405.435 g/mole. It is most selective $â_{1}$ receptor antagonist currently available for clinical use.



(Figure 1)

Developed Chromatographic parameters are as under

Mobile phase: Buffer-ACN (65: 35, v/v)
Buffer: 50 mM phosphate buffer with 2 ml triethylamine, pH 3.5 with H_3PO_4

Column: Phenomenex, C8 (250 mm x 4.6 mm i.d., 5µ particle size)

Flow rate: 1 ml/min

Wavelength: 280 nm

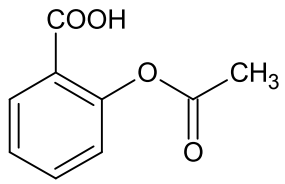
Injection volume: 20 µl

Diluent: Mobile phase

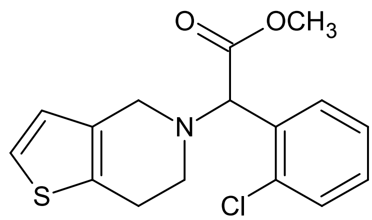
The developed procedure has been evaluated over the specificity, linearity, accuracy, precision, limit of detection, limit of quantification and robustness in order to ascertain the stability of the analytical method. It has been proved that it was specific, linear, precise, accurate and robust and stability indicating. Hence, the method is recommended for routine quality control analysis and also for stability of sample analysis.

Section-II: HPLC Method Development and Validation of Combine Dosage form of Aspirin and Clopidogrel

Aspirin (Acetylsalicylic acid) (Figure 2) is a non-steroidal anti-inflammatory drug that exhibits anti-inflammatory, analgesic and antipyretic activities. Clopidogrel [*S*-(*a*)(2-chlorophenyl)-6,7-dihydrothieno (3,2-C) pyridine-5 (4*H*) acetic acid methyl ester sulphate] (Figure 3) is a platelet aggregation inhibitor and act as a an anticoagulant.



(Figure 2)



(Figure 3)

Developed Chromatographic parameters are as under

Mobile phase: Buffer-ACN(65: 35, v/v)

Buffer: 0.3% orthophosphoric acid

Column: Phenomenex, C8 (250 mm x 4.6 mm i.d., 5µ particle size)

Flow rate: 1 ml/min

Wavelength: 226 nm

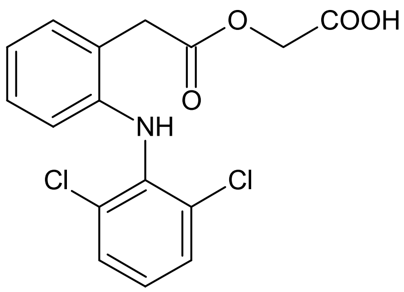
Injection volume: 20 µl

Diluent: Mobile phase

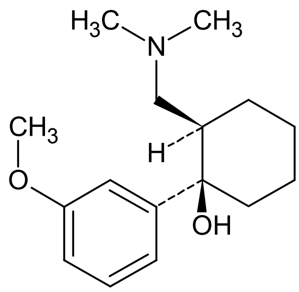
The developed procedure has been evaluated over the specificity, linearity, accuracy, precision, limit of detection, limit of quantification and robustness in order to ascertain the stability of the analytical method. It has been proved that it was specific, linear, precise, accurate and robust and stability indicating. Hence, the method is suitable for routine quality control analysis and also for stability sample analysis.

Section-III: HPLC Method Development and Validation of Combine Dosage form of Tramadol hydrochloride and Aceclofenac

Tramadol hydrochloride and aceclofenac are available in combined tablet dosage form. Aceclofenac (Figure 4) is 2-[(2,6-dichlorophenyl)amino] benzene acetic acid carboxy methyl ester has analgesic and anti – inflammatory activity. Tramadol (Figure 5) is (1*R*,2*R*)-rel-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol has analgesic activity.



(Figure 4)



(Figure 5)

Developed Chromatographic parameters are as under

Mobile phase:	Buffer-ACN (65: 35, v/v)
	Buffer: 0.01M ammonium acetate buffer with 2 ml triethylamine,
	pH 6.5 with glacial Acetic acid
Column:	Phenomenex, C18 (250 mm x 4.6 mm i.d., 5μ particle size)
Flow rate:	1 ml/min
Wavelength:	270 nm
Injection volume:	20 μl
Diluent:	Water: ACN (50: 50, v/v)

The developed procedure has been evaluated over the specificity, linearity, accuracy, precision, limit of detection, limit of quantification and robustness in order to ascertain the stability of the analytical method. It has been proved that it was specific, linear, precise, accurate and robust and stability indicating. Hence, the method is very useful for routine quality control analysis and also for stability sample analysis.

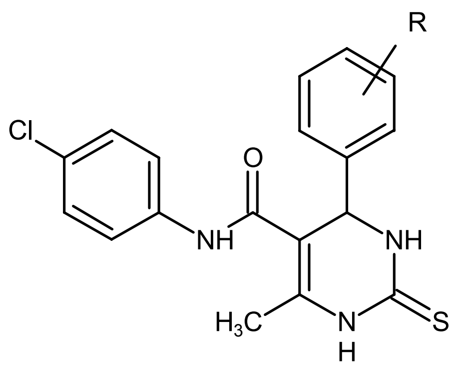
PART-[B]: STUDIES ON SYNTHESIS OF BIOACTIVE COMPOUNDS

Tetrahydropyrimidine and their derivatives represent one of the most active classes of calcium channel blockers and it also possessing wide spectrum of biological activities. In order to develop better medicinally important compounds, it was considered of interest to synthesize some new dihydropyrimidinonthione and their derivatives as under.

SECTION-I: Synthesis, characterization and antimicrobial screening of *N*-(4-chlorophenyl)-6-methyl-4-aryl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamides.

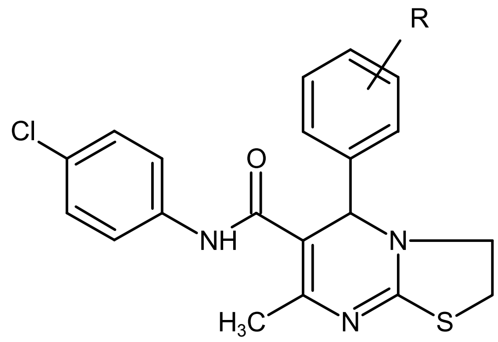
This section covers literature survey, mechanistic studies, and different alternative routes for the synthesis of parent compounds. DHPMs and their derivatives have attracted interest in medicinal chemistry, exhibiting pharmacological and therapeutic properties such as antiinflammatory, antiviral, antibacterial, antitumor, calcium channel antagonist antihypertensive agent and anticancer. Recently DHPMs have been implicated in the catabolism of pyrimidine base. Various catalyst for mild, rapid high yielding protocol, several ionic liquids mediated synthesis, solid phase synthesis, microwave and ultrasound assisted synthesis are described in detail in the introductory part of this section.

N-(4-chlorophenyl)-3-oxobutanamide react with thiourea and different aromatic aldehydes to give tetrahydropyrimidines. General structure of synthesized compounds is represented as under.



SECTION-II: Synthesis, characterization and antimicrobial screening of *N*- (4-chlorophenyl)-7-methyl-5-aryl-2,3-dihydro-5*H*-thiazolol [3,2-*a*]pyrimidine-6-carboxamides.

In this section we have carried out the synthesis of *N*-(4-chlorophenyl)-7-methyl-5-aryl-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamides by the reaction of *N*-(4-chlorophenyl)-6-methyl-4-aryl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamides with 1,2-dibromoethane using DMF as a solvent.



All the compounds are well characterized by ^{1}H NMR, FT-IR and mass spectral techniques. Selected compounds have been evaluated for their *in vitro* antimicrobial activity towards gram positive, gram negative bacterial strains and fungi.

Part-[A]

HPLC Method Development and Validation of some Pharmaceutical Formulation

INTRODUCTION

1. History and Instrumentation

The year 2003 was the year of centenary of chromatography .In fact it was LC-liquid chromatography/column chromatography from used by Tswett in 1903 and coined a name of CHROMATOGRAPHY to this new technique. In column liquid chromatography during last four decades, however, there has been an explosive spurt in activity to revitalize this apparently passive technique. The tremendous advancements in the researches in biochemistry, diagnostic medicine and pharmaceutical materials were mainly responsible for triggering the explosive growth of LC, as many of the chemical substances falling under these heads were nonvolatile, so that Gas Chromatography (GC) could not be exploited to study them. It is worth mentioning here that, in spite of the extraordinary success and wide applicability of GC, only about 15% of organic materials are amenable to direct GC analysis. Among the remaining 85% can be included most kinds of plastics and resins, and many pharmaceuticals, pollutants, biochemical materials, dyes, pigments, etc. Insufficient volatility and thermal instability of such compounds are mainly responsible for this unfortunate limitation imposed on GC.

On the contrary, LC does not suffer from the limitation of GC mentioned above. It is ideally suited for the separation of nonvolatile or unstable materials. The clear aim, therefore, in the surge of interest and activity in LC was to make it a complementary technique to GC. Naturally, a prerequisite for success in this line turned out to be the achievement for LC, many of the commendable and accepted features of GC, such as high resolving power, fast analysis, continuous monitoring of column effluent, ease and simplicity of operation, precise identification based on accurate measurement of retention parameters, accurate quantitative measurement, repetitive analysis with the same column and, finally, automation of the complete analysis and data handling operation.

During the last four decades, very fast development has been going on in what is now being described as **High Performance Liquid Chromatography** (HPLC) in columns. With the availability of pumps capable of producing pressures of a few thousand psi, a typical HPLC analysis takes only a few minutes, compared to several hours required by its “classical” form to achieve a similar result. This situation is essentially the culmination of the efforts put forward by instrument manufacturers. As a result, HPL chromatographs have taken their rightful place beside the gas chromatographs in almost every type of analytical laboratory.

HPLC today is the product of quarter century of refinement, driven by technical advances and economic competition in a USD 2 Billon plus equipment market. Recently, manufactures have improved HPLC'S performance easier. The proteomics researchers need high resolution and small sample capabilities and the pharmaceutical industries demand for high throughput screening for drug discovery. The HPLC instrumentation took on the basis of these demands.

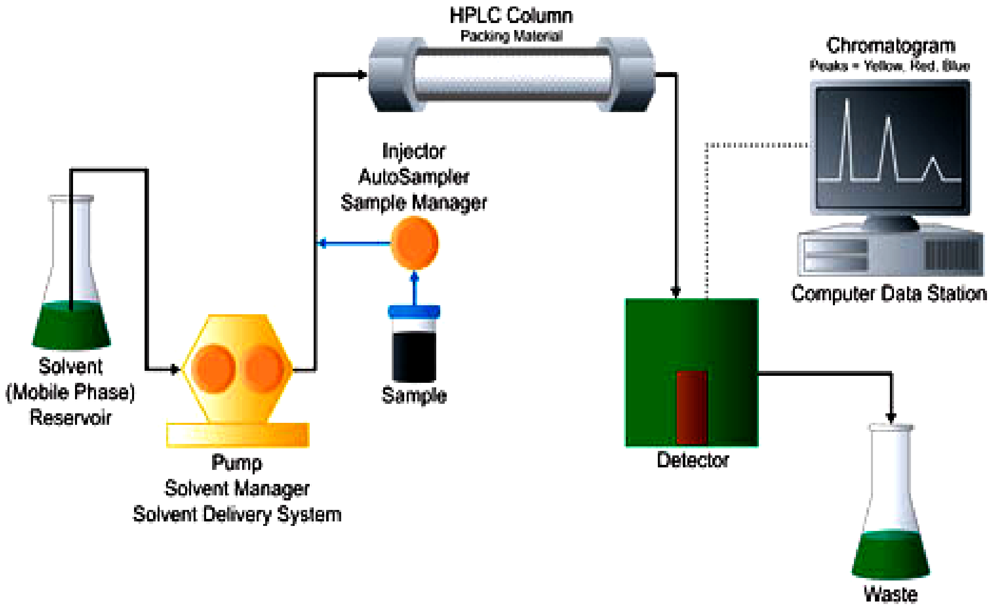
To increase the throughput, the systems with multi columns and more efficient stationary phases are being developed. It appears that the advances in column technology have significantly overtaken the advances in instrumental and hardware aspects of HPLC.

The temperature programming of columns in HPLC is a new trend emerging. The temperature programming adds a third dimension (in addition to mobile phase and stationary phase) to HPLC. This increases the speed, selectivity and efficiency of HPLC.

Lowering limits of detection and increasing method sensitivity have the goals of analysts since the advent of liquid chromatography. It was soon recognized that using smaller bore columns would improve sensitivity, but a lack of affordable and suitable HPLC instrumentation meant that micro HPLC was the province of research departments. Only recently has micro HPLC become popular, due mainly to the increasing popularity of LCMS and to the improvements in instrument technology. This demand was largely driven by chromatographers working with small complex biological samples. It requires highly sensitive separation techniques.

The choice of multi detectors and diode array and mass spectrometers to analyze the stream from the chromatography column means the results with more data per sample and hence data handling and interpretation tools are required.

A schematic diagram of HPLC equipment is given in Figure 1 [1].



Figer-1 Schematic diagram of HPLC equipment

The basic components of HPLC are: [2-6]

(i) Pumping System

(ii) Sample Introduction Device

(iii) Chromatographic Column

(iv) Detector

(v) Data handling Device

(i) Pumping System

The HPLC pump is very important component of the system. It delivers the constant flow of the mobile phase or phases so that the separation of the components of the mixture occur in a reasonable time. Its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system are as under

a. ***Displacement pump:*** It produces a flow that tends to independent of viscosity and backpressure and also output is pulse free. But it possesses limited capacity (250 ml).

b. ***Reciprocating pump:*** It has small internal volume (35 to 400 μl). It has high output pressure (up to 10,000 psi) and constant flow rates. But it produces a pulsed flow.

c. ***Pneumatic or constant pressure pump:*** They are pulse free, suffer from limited capacity as well as a dependence of flow rate on solvent viscosity and column backpressure. They are limited to pressure less than 2000 psi.

There are two type of elution process, i.e. *isocratic* and *gradient*,

Isocratic: In this system, the things are kept constant throughout the run. In the case of pumping of mobile phase, the mobile phase composition is kept constant throughout the run. The nominal flow rate accuracy required is ±1% of the set flow

Gradient: There is some change purposely incorporated during the particular sample run to achieve a better or/and faster separation. In case of pumping mobile phase, the composition of mobile phase is continuously varied during the particular run. The gradient accuracy of ±1% of the step gradient composition is typical.

(ii) Sample Introducing Device

It is not possible to use direct syringe injection on column like GC, as the inlet pressure in LC is too high. Insertion of the sample onto the pressurized column must be as a narrow plug so that the peak broadening attributable to this step is negligible. The injection system itself should have no dead (void) volume. There are three important ways of introducing the sample into injection port.

a. ***Loop injection:*** In which, a fixed amount of volume is introduced by making use of fixed volume loop injector.

b. ***Valve injection:*** In which, a variable volume is introduced by making use of an injection valve.

c. ***On column injection:*** In which, a variable volume is introduced by means of a syringe through a septum.

(iii) Chromatographic Column

Column is a heart of chromatography. The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 μm or less. Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

Column packing:

The packing used in modern HPLC consists of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

a. ***Porous, polymeric beds:*** Porous, polymeric beds based on styrene divinyl benzene co-polymers used for ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

b. ***Porous layer beds:*** Consisting of a thin shell (1-3 μm) of silica or modified silica on an spherical inert core (e.g. Glass). After the development of totally porous micro particulate packings, these have not been used in HPLC.

c. ***Totally Porous silica particles (dia. < 10 μm):*** These packing have widely been used for analytical HPLC in recent years. Particles of diameter > 20 μm are usually dry packed. While particles of diameter < 20 μm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.

(iv) Detector

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. There are several detectors available in the market. However UV-Visible detector, photo diode array detector, fluorescence detector, conductometric and coulometric detector are more commonly used. The new ELSD detector is proving to be important detector, while the MS detector is outstanding. Detectors are usually of two types:

a. ***Bulk property detectors:*** It compares overall changes in a physical property of the mobile phase with and without an eluting solute e.g. refractive index, dielectric constant or density.

b. ***Solute property detectors:*** It responds to a physical property of the solute, which is not exhibited by the pure mobile phase e.g. UV absorbance, fluorescence or diffusion current.

2. Drug Analysis

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one [7]. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standard samples and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Also quality is important in every product or service but it is vital in medicines as it involves in human life. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stage of production. The decision to release or reject a product is based on one or more type of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation. Providing simple analytical procedure for complex formulation is a matter of most importance.

In brief, the reasons for the development of newer methods of drug analysis are:

- The drug or drug combination may not be official in any pharmacopoeias.
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for the quantitation of the drug in biological fluids may not be available.
- Analytical methods for a drug in combination with other drugs may not be available.
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

(I) ***Introduction to HPLC Methods of Analysis for Drugs [7-9]***

Most of the drugs in single/multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are:

- Speed (analysis can be accomplished in 20 minutes or less).
- Greater sensitivity (various detectors can be employed).
- Improved resolution (wide variety of stationary phases).
- Reusable columns (expensive columns but can be used for many analysis).
- Ideal for the substances of low volatility.
- Easy sample recovery, handling and maintenance.
- Instrumentation tends itself to automation and quantitation (less time and less labour).
- Precise and reproducible.
- Calculations are done by integrator itself.
- Suitable for preparative liquid chromatography on a much larger scale.

There are different modes of separation in HPLC. They are normal phase mode, reversed phase mode, reverse phase ion pair chromatography, affinity chromatography and size exclusion chromatography (gel permeation and gel filtration chromatography).

In the normal phase mode, the stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence takes longer time to elute.

Reversed phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are Octa Decyl Silane (ODS) or C18, C8, C4, etc. (in the order of increasing polarity of the stationary phase).

In ion exchange chromatography, the stationary phase contains ionic groups like NR_3^+ or SO_3^{--}, which interact with the ionic groups of the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and salt concentration can modulate the retention.

Ion pair chromatography may be used for the separation of ionic compounds and this method can also substitute for ion exchange chromatography. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (coulumbic association species formed between two ions of opposite electric charge) with suitable counter ions. This technique is referred to as reversed phase ion pair chromatography or soap chromatography.

Affinity chromatography uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can adsorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

Size exclusion chromatography separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

(II) *Method Development and Design of Separation Method*

Methods for analyzing drugs in single or multi component dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water-soluble.

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 5-10% organic phase in the mobile phase and the organic phase concentration (methanol or acetonitrile) can be increased up to 100% within 30-45 min. Separation can then be optimized by changing the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, namely at what mobile phase composition.

Changing the polarity of mobile phase can alter elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic samples (acidic or basic) can be separated, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by the proper selection of pH.

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase of the organic phase concentration is needed.

In UV detection, good analytical results are obtained only when the wavelength is selected carefully. This requires knowledge of the UV spectra of the individual components present in the sample. If analyte standards are available, their UV spectra can be measured prior to HPLC method development.

The molar absorbance at the detection wavelength is also an important parameter. When peaks are not detected in the chromatograms, it is possible that the sample quantity is not enough for the detection. An injection of volume of 20 µl from a solution of 1 mg/ml concentration normally provides good signals for UV active compounds around 220 nm. Even if the compounds exhibit higher λ_{max}, they absorb strongly at lower wavelength. It is not always necessary to detect compounds at their maximum absorbance. It is, however, advantageous to avoid the detection at the sloppy part of the UV spectrum for precise quantitation. When acceptable peaks are detected on the chromatogram, the investigation of the peak shapes can help further method development.

The addition of peak modifiers to the mobile phase can affect the separation of ionic samples. For examples, the retention of the basic compounds can be influenced by the addition of small amounts of triethylamine (a peak modifier) to the mobile phase. Similarly for acidic compounds small amounts of acids such as acetic acid can be used. This can lead to useful changes in selectivity.

When tailing or fronting is observed, it means that the mobile phase is not totally compatible with the solutes. In most case the pH is not properly selected and hence partial dissociation or protonation takes place. When the peak shape does not improve by lower (1-2) or higher (8-9) pH, then ion-pair chromatography can be used. For acidic compounds, cationic ion pair molecules at higher pH and for basic compounds, anionic ion-pair molecules at lower pH can be used. For amphoteric solutes or a mixture of acidic and basic compounds, ion-pair chromatography is the method of choice.

The low solubility of the sample in the mobile phase can also cause bad peak shapes. It is always advisable to use the same solvents for the preparation of sample solution as the mobile phase to avoid precipitation of the compounds in the column or injector.

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that more or less symmetrical peaks on the chromatogram detect all the compounds. By sight change of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

The peak resolution can be increased by using a more efficient column (column with higher theoretical plate number, N) which can be achieved by using a column of smaller particle size, or a longer column. These factors, however, will increase the analysis time. Flow rate does not influence resolution, but it has a strong effect on the analysis time.

Unfortunately, theoretical predictions of mobile phase and stationary phase interactions with a given set of sample components are not always accurate, but they do help to narrow down the choices for method development. The separation scientist must usually perform a series of trial and error experiments with different mobile phase compositions until a satisfactory separation is achieved.

The parameters that are affected by the changes in chromatographic conditions are:

A. Resolution (R_S).

B. Capacity factor (k').

C. Selectivity (α).

D. Column efficiency (N).

E. Peak asymmetry factor (A_S).

A. Resolution (R_S): Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture. The resolution (R_S), of two neighboring peaks were defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_S is 1.5 It is calculated by using the formula,

$$R_S = Rt_2 - Rt_1 / 0.5 (W_1 + W_2)$$

Where, Rt_1 and Rt_2 are the retention times of components 1 and 2.

W_1 and W_2 are peak width of components 1 and 2.

B. Capacity factor (k'): Capacity factor is the ratio of the reduced retention volume to the dead volume. Capacity factor (k'), is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column during an isocratic separation. The ideal value of k' ranges from 2-10. Capacity factor can be determined by using the formula,

$$k' = V_1 - V_0 / V_0$$

Where, V_1 = retention volume at the apex of the peak (solute) and

V_0 = void volume of the system.

The values of k' of individual bands increase or decrease with changes in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in water/ organic mobile phase. Typically an increase in percentage of the organic phase by 10% by volume will decrease k' of the bands by a factor of 2-3.

C. *Selectivity (α):* The selectivity, (or separation factor) is a measure of relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times. Selectivity represents the separation power of particular adsorbent to the mixture of these particular components.

This parameter is independent of the column efficiency, it only depends on the nature of the components, eluent type, eluent composition and adsorbent surface chemistry. In general, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency.

The ideal value of α is 2. It can be calculated by using formula,

$$\alpha = V_2 - V_1 / V_1 - V_0 \quad = k_1'/ k_2'$$

Where, V_0 = the void volume of the column,

V_1 and V_2 = the retention volumes of the second and the first peak respectively.

D. *Column efficiency (N):* Efficiency, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Similar the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16\ Rt^2 / W^2$$

Where, Rt is the retention time.

W is the peak width.

E. Peak asymmetry factor (A_S): Peak asymmetry factor, can be used as a criterion of column performance. The peak half width, b, of a peak at 10 % of the peak height, divided by the corresponding front half width, a, gives the asymmetry factor.

$$A_S = b / a$$

For a well-packed column, an asymmetry factor of 0.9 to 1.1 should be achievable.

(III) ***Validation of Analytical Method***

HPLC method validation is the process used to confirm that the HPLC procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of HPLC results, and it is an integral part of any good analytical practice.

Method validation has received considerable attention in literature and from industrial committees and regulatory agencies. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use [10] has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics. ICH has also developed appendices with more detailed methodology [11].

The United States Food and Drug Administration (US FDA) has proposed guidelines on submitting samples and analytical data for methods validation [12,13]. The United States Pharmacopoeia (USP) has published specific guidelines for method validation for compound evaluation [14].

The US FDA has added section 211.222 on 'methods validation' to cGMP(Current Good Manufacturing Practices)regulations [15]. This requires the manufacturer to establish and document the accuracy, sensitivity, specificity, reproducibility and any other attribute necessary to validate test methods. The validation is also required to meet the existing requirements for laboratory records provided at Sec. 211.194 (a). These requirements include a statement of each method used in testing the sample to meet proper standards of accuracy and reliability, as applied to the tested product.

Representatives of the pharmaceutical and chemical industry have published papers on the validation of analytical methods. Hokanson [16,17] applied the life cycle approach, developed for computerized systems, to the validation and revalidation of methods. Green [18] gave a practical guide for analytical method validation, with a description of a set of minimum requirements for a method. Renger and his colleagues [19] described the validation of a specific analytical procedure, for the analysis of theophylline in a tablet using High Performance Thin Layer Chromatography (HPTLC). The validation procedure in this particular article is based on requirements for European Union multi-state registration. Winslow and Meyer [20] recommend the definition of a master plan for validating analytical methods.

A. *Scope of the method and validation parameters*

The scope of the method and its validation parameters and acceptance criteria should be defined early in the process. These includes:

- What analytes should be detected?
- What are the expected concentration levels?
- What are the sample matrices?
- Are there interfering substances expected and, if so, should they be detected and quantified?
- Are there any specific legislative or regulatory requirements?
- Should information be qualitative or quantitative?
- What are the required detection and quantitation limits?
- What is the expected concentration range?
- What precision and accuracy is expected?
- How robust should the method be? For example, should the method work at a specific room temperature or should it run independent from room temperatures?
- Which type of HPLC should be used, is the method for one specific model from a specific vendor or should it be used by all models from all vendors. This is especially important for HPLC gradient methods, because different instrument may have different delay volumes ranging from 0.5 up to 8 ml. This can have a tremendeous impact the separation and elution order of the compounds.
- Will the method be used in one specific laboratory or should it be applicable in all laboratories in your organization?
- What skills should the anticipated users of the HPLC method have?

The method's performance characteristics and acceptance criteria should be based on the intended use of the method. It is not always necessary to validate all parameters that are available for HPLC. For example, if the method is to be used for qualitative trace level analysis, there is no need to test and validate the method's limit of quantitation, or the linearity, over the full dynamic range of the equipment. Initial parameters should be chosen according to the chromatographer's experience and best judgment. Final parameters should be agreed between the lab or analytical chemist performing the validation and the lab or individual applying the method.

Before an HPLC is used to validate a method, its performance specifications should be verified using generic chemical standards. Satisfactory results for a method can only be obtained with HPLC equipment that is performing well. Special attention should be paid to those equipment characteristics that are critical for the method. For example, if detection limit is critical for a specific method, the instrument's specification for baseline noise and, for certain detectors, also the response to specified compounds, should be verified.

Any chemicals used to determine critical validation parameters, such as reagents and reference standards, should be

- Available in sufficient quantities
- Accurately identified
- Sufficiently stable and
- Checked for exact composition and purity.

Any other materials and consumables, for example HPLC columns, should be new. This ensures that one set of consumables can be used for most experiments and avoids unpleasant surprises during method validation.

If there is little or no information on the method's performance characteristics, it is recommended to prove the suitability of the method for its intended use in initial experiments. These studies should include the approximate precision, working range and detection limits. If the preliminary validation data appear to be inappropriate, the method itself, the HPLC equipment or the acceptance limits should be changed. HPLC method development and validation is therefore an iterative process. For example, selectivity is achieved through selection of mobile phase composition. For quantitative measurements, the resolution factor between two peaks should be 2.5 or higher. If this value is not achieved, the mobile phase composition needs further optimization.

B. Sequence of validation experiments [21-24]

There are no official guidelines on the correct sequence of validation experiments and the optimal sequence may depend on the method itself. Based on our experience, for a liquid chromatographic method, the various validation parameters are as under:

B*i*. Specificity

B*ii*. Linearity

B*iii*. Precision

a) Repeatability

b) Intermediate Precision

c) Reproducibility

B*iv*. Accuracy

B*v*. Solution Stability

B*vi*. Limit of detection (LOD)

B*vii*. Limit of quantitation (LOQ)

B*viii*. Robustness

All the work covered under current research topic have method development for some pharmaceutical formulation including validation of the developed method .A common method validation protocol was followed for all the method developed during the research work (FDA, ICH Q_2A & Q_2B, 2005).

Bi. Specificity

The evaluation of the specificity of the method was determined against placebo. The interference of the excipients of the claimed placebo present in pharmaceutical dosage form was derived from placebo solution. Further the specificity of the method toward the drug was established by means of checking the interference of the degradation products in the drug quantification for assay during the forced degradation study. The peak purity of analyte peak was evaluated in each degraded sample with respect to total peak purity and three point peak purity. The peak purity value must be more than 0.9999 in every case.

Force degradation study

These study were undertaken to elucidate inherent stability characteristics. Such testing is part of the development strategy and is normally carried out under more sever condition than those used for accelerated stability studies. Force degradation of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved.

Stress testing is likely to be carried out on a single batch of the drug substance. It should include the effect of temperatures (in 10°C increments (e.g., 50°C, 60°C, etc.) above that for accelerated testing), humidity where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values. Photo stability testing should be an integral part of stress testing. Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical procedures.

So, as per the guidelines the tress studies for all the drug under investigation were done in the same conditions, the only difference were in temperature and the time required for each drug to degrade up to 10-15% level. In general, the drug were kept at solution and solid state stability in the following manner,

Solution state stability:

- Acidic hydrolysis: - Drug solution in 1-5N HCl solution
- Alkaline hydrolysis: - Drug solution in 1-5N NaOH solution
- Oxidative degradation: - Drug solution in 3-5% H_2O_2 (aqueous)

Solid state stability

- Thermal degradation: - Solid drug were exposed at 80 ^{0}C for 72 h.
- Photolytic degradation: - Solid drug were exposed in UV-light for 72 h.

All the above mentioned sample were used for method development trials to develop a stability indicating assay method and also used to evaluated the specificity of the method.

Bii. 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.

A linear relationship should be evaluated across the range of the analytical procedure. It was demonstrated directly on the drug substance by dilution of a standard stock solution of the drug product components, using the proposed procedure. For the establishment of linearity, minimum of five concentrations are recommended by ICH guideline. Here, linearity test solutions for the assay method were prepared at seven concentration levels from 40, 60, 80, 100, 120, 140 and 160% of assay analyte concentration. The peak areas versus concentration data were evaluated by linear regression analysis. Intercept and correlation coefficient (r^2) was evaluated. The Value of r^2 should fall around 0.9999.

Biii. 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. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

a) Repeatability: Repeatability study was performed by preparing a minimum of 6 determinations at 100% of the test concentration and analyzed as per the respective methodology. The assay values were evaluated for %RSD, which should not more than 2%.

b) *Intermediate Precision:* The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. Here, Intermediate precision of the method was checked by carrying out six independent assays of test sample preparation on the different day by another person under the same experimental condition and calculated the %RSD of assays. It should not be more than 2%.

c) *Reproducibility:* Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier.

Biv. 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. The evaluation of accuracy has got very prime importance as it deliberately force the method to extract the drug in higher and lower level. Here the known amount of drug(s) was/were spiked with identical amount of placebo preparation. The drug spiking was done at three different concentration levels, that is 50, 100 and 150% of assay concentration level. The spike placebos were then treated as per sample procedure and assay of the synthetic mixture was performed in triplicate. The mean recovery at each concentration level was evaluated and it should be lie between the range of 98-102%. The % RSD of % recovery at each level should not be more than 2.0.

Bv. Solution Stability

The stability of solution for test preparation was evaluated. The solution was stored at ambient temperature and 2-8 ^{0}C and tested at interval of 12 h, 24 h, 36 h and 48 h. The assays for the aged solution were evaluated using a freshly prepared standard solution. The assay value of initial time point was compared with the assay of the aged solution. The difference between assays should not be more than 2% from the initial value for formulations. Overall %RSD of peak area of standard preparation injected at initial at stage and injected after different time intervals should not be more than 2.0.

Bvi. Limit of detection (LOD)

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. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit. The limit of detection was evaluated by serial dilutions of analyte stock solution in order to obtain signal to noise ratios of 3:1.

Bvii. Limit of quantitation (LOQ)

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. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1. The limit of quantification was evaluated by serial dilutions of analyte stock solution in order to obtain signal to noise ratios of 10:1.

Bviii. 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.

In the case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in a mobile phase;
- Influence of variations in mobile phase composition;
- Different columns (different lots and/or suppliers);
- Temperature;
- Flow rate.

The factors chosen for all the drugs under investigation were the flow rate, mobile phase composition, pH of a mobile phase and using different lot of LC column. The observation shall be summarized and critical parameters shall be listed out in the validation report. System suitability parameter must be within the limit of acceptance criteria as mentioned in the method.

C. Advantages of Analytical method Validation

- The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user.
- Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end.
- Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

3. Current Trend

The Reverse Phase High Performance Liquid Chromatography (RP-HPLC) analysis has stolen the show from all other techniques. It finds more acceptability with regulatory authorities. The RP-HPLC has got very large application in current pharmaceuticals growth towards the generic market due to its versatility for the determination of purity of the compound and its related impurities (degradation of products) as well. RP-HPLC is versatile for the detectors also as various types of detectors can be attached as per the chemical nature of the components. Various detectors like UV-Visible, Photo Diode Array (PDA), RI, Fluoresce, ELSD and MS detectors are applied to determine the assay and related impurities formed during the stressed and routine stability study.

The development and sales of combined dosage form in Indian pharmaceutical market is promising. The methods suggested and published for the combined dosage forms are, in many cases, suitable only for the quantification of each component. In another major approach, the pharmaceutical giants are trying to develop the generic version of the innovator formulation in the USA, UK and other developed countries. In this regard the method developed for quality equation of the proposed formulation should have capability to resolve all the major degradation products formed during the routine stability study designed as per ICH guidelines. The routine stability study takes more time to evaluate the formulation for its quality parameters at each time interval using the developed method.

Forced degradation (Stress): Study of the drug component has become an integral part of method development. Forced degradation or stress testing is undertaken to demonstrate specificity when developing stability-indicting methods, particularly when little information is available about potential products. These studies also provide information about the degradation pathways and degradation products that could be formed during storage. Forced degradation studies may help facilitate pharmaceutical development as well in areas such as formulation development, manufacturing and packaging, in which knowledge of chemicals behavior can be used to improve quality of the product.

These studies established the inherent stability characteristics of the molecules, such as degradation pathways and lead to identification of degradation products, which renders support to the suitability of the proposed analytical procedure. The detailed nature of the studies will depend on the individual drug substances and type of drug product.

It is recognized that some degradation may useful in developing and validating suitable analytical method, but it may not always be necessary to examine specifically for all degradation products, if it has been demonstrated that in practice these are not forced.

This method development using the forced degradation study of each component present in the formulation is very useful and it saves time to judge the major degradation products in a very short time. These methods, which are developed and tested for their robustness and specificity by studying post degradation samples, can be uniformly applied for all the analysis involving the API. This though seems to be consuming and expensive, is, in reality, very advantageous as there will be no need to further modify the method whenever a new impurity is detected.

4. Objective

The specific and main objectives of the work are:

- Development and validation of a stability indicating HPLC assay method for determination of nebivolol in tablet formulation.
- Development and validation of HPLC assay method for determination of aspirin and clopidogrel in combined dosage form in presence of degradation product formed under ICH recommended stress condition.
- Development and validation of stability indicating HPLC assay method for the simultaneous determination of tramadol HCl and aceclofenac components from combine dosage form.

5. Instrument Used for Whole Research Work

A. Chromatographic system:

The chromatographic system used to perform development and validation of this assay method was comprised of a LC-10AT*vp* binary pump, a SPD-M10A*vp* photo diode array detector and a rheodyne manual injector model 7725i with 20il loop (Shimadzu, Kyoto, Japan) connected to a multi-instrument data acquisition and data processing system (Class-*VP* 6.13 SP2, Shimadzu).

B. Other instruments:

- UV-Visible Spectrophotometer (Phamaspec-UV-1700, Shimadzu, Kyoto, Japan).
- Ultrasonic Bath (SONICA, Spincotech Pvt. Ltd., Mumbai).
- pH-Meter (Li-610, Elico Ltd. India).
- Hot Air Oven (Nova Instruments, India).

REFERENCES

[1] R. Meyer Veronica, *Practical High Performance Liquid Chromatography*, **2nd Ed** (1993) 26, 27, 40, 222, 246 and 258.

[2] http://hplc.chem.shu.edu/NEW/HPLC_Book/

[3] Szepei Gabor., *HPLC in pharmaceutical Analysis*, Vol**. I** (1990) 101-173.

[4] G. H. Jeffery, J. Bassett, *Vogel's textbook of Quantitative Chemical Analysis*, **5th Ed** (1991) 217-235.

[5] H. Willard Hobart, L. L. Merritt, A. Dean John, *Instrumental Methods of Analysis*, **7th Ed**, CBS Publishers, 580-610.

[6] B.K. Sharma, *Instrumental Methods of Chemical Analysis*, **20th Ed** (2001) 54-83.

[7] Swarbrick James, C. Boylan James, *Encyclopedia of pharmaceutical technology*, Vol. **I** (1998) 217-224.

[8] Lindsay Sandy, HPLC by open learning, (1991) 30-45.

[9] W. J. Lough, I. W. W. Wainer, *HPLC fundamental principles and practices*, (1991) 52-67.

[10] International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, *Validation of analytical procedures: definitions and terminolog*y, Geneva (1996).

[11] International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, *Validation of Analytical Procedures: methodolog*y, adopted in (1996) Geneva.

[12] US FDA Technical Review Guide: Validation of Chromatographic Methods, Center for Drug Evaluation and Research (CDER), Rockville, MD, (1993).

[13] US FDA, Guidelines for submitting samples and analytical data for method validation, Rockville, MD, Center for Drugs and Biologics Department of Health and Human Services (1987).

[14] General Chapter <1225>, Validation of compendial method*s, United States Pharmacopeia XXII*I, National Formulary, XVIII, Rockville, MD, The United States Pharmacopeial Convention, Inc, (1995) 1982-1984.

[15] US FDA HSS, 21 CFR Part 211, Current Good Manufacturing Practice of Certain Requirements for Finished Pharmaceuticals; Rockville (1997).

[16] G. C. Hokanson, A life cycle approach to the validation of analytical methods during pharmaceutical product development, part I: The initial validation process, *Pharm. Tec*h. (1994) 118-130.

[17] G.C. Hokanson, A life cycle approach to the validation of analytical methods during pharmaceutical product development, part II: Changes and the need for additional validation, *Pharm.Tec*h. (1994) 92-100.

[18] J. M. Green, A practical guide to analytical method validation, Anal. Chem. News & Features, May 1 (1996) 305A-309A.

[19] Renger, H. Jehle, M. Fischer and W. Funk, *J. Planar. Chrom*. (1995) 269-278.

[20] P. A. Winslow, R. F. Meyer, *J. Validation Technology***.** (1997) 361-367.

[21] ICH, Q2 (R1) Validation of analytical procedure: Text and methodology, in: International Conference on Harmonization, IFPMA, Geneva, (2005).

[22] (A) United States Pharmacopoeia, **27 th Ed**. (2004) United states Pharmacopeial Convention, Rockville, MD.

[23] Quality Assurance of Pharmaceuticals, (A compendium of guidelines and related materials) WHO, Geneva, Vol. **I** (1997)119-124.

[24] Official methods of Analysis, **17th Ed**. (2003) AOAC INTERNATIONAL, Gaithersburg, MD.

Part-[A]

(Section-1)

HPLC Method Development and
Validation of Nebivolol

HPLC METHOD DEVELOPMENT AND VALIDATION OF NEBIVOLOL

1. Introduction

In Western societies, 15-20% of the adult population has hypertension [1]. According to the 1996 health survey, 23% of all adults in England had high blood pressure. Among those subjects who had hypertension, 59% were receiving anti-hypertensive medication [2]. Of the populations on antihypertensive medication 64% had their blood pressure under control, but the other 36% were inadequately treated [2]. Untreated, sustained hypertension is a risk factor for the development of cardiovascular diseases heart failure, stroke, coronary heart disease and its sequelae, and renal failure. Beta-blockers are a well-established class of drugs for treating hypertension. There is a substantial amount of evidence from randomized controlled trials demonstrating their benefit in reducing morbidity and mortality in hypertensive patients.

Nebivol first introduced in the UK in 1999. Third generation, nebivolol is highly selective â1-adrenoceptor antagonist indicated for the treatment of essential hypertension. In addition to its â-blocking effects, nebivolol also has an endothelium dependent mild vasodilatory action that may slow or prevent some of the vascular complications associated with hypertension [3]. This review examines the pharmacological properties of nebivolol and its efficacy in controlled clinical trials, particularly when compared with the other available treatments for hypertension, including other â -blockers, angiotensin converting enzyme (ACE) inhibitors and calcium channel blockers.

1.1 Description

Nebivolol is chemically 1-(6-fluorochroman-2-yl)-2-[(2-(6- fluorochroman-2-yl)-2-hydroxy-ethyl] amino] ethanol (Figure 1). Its molecular formula is $C_{22}H_{25}F_2NO_4$ having molecular weight 405.435 g/mole [4].

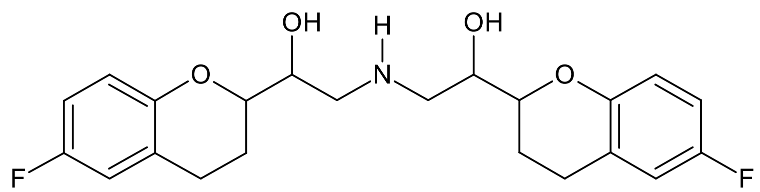


Figure 1: Chemical structure of nebivolol

1.2 Pharmacology

Chemistry: Nebivolol is structurally distinct from older â-blockers and is highly lipophilic [5]. The therapeutic formulation of nebivolol is a racemic mixture containing equal proportions of the D-and L-enantiomers [6]. The pharmacological properties of the enantiomers differ, with D-nebivolol largely responsible for the â -adrenergic blocking effects of the drug whilst both enantiomers are associated with its mild vasodilatory properties [5-7].

Mechanism of action: Nebivolol is the most selective â1-adrenoceptor antagonist currently available in the UK for clinical use and has no á 1-blocking action at therapeutic doses [8,9]. In addition to its classical â-blocking effects upon the sympathetic nervous system, heart rate and cardiac contractility, nebivolol has additional mild vasodilatory properties that cannot simply be ascribed to â1-adrenoceptor.

Vasodilatory effects: Affinities of various â-blockers for â1- and â2-adrenergic receptors from lung tissue There is considerable experimental evidence to suggest that the vasodilatory effect of nebivolol results from its ability to stimulate the release of the potent vasodilator, NO(nitric oxide), from endothelial cells [10-14]. One of the key studies implicating NO release determined that the endothelium must be intact for nebivolol induced relaxation of canine coronary artery strips to occur and this effect was blocked by the NO synthesis inhibitor, NG-monomethyl-L-arginine (L-NMMA) [15]. Although the exact mechanism is still under investigation, *in vitro* studies have suggested a number of possible mechanisms: the involvement of ATP efflux, a nebivolol mediated increase in endothelial free calcium ions leading to an increase in NO production by NO synthase, the involvement of endothelial 5-HT_{1A} receptors, interaction with oestrogen receptors, a free radical scavenging effect of nebivolol [10,12,14,16,17].

Studies in humans: Human studies in small numbers of healthy volunteers where nebivolol was infused into phenylephrine preconstricted superficial hand veins of eleven volunteers [18] or into the brachial artery in five groups of eight volunteers [19] have confirmed that nebivolol has nitric oxide mediated venodilator effects. These studies were extended in a series of eight patients with essential hypertension, and similar results were obtained.

1.3 Pharmacokinetics

The rate of metabolism of nebivolol to its active hydroxyl metabolites is dependent on the presence or absence of a genetic polymorphism in the gene encoding the cytochrome P450 (CYP) 2D6 isoenzyme, leading to two distinct metaboliser phenotypes, 'poor' and 'extensive' [5,6]. At steady state, the peak plasma concentration of unchanged nebivolol is 23 times higher in poor metabolisers than in extensive metabolisers. However, when plasma concentrations of unchanged nebivolol and its hydroxylated metabolites are considered together, plasma levels are comparable between phenotypes, which explains the similar clinical effects observed in both groups and also excludes the need for dosage adjustment.

Although the pharmacokinetic profile of nebivolol is not affected by the age of the patient, the recommended starting dose in patients aged over 65 years is half that of the normal starting dose (i.e.2.5 mg *vs.* 5 mg) [6]. This is consistent with similar recommendations for prescribing other antihypertensives to the elderly. The recommended starting dose of nebivolol in patients with renal insufficiency is also 2.5 mg, whilst a lack of data precludes the use of nebivolol in patients with hepatic insufficiency or impaired liver function [6]. Despite its relatively high lipophilicity, nebivolol demonstrates limited distribution in adipose tissue and consequently, there is no need for dosage adjustment in obese patients [20].

1.4 Efficacy

In a multicenter, double blind, randomized, parallel group dose finding study in 509 patients with primary essential hypertension, nebivolol 2.5, 5 and 10 mg, but not 0.5 or 1mg, for 4 weeks significantly reduced mean supine diastolic BP (by 7.1 to 10.2 mmHg; $p<0.05$) at trough drug levels (23-25 h post dosing), compared with placebo. There was no significant difference between the nebivolol 5 mg and 10 mg groups.

The trough-to-peak ratio for supine diastolic BP with nebivolol 5 mg once daily was 0.894 [21]. In a non-comparative study nebivolol 5 mg was given once daily to 37 patients with mild to moderate essential hypertension (diastolic BP between 95 and 114 mmHg). Significant ($p<0.0001$ vs baseline) reductions were maintained during therapy over 12 months [22]. Other comparative studies found a similar reduction in 24 h ambulatory BP with nebivolol 2.5 to 10 mg daily compared with lisinopril 10 to 40 mg daily, enalapril 10 mg daily, atenolol 100 mg daily and nifedipine 20 mg bd for up to 12 weeks ($p<0.01$ for systolic and diastolic BP for all drugs vs baseline or placebo) [23].

1.5 Adverse Effect

Nebivolol has been studied in over 3000 patients with hypertension, who have received the drug for at least one month, and some for 3 years [24]. The most frequent adverse events (incidence between 1-10%) were headache, dizziness, tiredness and paraesthesia. Other adverse events reported by at least 1% of patients were: diarrhoea, constipation, nausea, dyspnoea and oedema [25]. Other adverse events have been reported with a frequency of less than 1%.

Generally, in comparative trials there were no statistically significant differences reported between the severity and frequency of adverse events in patients receiving once daily nebivolol 5 mg (20 to 48.6% of patients reported adverse events), placebo (25 to 36%), atenolol 50 mg (13%) or enalapril 10mg (55%). [24] To date, no significant adverse effects on plasma lipids or glucose metabolism have been demonstrated in patients with hypertension, although rare cases of raised triglyceride levels have been reported [23].

1.6 Contraindications

The use of nebivolol (and â-blockers in general) is contra indicated in patients with:

- Cardiogenic shock
- Uncontrolled heart failure
- Sick sinus syndrome
- Second and third degree heart block
- History of bronchospasm and bronchial asthma
- Untreated phaeochromocytoma
- Metabolic acidosis
- Hepatic insufficiency or impaired liver function
- Bradycardia
- Hypotension

- Pregnancy or lactation
- Severe peripheral circulatory disturbances

Furthermore, nebivolol should be used with caution in patients with:

- Peripheral circulatory disorders (e.g. Raynaud's disease)
- First degree heart block
- Prinzmetal's angina
- Diabetes (treatment may mask symptoms of hypoglycaemia)
- Hyperthyroidism (treatment may mask symptoms of tachycardia)
- COPD
- History of psoriasis

1.7 Dosing

The dose is one tablet daily, preferably at the same time of the day. Tablet may be taken with meals.

2. Literature Review

The literature reviews regarding nebivolol suggest that various analytical methods were reported for its determination as drug, in pharmaceutical formulation and in various biological fluids. The literature reviews for analysis of nebivolol are as under

2.1 **M. M. Kamila, N. Mondal, L. K. Ghosh, B. K. Gupta** have developed and validated a simple UV spectrophotometric method for the determination of assay of nebivolol hydrochloride in raw material and tablets. The absorbance was measured at 282 nm for nebivolol hydrochloride tablet solution. The developed method was applied directly and easily to analyze bulk and pharmaceutical formulations [26].

2.2 **N. V. S. Ramkrishna, K. N. Vishwottam, M. Koteshwara, S. Manoj, M. Santosh, D. P. Varma** have developed and validated a rapid liquid chromatographic /electro spray ionization tandem mass spectrometric method for the quantification of nebivolol in human plasma. The method involved a simple single step liquid-liquid extraction with diethyl ether/dichloromethane (70/30). The analyte was chromatograph on C18 column by isocratic elution with water-acetonitrile-formic acid (30:70:0.03,v/v/v) and analyzed by mass spectrometry. The method can be considered suitable for application to pharmacokinetic studies of nebivolol [27].

2.3 **K. R. Rajeswari, G. G. Sankar, A. L. Rao, D. B. Raju, J. V. L. N. Seshagiri Rao** have developed and validated RP-HPLC method for the estimation of nebivolol in bulk drug and pharmaceutical formulation. Nebivolol was chromatograph on C18 column in a mobile phase consisting of acetonitrile and 30 mM KH_2PO_4 buffer (pH 3.1) in the ratio of 55: 45 (v/v). Flow rate was 0.8 ml/min and the eluent were monitored at 286 nm. The method can be used for the routine quality control analysis of nebivolol formulation [28].

2.4 **T. S. Reddy, P. S. Devi** have been established and validated a quantitative densitometric high performance thin layer chromatographic method for determination of nebivolol hydrochloride in pharmaceutical preparations. Nebivolol hydrochloride from the formulations was separated and identified on silica gel 60 F_{254} HPTLC plates with toluene-ethyl acetate-methanol-formic acid (8:6:4:1,v/v/v), as mobile phase. The plates were developed to a distance of 8 cm. Densitometric quantification was performed at 285 nm by reflectance scanning. The method could find application in routine quality control analysis of pharmaceutical formulations [29].

2.5 **P. S. Selvan, K. V. Gowda, U. Mandal, W. D. S. Solomon, T. K. Pal** have developed liquid chromatographic tandem mass spectrometry method for the simultaneous determination of nebivolol and valsartan in human plasma. Nebivolol and valsartan were extracted from plasma using acetonitrile and separated on a C18 column. The mobile phase consisting of a mixture of acetonitrile - 0.05 mM formic acid (50:50 v/v, pH 3.5) was delivered at a flow rate of 0.25 ml/min. This method can be applied to the pharmacokinetic study of fixed dose combination (FDC) of nebivolol and valsartan formulation product [30].

2.6 **L. J. Patel, B. N. Suhagia, P. B. Shah** developed HPLC and HPTLC method for the estimation of nebivolol hydrochloride in tablet formulation. In HPLC method they have used C18 column and mobile phase consisting of 50 mM KH_2PO_4 buffer (pH 3.0)- acetonitrile (45:55,v/v). The flow rate was 1.0 ml/min and effluent was monitored at 282 nm. For HPTLC method, precoated silica gel $60F_{254}$ used as a stationary phase and mobile phase consisting of ethyl acetate-toluene-methanol-ammonium hydroxide (1:6:2:0.1,v/v/v/v) were used. The detection of spot was carried out at 282 nm. The method can be used for estimation of nebivolol hydrochloride in tablet dosage form [31].

3. Aim of Present Work

As per discussion in the literature review, one UV, two HPTLC, two LC-MS and two HPLC methods for the determination of nebivolol in pharmaceutical dosage forms or in biological fluids are reported. UV, HPLC and HPTLC methods simply used for estimation of nebivolol from pharmaceutical formulation and LC-MS methods applied to the pharmacokinetic studies of nebivolol. So far to our present knowledge, no validated stability indicating HPLC assay method for the determination of nebivolol in pharmaceutical formulation was available in literature. Our work deals with the forced degradation of nebivolol under stress condition like acid hydrolysis, base hydrolysis, oxidation, thermal and photolytic stress. This work also deals with the validation of the developed method for the assay of nebivolol from its dosage form (tablets). Hence, the method is recommended for routine quality control analysis and also stability sample analysis.

The aim and scope of the proposed work are as under

- To developed suitable HPLC method for nebivolol.
- Forced degradation study of nebivolol under stress condition.
- To resolve all major impurities generated during the force degradation studies of nebivolol.
- Perform the validation for the developed method.

4. Experimental

4.1 Materials

Nebivolol hydrochloride standard of was provided by Cadila Pharmaceuticals Ltd., (India). Nebivolol tablets and the inactive ingredient used in drug matrix were obtained from market. Each tablet contains 5 mg nebivolol as label claim. Analytical grade potassium dihydrogen orthophosphate and triethylamine were purchased from Sisco Research Pvt. Ltd., Mumbai (India) and Spectrochem Pvt. Ltd., Mumbai (India) respectively. HPLC grade acetonitrile, methanol and water were obtained from Spectrochem Pvt. Ltd., Mumbai (India). Analytical grade hydrochloric acid, sodium hydroxide pellets, orthophosphoric acid and 30% v/v hydrogen peroxide solution were obtained from Ranbaxy Fine Chemical, New Delhi (India).

4.2 Instrumentation

LC-10AT*vp* HPLC system was used as describe as Part-[A] (5.A).

4.3 Mobile Phase Preparation

The mobile phase consisted of acetonitrile-50mM phosphate buffer pH 3.5 (35: 65, v/v). To prepare the buffer solution, 6.8 g potassium dihydrogen phosphates was weighed and dissolved in 1000 ml HPLC grade water, 2 ml triethylamine was added and then adjusted to pH 3.5 with orthophosphoric acid. Mobile phase was filtered through a 0.45 ìm nylon membrane (Millipore Pvt. Ltd. Benglore, India) and degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai).

4.4 Diluent Preparation

Mobile phase used as a diluent.

4.5 Standard Preparation

A nebivolol standard solution containing 0.1 mg/ml, was prepared in a 250 ml volumetric flask by dissolving 27.25 mg of nebivolol hydrochloride (equivalent to 25 mg nebivolol) in 10 ml methanol and then diluted to volume with mobile phase.

4.6 Test Preparation

Twenty tablets were weighed and the average weight of tablet was determined. From these, five tablets were weighed and transfered into a 250 ml volumetric flask. About 10 ml methanol and 150 ml mobile phase was added and sonicated for a minimum 30 min with intermittent shaking. Then content was brought back to room temperature and diluted to volume with mobile phase. The sample was filtered through 0.45 μm nylon syringe filter. The concentration obtained was 0.1 mg/ml of nebivolol.

4.7 Chromatographic Conditions

Chromatographic analysis was performed on a Phenomenex Luna C8 (2) (250 mm × 4.6 mm i.d., 5 ìm particle size) column. The mobile phase consisted of acetonitrile-50 mM phosphate buffer pH 3.5 (35: 65, v/v). The flow rate of the mobile phase was adjusted to 1.0 ml/min and the injection volume was 20 μl. Detection was performed at 280 nm.

5. Result and Discussion

5.1 Development and Optimization of the HPLC Method

Proper selection of the methods depends upon the nature of the sample, (ionic or ionisable or neutral molecule) its molecular weight and solubility. Nebivolol is dissolved in polar solvent hence RP-HPLC was selected to estimate them. To develop a rugged and suitable HPLC method for the quantitative determination of nebivolol, the analytical condition were selected after testing the different parameters such as diluents, buffer, buffer concentration, organic solvent for mobile phase and mobile phase composition and other chromatographic conditions. Our preliminary trials using different composition of mobile phases consisting of water with methanol or acetonitrile, did not give good peak shape. By using 50 mM KH_2PO_4 buffer with addition of 2 ml triethylamine per 1000 ml of buffer, adjusted to pH 3.5 with orthophosphoric acid and keeping mobile phase composition as acetonitrile-phosphate buffer (35: 65, v/v), best peak shape was obtained. Triethylamine was added to buffer to lower the peak asymmetry. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. Figure 2 and Figure 3 represent the chromatograms of standard and test prepa

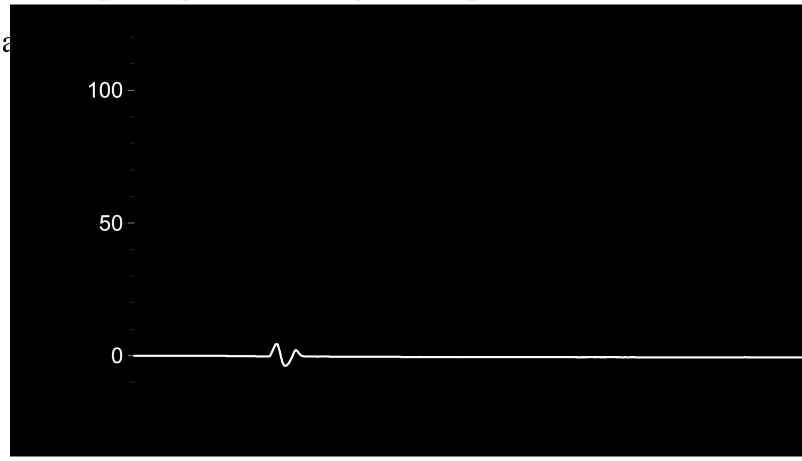


Figure 2: Chromatogram of standard preparation

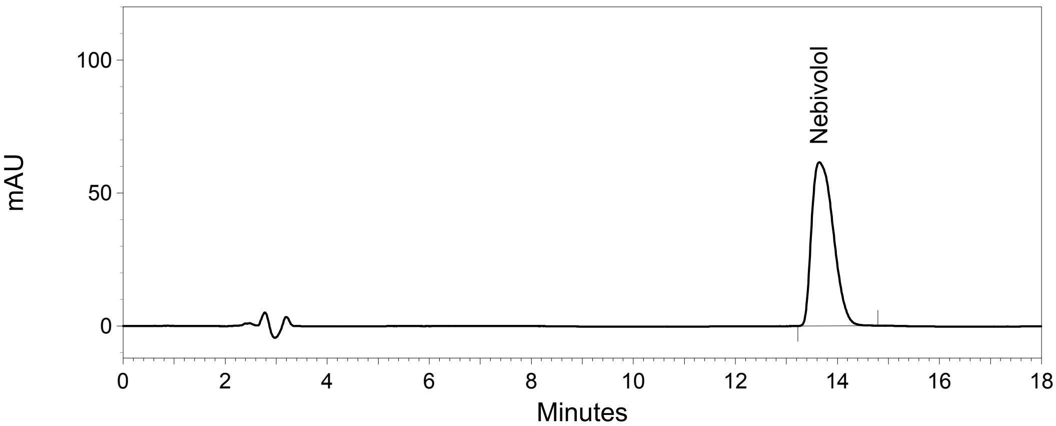


Figure 3: Chromatogram of test preparation

5.2 Degradation Study

The degraded samples were prepared by transferring powdered tablets, equivalent to 25 mg nebivolol into a 250 ml round bottom flask. Then prepared samples were employed for acidic, alkaline and oxidant media and also for thermal and photolytic conditions. After the degradation treatments were completed, the stress content solutions were allowed to equilibrate to room temperature and diluted with mobile phase to attain 0.1 mg/ml concentration. Specific conditions were described as follows.

5.2.1 Acidic condition: Acidic degradation study was performed by heating the drug content in 5 N HCl(50 ml) at 80 ^{0}C for 3 h and mixture was neutralized with 5 N NaOH solutions. Nebivolol was found to be degrading up to 10% in acidic condition. (Figure 4).

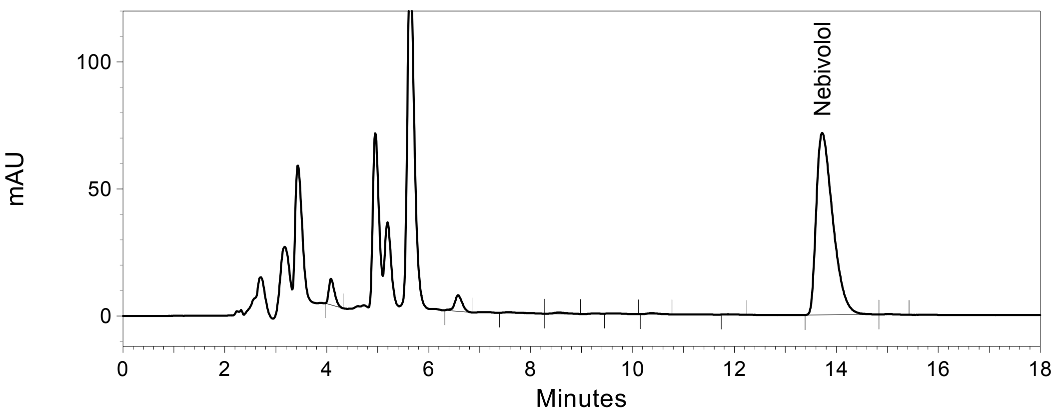


Figure 4: Chromatogram of acidic forced degradation study

5.2.2 *Alkaline condition:* Alkaline degradation study was performed by heating the drug content in 5 N NaOH(50 ml) at 80 ^{0}C for 3 h and mixture was neutralized with 5 N HCl solutions. In alkali degradation, it was found that around 8-10% of the drug degraded (Figure 5).

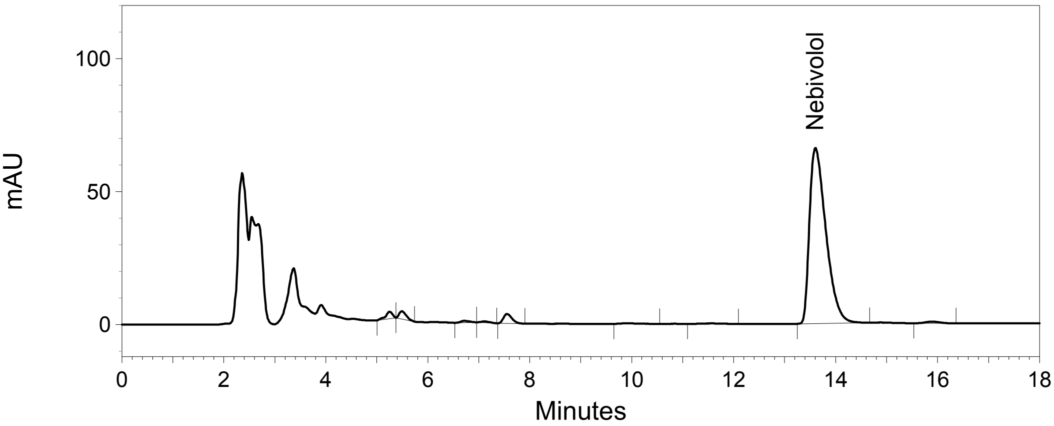


Figure 5: Chromatogram of alkali forced degradation study

5.2.3 *Oxidative condition:* Oxidation degradation study was performed by heating the drug content in 3% v/v H_2O_2(50 ml) at 80 ^{0}C for 3 h. Major degradation was found in oxidative condition that product was degraded up to 50%. The major impurity peaks were found at 8.63 min and 53.46 min (Figure 6).

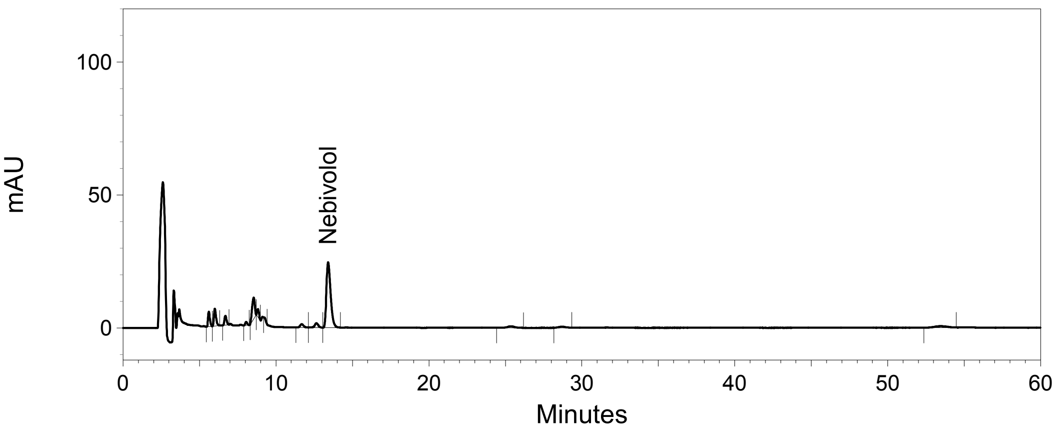


Figure 6: Chromatogram of oxidative forced degradation study

5.2.4 *Thermal condition:* Thermal degradation was performed by exposing solid drug at 80 ^{0}C for 72 h. Nebivolol was found to be stable under thermal degradation condition (Figure 7).

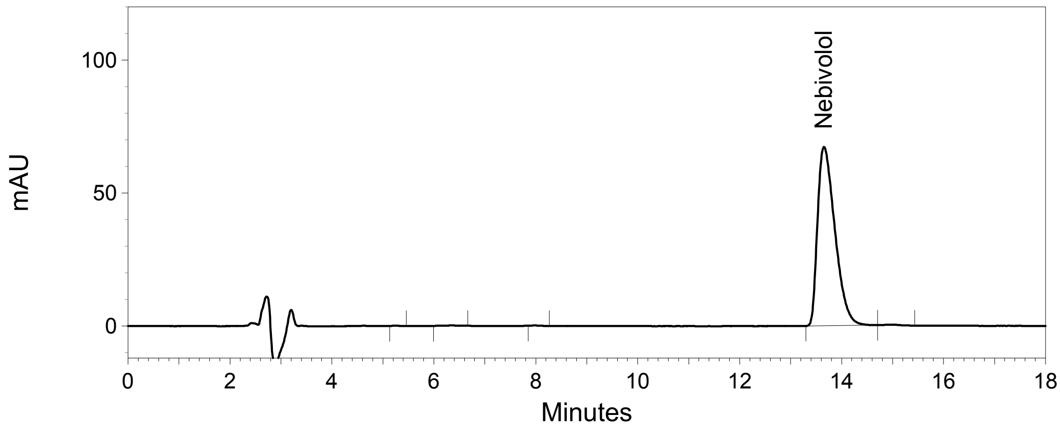


Figure 7: Chromatogram of thermal degradation study

5.2.5 Photolytic condition: Photolytic degradation study was performed by exposing the drug content in UV-light for 72 h. There was no degradation observed in above specific photolitic condition. Nebivolol was found to be stable in UV-light (Figure 8).

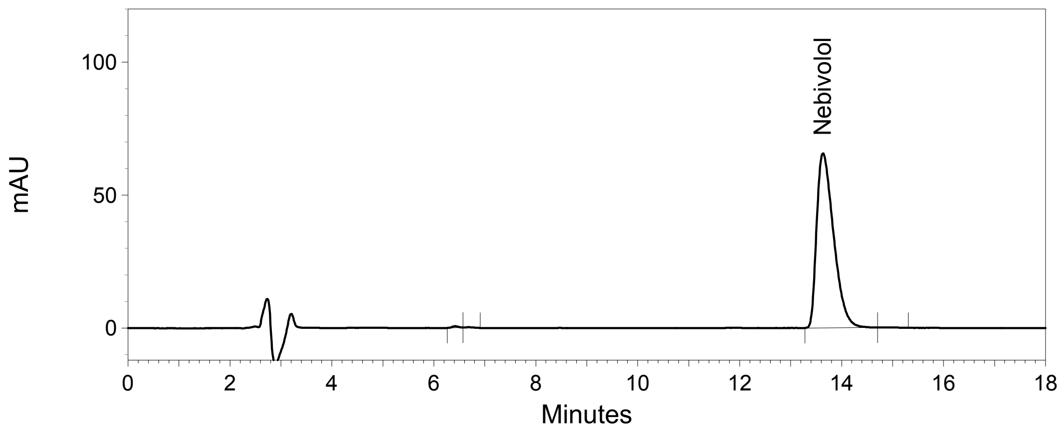


Figure 8: Chromatogram of UV-light degradation study

5.3 Method Validation

5.3.1 Specificity: The specificity of the method was determined by checking the interference of placebo with analyte and the proposed method was eluted by checking the peak purity of nebivolol during the force degradation study. The peak purity of the nebivolol was found satisfactory (0.9999) under different stress condition. There was no interference of any peak of degradation product with drug peak.

5.3.2 Linearity: Seven points calibration curve were obtained in a concentration range from 0.04-0.16 mg/ml for nebivolol. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation was y =16749949.98x +3974.44 with correlation coefficient 0.9999. (Figure 9) Chromatogram obtain during linearity study were shown in Figure 10-16.

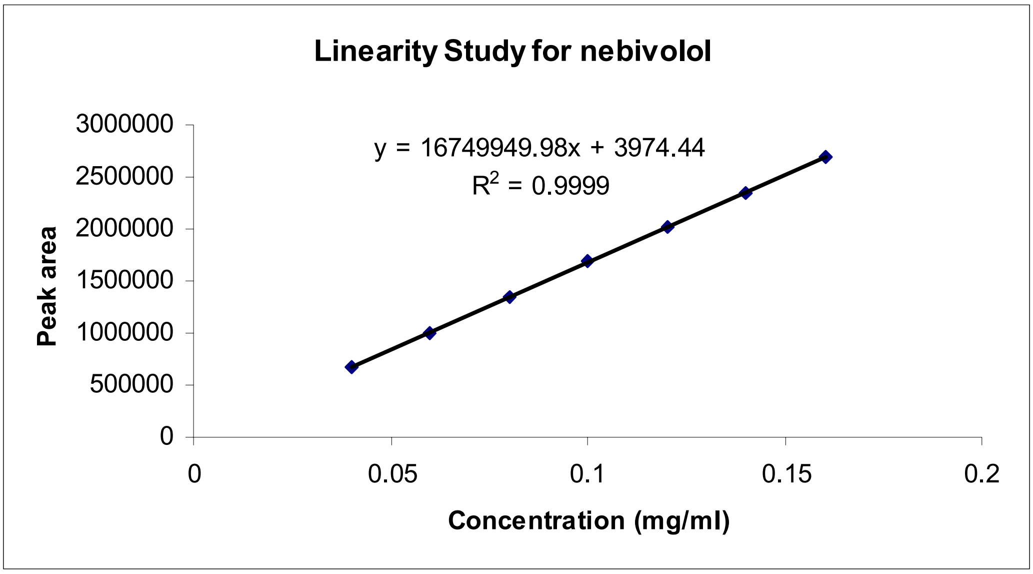


Figure 9: Linearity curve for nebivolol

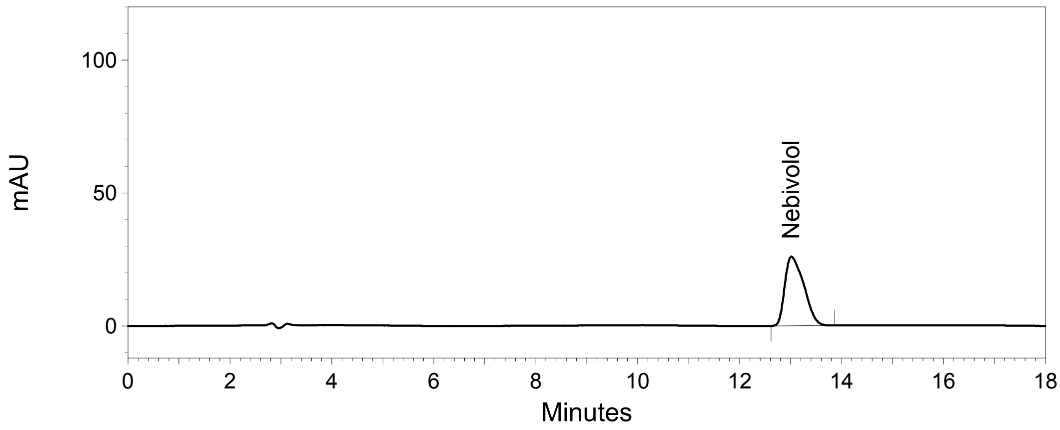


Figure 10: Linearity study chromatogram of level-1 (40%)

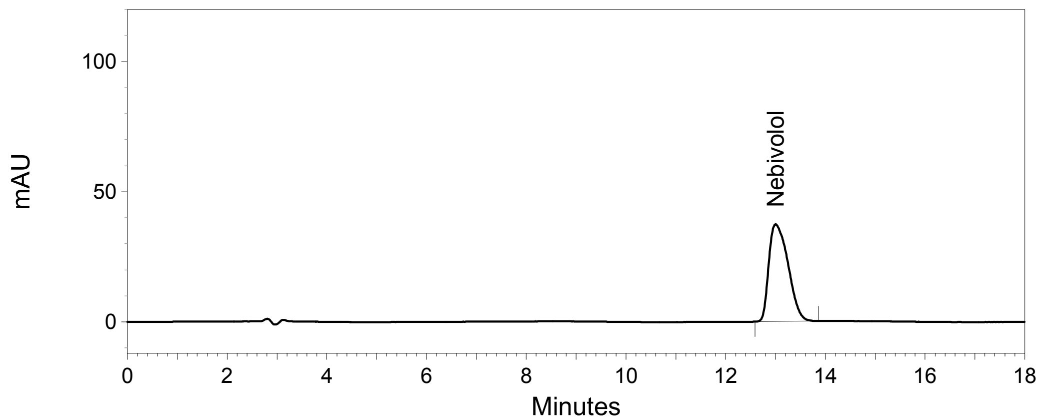


Figure 11: Linearity study chromatogram of level-2 (60%)

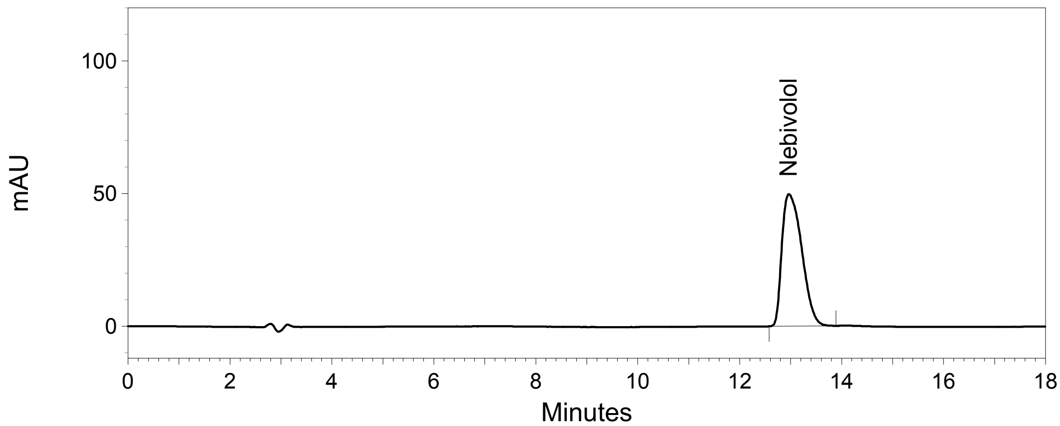


Figure 12: Linearity study chromatogram of level-3 (80%)

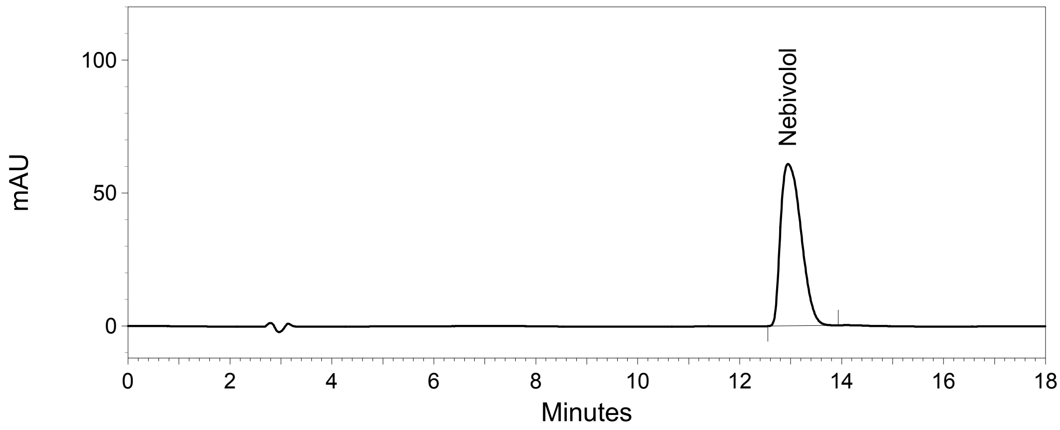


Figure 13: Linearity study chromatogram of level-4 (100%)

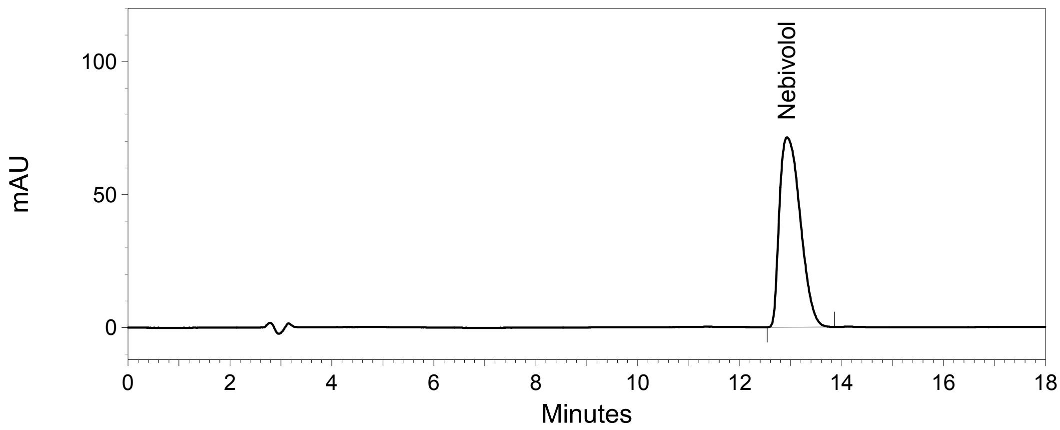


Figure 14: Linearity study chromatogram of level-5 (120%)

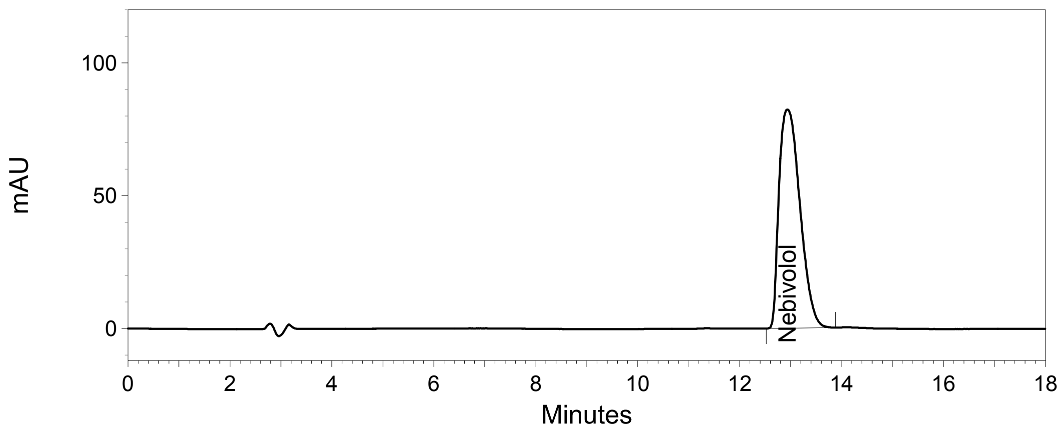


Figure 15: Linearity study chromatogram of level-6 (140%)

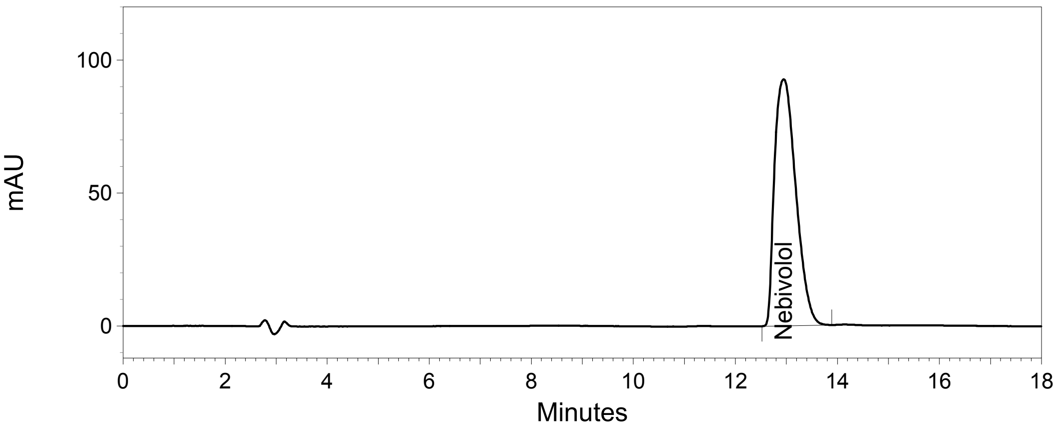


Figure 16: Linearity study chromatogram of level-7 (160%)

5.3.3 Precision: The result of repeatability and intermediate precision study are shown in Table 1. The developed method was found to be precise as the %RSD values for the repeatability and intermediate precision studies were < 0.69% and < 1.39 %, respectively, which confirm that method was precise.

Table 1: Evaluation data of precision study

Set	Intraday (n = 6)	Interday (n = 6)
1	100.5	99.9
2	100.9	98.8
3	102.1	101.8
4	101.1	99.7
5	101.2	102.3
6	102.3	99.5
Mean	101.4	100.3
Standard deviation	0.70	1.39
% RSD	0.69	1.39

5.3.4 Accuracy: The HPLC area responses for accuracy determination are depicted in Table 2. The result shown that best recoveries (98.57-99.55 %) of the spiked drug were obtained at each added concentration, indicating that the method was accurate. Chromatogram obtain during accuracy study were shown in Figure 17-19.

Table 2: Evaluation data of accuracy study

Level (%)	Amount Added Concentration [a] (mg/ml)	Amount Found Concentration [a] (mg/ml)	% Recovery	% RSD
50	0.05387	0.05362	99.55	0.60
100	0.10707	0.10554	98.57	0.09
150	0.16187	0.16102	99.48	0.53

[a] Each value corresponds to the mean of three determinations.

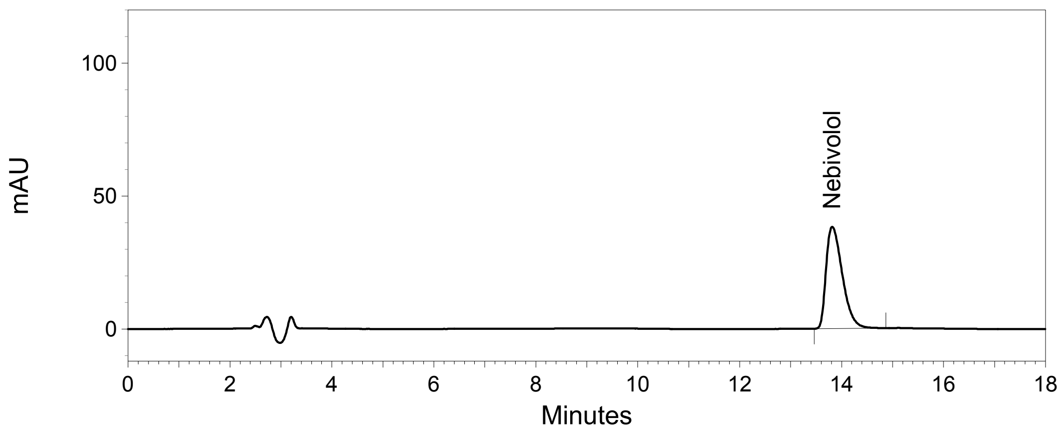


Figure 17: Accuracy study chromatogram of level-1 (50%)

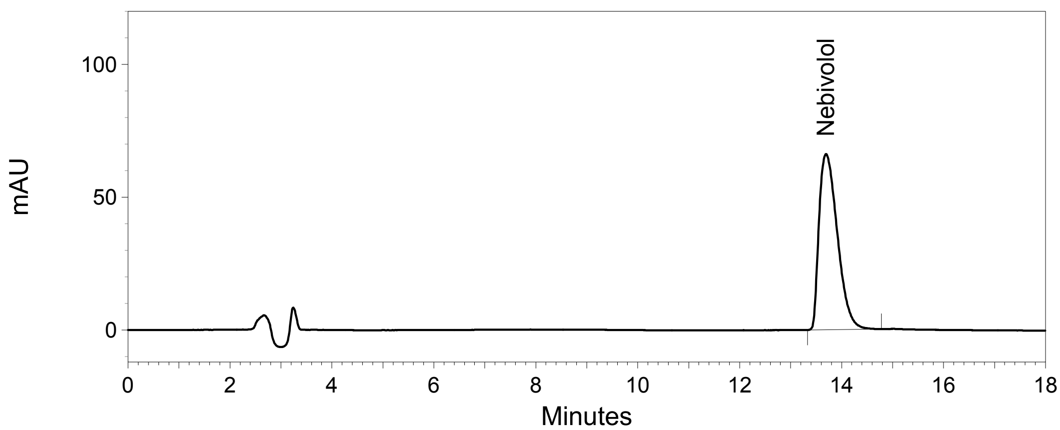


Figure 18: Accuracy study chromatogram of level-2 (100%)

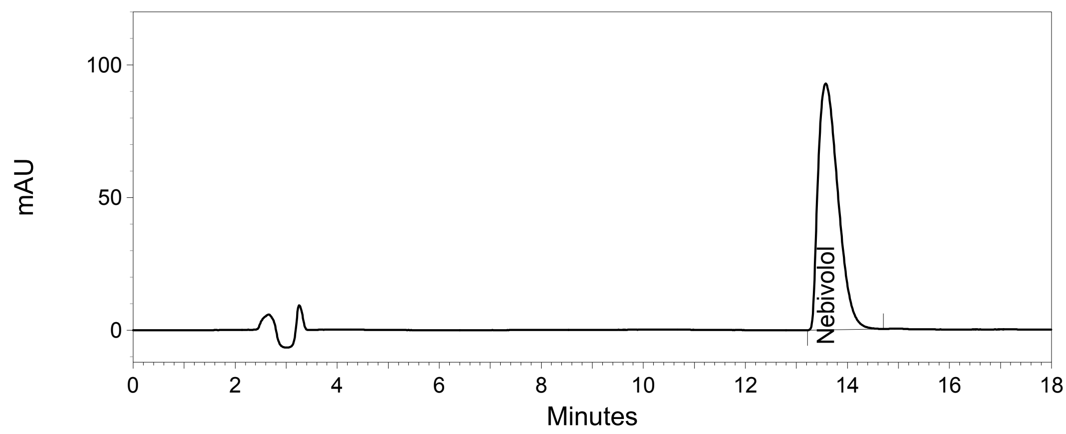


Figure 19: Accuracy study chromatogram of level-3 (150%)

5.3.5 ***Solution stability study:*** Table 3 shows the results obtain in the solution stability study at different time intervals for test preparation. It was concluded that the test preparation solution was found stable up to 48 h at 2-5 ^{0}C and ambient temperature, as during this time the result was not decrease below the minimum percentage.

Table 3: Evaluation data of solution stability study

Intervals	% Assay for Test Preparation Solution Stored at 2-5 ^{0}C	% Assay for Test Preparation Solution Stored at Ambient Temperature
Initial	99.2	99.2
12 h	99.0	99.1
24 h	98.6	98.5
36 h	98.3	98.1
48 h	98.1	98.0

5.3.6 ***Robustness:*** The result of robustness study of the developed assay method was established in Table 4. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory, hence the analytical method would be concluded as robust. Chromatogram obtain during robustness study were shown in figure 20-26.

Table 4: Evaluation data of robustness study

Robust conditions	% Assay	System Suitability Parameters	
		Theoretical Plates	Asymmetry
Flow 0.9 ml/min	101.0	7657	1.68
Flow 1.1 ml/min	100.4	7803	1.66
Buffer pH 3.3	100.0	9062	1.72
Buffer pH 3.7	99.8	7534	1.70
Buffer-ACN (63: 37,v/v)	100.3	7723	1.70
Buffer-ACN (67: 33,v/v)	99.8	7937	1.60
Column change	99.9	6993	1.69

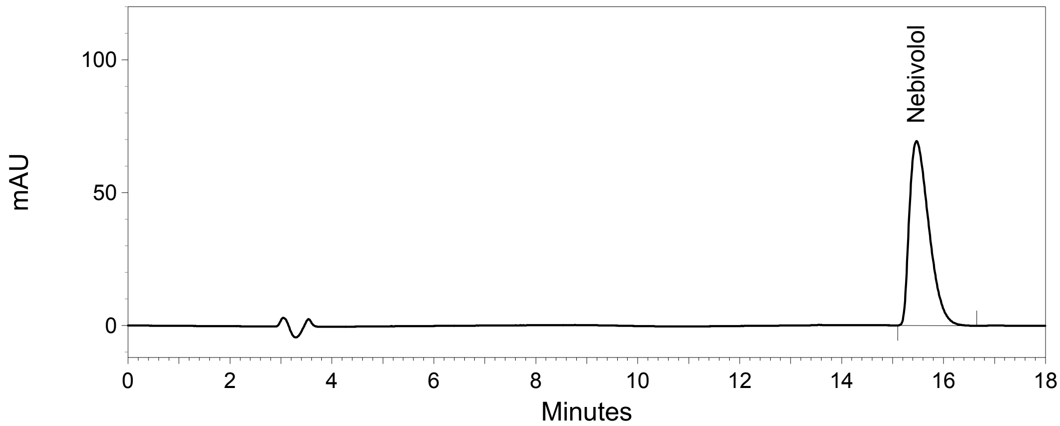


Figure 20: Standard chromatogram (0.9 ml/min flow rate)

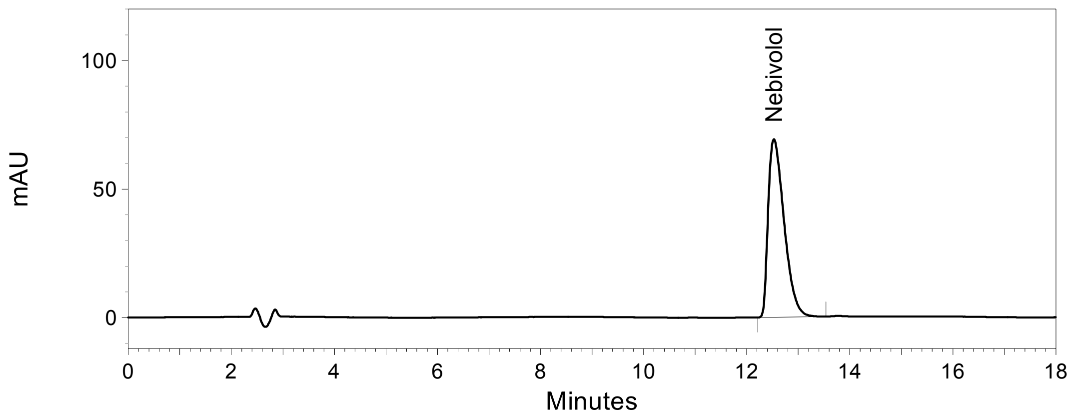


Figure 21: Standard chromatogram (1.1 ml/min flow rate)

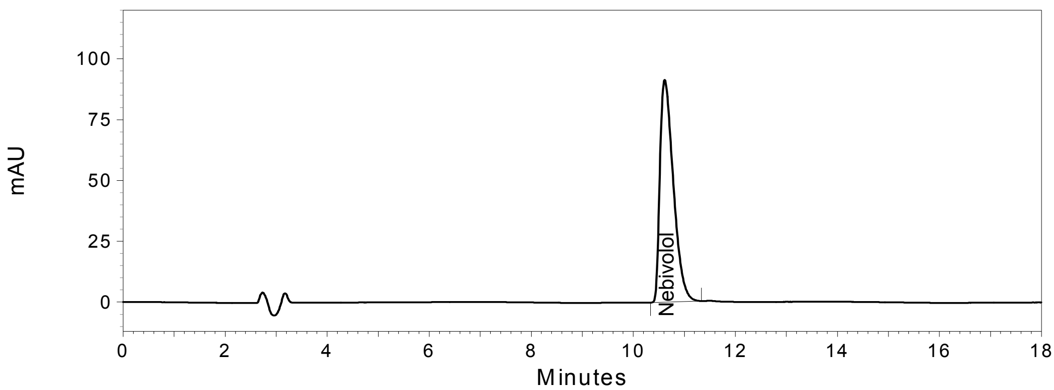


Figure 22: Standard chromatogram [Buffer-ACN (63: 37,v/v)]

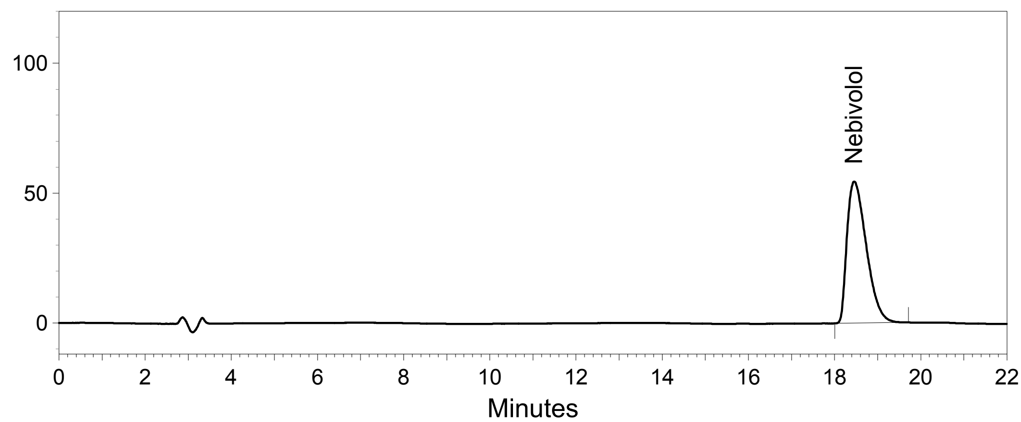


Figure 23: Standard chromatogram [Buffer-ACN (67: 33, v/v)]

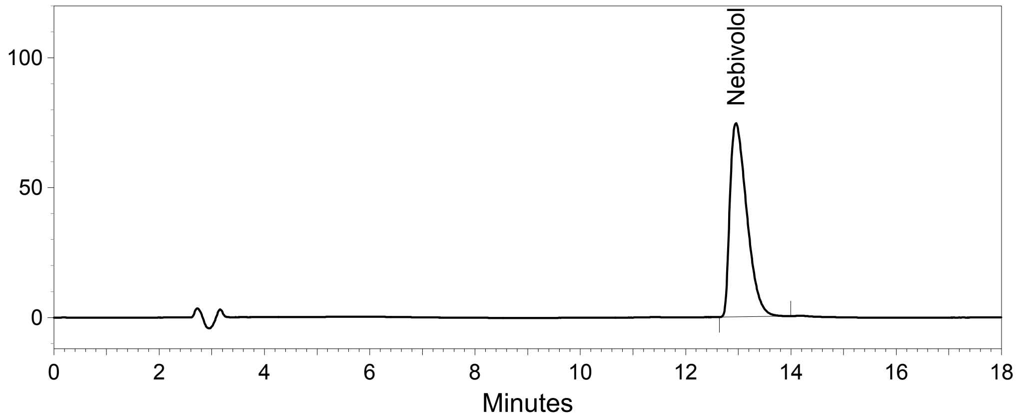


Figure 24: Standard chromatogram (pH 3.7)

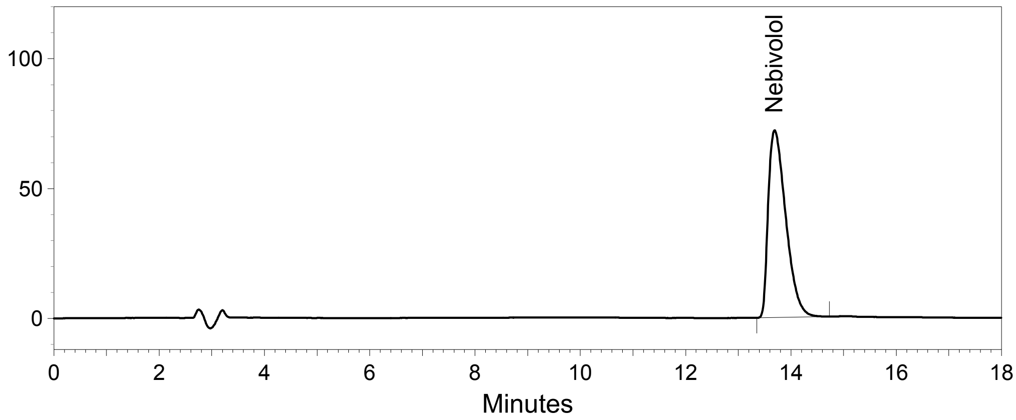


Figure 25: Standard chromatogram (pH 3.3)

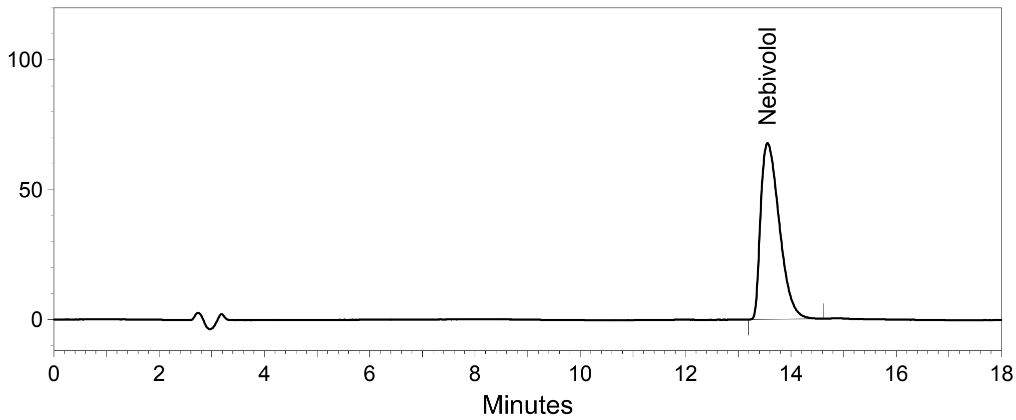


Figure 26: Standard chromatogram (Column change)

***5.3.7 System suitability*:** A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same. Acceptance criteria for system suitability, Asymmetry not more than 2.0, theoretical plate not less then 5000 and % RSD of peak area not more then 2.0, were full fill during all validation parameters.

6. Calculation and Data

Calculation formula used:

1. Calculation formula for % assay of nebivolol

$$\%\ \text{Assay} = \frac{\text{Mean Test Area}}{\text{Mean Standard Area}} \times \frac{\text{Standard Weight}}{250} \times \frac{405.48}{441.94}$$

$$\times \frac{250}{\text{Test Weight}} \times \frac{\text{Average Weight}}{\text{Lable Claim}} \times \text{Potency of Standard}$$

2. Relative standard deviation

$$\%\ \text{RSD} = \frac{\text{Standard Deviation of Measurments}}{\text{Mean Value of Measurments}} \times 100$$

3. Recovery

$$\%\ \text{Recovery} = \frac{\text{Amount found}}{\text{Amount Added}} \times 100$$

4. Amount found

$$\text{Amount Found (mg/ml)} = \frac{\text{Mean Test Area}}{\text{Mean Standard Area}} \times \text{Standard Concentration}$$

5. Amount added

$$\text{Amount Added (mg/ml)} = \frac{\text{Weight}}{\text{Volume}}$$

Specificity Study for Analytical Method Validation of Nebivolol Tablets

Standard Weight (mg)	26.8
Standard Dilution	250
Standard Potency	98.59 %
Factor 1	405.48
Factor 2	441.94
Standard Concentration (mg/ml)	0.0984

Replicate	1	2	3	4	5
Standard Area	1654331	1649549	1653034	1653003	1653616
Mean Standard Area	1652707				
Stdev.	1846.02				
% RSD	0.11				

Replicate	**Test Area**
1 2	1744413 1813285
Mean Test Area	1778849
Test Weight (mg)	1041.2
Label claim (mg)	5
Mean Test Weight (mg)	204.1
% Assay	102.2 %

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{1778849}{1652707} \times \frac{26.8}{250} \times \frac{405.48}{441.94} \times \frac{250}{1041.2} \times \frac{204.1}{5} \times 98.59$$

$$= 102.2\ \%$$

Linearity Study for Analytical Method Validation of Nebivolol Tablets

Standard Weight (mg)	26.8
Standard Dilution	250
Standard Potency	98.59 %
Factor 1	405.48
Factor 2	441.94
Standard Concentration (mg/ml)	0.0984
Concentration of Linearity Stock Solution (mg/ml)	0.5460

Replicate	1	2	3	4	5
Standard Area	1686152	1681540	1682487	1679841	1680488
Mean Standard Area	1682102				
Stdev	2217.37				
% RSD	0.13				

Concentration Level (%)	**Volume of Linearity stock solution taken (ml)**	**Diluted to (ml)**	**Final Concentration (mg/ml)**	**Mean Area**
40	2.0	25	0.0401	673255
60	3.0	25	0.0601	1001175
80	4.0	25	0.0802	1347328
100	5.0	25	0.1002	1701055
120	6.0	25	0.1202	2024118
140	7.0	25	0.1403	2345866
160	8.0	25	0.1603	2683441
		Correlation co-efficient		0.9999
		Slope		16749949.98
		Intercept		3974.44

Precision Study for Analytical Method Validation of Nebivolol Tablets

Description	Mean area	Tes[redacted]	[redacted]
Set 1	1722946	[redacted]	[redacted]
Set 2	1713205	[redacted]	[redacted]
Set 3	1714246	[redacted]	[redacted]
Set 4	1698890	[redacted]	[redacted]
Set 5	1736411	[redacted]	[redacted]
Set 6	1752798	[redacted]	[redacted]
		Confid [redacted]	[redacted]

Standard Dilution	250		
Standard Potency			
Label Claim (mg)	5		
Mean Test Weight (mg)	204.1		
Factor 1	405.48		
Factor 2	441.94		
Standard Concentration (mg/ml)			
Replicate	1	2	3
Standard Area	1651094	1650113	164
Mean Standard Area	1647724		
Stdev.	3238.79		
% RSD	0.20		

Calculation:

Prototype calculation for one set:

$$\% \ \text{Assay} = \frac{1722946}{1647724} \times \frac{26.8}{250} \times \frac{405.48}{441.94} \times \frac{250}{1029.2} \times \frac{204.1}{5} \times 98.59$$

$$= 100.5 \ \%$$

IntermediatePrecision Study for Analytical Method Validation of Nebivolol Tablet

Standard Weight (mg)	26.9
Standard Dilution	250
Standard Potency	98.59 %
Label Claim (mg)	5
Mean Test weight (mg)	204.1
Factor 1	405.48
Factor 2	441.94
Standard Concentration (mg/ml)	0.1076

Replicate	1	2	3	4	5
Standard Area	1642429	1641707	1645698	1644263	1646619
Mean Standard Area	1644143				
Stdev.	2087.76				
% RSD	0.13				

Description	Mean area	Test Weight (mg)	% Assay
Set 1	1700283	1027.8	99.9
Set 2	1649225	1008.4	98.8
Set 3	1734995	1030.1	101.8
Set 4	1664862	1008.7	99.7
Set 5	1707946	1008.4	102.3
Set 6	1661793	1009.4	99.5
		Mean	100.3
		Stdev	1.39
		% RSD	1.39
		Confidence Level (95.0%)	1.46

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{1700283}{1644143} \times \frac{26.9}{250} \times \frac{405.48}{441.94} \times \frac{250}{1027.8} \times \frac{204.1}{5} \times 98.59$$

$$= 99.9\ \%$$

Comparison for Precision and Intermediate Precision Study for Analytical Method Validation for Nebivolol Tablets

	Set	%Assay
Precision study	1	100.5
	2	100.9
	3	102.1
	4	101.1
	5	101.2
	6	102.3
Intermediate precision study	1	99.9
	2	98.8
	3	101.8
	4	99.7
	5	102.3
	6	99.5
	Mean	100.8
	Stdev	1.18
	% RSD	1.17

Accuracy Study for Analytical Method Validation of Nebivolol Tablets

Standard Weight (mg)	28.5
Standard Dilution	250
Standard Potency	98.59 %
Factor 1	405.48
Factor 2	441.94
Standard Concentration (mg/ml)	0.1046

Replicate	1	2	3	4	5
Standard Area	1644642	1638915	1637776	1635107	1634085
Mean Standard Area	1638105				
Stdev	4142.62				
% RSD	0.25				

Accuracy Study for Analytical Method Validation of Nebivolol Tablets

Recovery Level	Set No.	Mean area	Weight (mg)	Volume (ml)	*Amount added* Concentration (mg/ml)	*Amount found* Concentration (mg/ml)	% Recovery	Mean % Recovery	Stdev	% RSD
50%	Set 1	838944	13.4	250	0.05360	0.05357	99.94	99.55	0.60	0.60
	Set 2	842247	13.6	250	0.05440	0.05378	98.86			
	Set 3	838201	13.4	250	0.05360	0.05352	99.85			
100%	Set 1	1654057	26.8	250	0.10720	0.10561	98.52	98.57	0.09	0.09
	Set 2	1650489	26.7	250	0.10680	0.10539	98.68			
	Set 3	1654214	26.8	250	0.10720	0.10562	98.53			
150%	Set 1	2521521	40.7	250	0.16280	0.16100	98.89	99.48	0.53	0.53
	Set 2	2522776	40.3	250	0.16120	0.16108	99.93			
	Set 3	2521314	40.4	250	0.16160	0.16099	99.62			

Calculation:

Prototype calculation for one set:

$$\%\ \text{Recovery} = \frac{0.05357}{0.05360} \times 100 \qquad = 99.94\ \%$$

$$\text{Amount Found (mg/ml)} = \frac{838944}{1638105} \times 0.1046 \qquad = 0.05357\ \text{mg/ml}$$

$$\text{Amount Added (mg/ml)} = \frac{13.4}{250} \qquad = 0.05360\ \text{mg/ml}$$

Solution Stability Study for Analytical Method Validation of Nebivolol Tablets

System suitability of standard preparation for solution stability					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	1678207	1662724	1687950	1697508	1667885
2	1676828	1661210	1688152	1685856	1666619
3	1677108	1658816	1687480	1688203	1662877
4	1672384	1662791	1694160	1690180	1664324
5	1675192	1663227	1681072	1695345	1671921
Mean	1675944	1661754	1687763	1691418	1666725
Stdev	2263.66	1810.65	4635.66	4881.45	3497.40
%RSD	0.14	0.11	0.27	0.29	0.21

Solution stability for standard preparation at 2 -8°C				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	1678207	1678207	1678207	1678207
2	1676828	1676828	1676828	1676828
3	1677108	1677108	1677108	1677108
4	1672384	1672384	1672384	1672384
5	1675192	1675192	1675192	1675192
1	1675603	1675731	1673748	1678891
2	1672995	1671705	1681093	1674779
Mean	1675474	1675308	1676366	1676198
Stdev	2151.06	2438.52	2903.63	2239.25
%RSD	0.13	0.15	0.17	0.13

Solution stability for standard preparation at room temperature				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	1678207	1678207	1678207	1678207
2	1676828	1676828	1676828	1676828
3	1677108	1677108	1677108	1677108
4	1672384	1672384	1672384	1672384
5	1675192	1675192	1675192	1675192
1	1672051	1670649	1671078	1677944
2	1671502	1675685	1673611	1670266
Mean	1674753	1675150	1674915	1675418
Stdev	2752.47	2713.93	2652.41	3022.22
%RSD	0.16	0.16	0.16	0.18

Solution stability for test preparation			
	Initial	***After 12 hours***	***After 24 hours***
	Standard	***Standard***	***Standar***
Replicate	Peak area	Peak area	Peak are
1	1678207	1662724	1687950
2	1676828	1661210	1688152
3	1677108	1658816	1687480
4	1672384	1662791	1694160
5	1675192	1663227	1681072
Replicate	Test Area	Test Area	Test Are
1	1618776	1614362	1614791
2	1616214	1610975	1614011
Mean	1617495	1612669	1614401
% Assay	99.2	99.0	98.6
Standard weight (mg)	28.1	27.9	28.2
Test weight (mg)	1009.9	1009.9	1009.9
% Absolute difference compare to that of initial		0.2	0.6

Solution stability for test preparation at room temperature					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	1678207	1662724	1687950	1697508	1667885
2	1676828	1661210	1688152	1685856	1666619
3	1677108	1658816	1687480	1688203	1662877
4	1672384	1662791	1694160	1690180	1664324
5	1675192	1663227	1681072	1695345	1671921
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	1618776	1615279	1612824	1616163	1616471
2	1616214	1614736	1613258	1612157	1620568
Mean	1617495	1615008	1613041	1614160	1618520
% Assay	99.2	99.1	98.5	98.1	98.0
Standard weight (mg)	28.1	27.9	28.2	28.1	27.6
Test weight (mg)	1009.9	1009.9	1009.9	1009.9	1009.9
% Absolute difference compare to that of initial		0.1	0.7	1.1	1.2

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{1617495}{1675944} \times \frac{28.1}{250} \times \frac{405.48}{441.94} \times \frac{250}{1009.9} \times \frac{204.1}{5} \times 98.49$$

$$= 99.2\ \%$$

Robustness Study for Analytical Method Validation of Nebivolol Tablets

	Flow Rate at 0.9ml/min	**Flow Rate at 1.1ml/min**	**Buffer-ACN 63: 37**	**Buffer- ACN 67: 33**	**Buffer pH 3.7**	**Buffer pH 3.3**	**Column Change**
Replicate	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area
1	1847616	1491015	1685531	1720433	1661058	1679262	1674703
2	1846682	1490200	1671047	1705267	1662446	1670135	1670401
3	1844559	1490957	1656672	1702791	1660010	1664996	1669246
4	1848685	1493043	1655294	1693139	1658041	1665068	1669566
5	1853369	1489485	1654541	1690811	1659592	1669327	1669856
Mean	1848182	1490940	1664617	1702488	1660229	1669758	1670754
Stdev	3273.17	1331.65	13512.10	11764.29	1646.47	5816.50	2247.83
% RSD	0.18	0.09	0.81	0.69	0.10	0.35	0.13
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area
1	1896568	1466612	1616882	1625078	1627315	1627315	1642753
2	1893215	1467884	1615982	1626737	1629228	1629228	1637531
Mean	1894892	1467248	1616432	1625908	1628272	1628272	1640142
Standard Weight (mg)	27.5	27.9	28.2	28.3	28.2	28.3	28.2
Test Weight (mg)	1031.2	1010.1	1008.1	1000.1	1018.2	1019.2	1023.2
Label Claim (mg)	5	5	5	5	5	5	5
Mean Test Weight (mg)	204.1	204.1	204.1	204.1	204.1	204.1	204.1
% Assay	101.0	100.4	100.3	99.8	99.8	100.0	99.9

Calculation:

Prototype calculation for one set: $\% \text{ Assay} = \frac{1894892}{1848182} \times \frac{27.5}{250} \times \frac{405.48}{441.94} \times \frac{250}{1031.2} \times \frac{204.1}{5} \times 98.49 = 101.0\ \%$

REFERENCES

[1] Y. Bonomo, P. Phillips, Epidemiology of Hypertension. *Medicine* **26** (1998) 134.

[2] P. Prescott-Clarke, P. Primatesta, Health Survey for England 1996: a survey carried out on behalf of the Department of Health. London Stationery Office (1998) (seried HS; no 6).

[3] S. Bodavari, Eds. In; The Merck Index, 14th Edn., Merck & Co., Inc., Whitehouse Station, NJ. (2006) 6432.

[4] J. Cockcroft, Nebivolol: a review. *Expert. Opin. Pharmacother*. **5** (2004) 893-899.

[5] W. McNeely, K. L. Goa, Nebivolol in the management of essential hypertension: *a review. Drugs*. **57** (1999) 633-651.

[6] *Nebilet*(nebivolol). Summary of product characteristics.* A. Menarini Pharmaceuticals UK Ltd. (2003).

[7] A. Van de Water, R. Xhonneux, R. S. Reneman, P. A. Janssen, *Eur. J. Pharmacol.* **156** (1988) 95-103.

[8] P. J. Pauwels, W. Gommeren, G. Van Lommen, P. A. Janssen, J. E. Leysen, *Mol. Pharmacol.* **34** (1988) 843-851.

[9] A. Van de Water, W. Janssens, J. Van Neuten, *J. Cardiovasc. Pharmacol.* **11** (1988) 552 -563.

[10] M. A. Broeders, P. A. Doevendans, B. C. Bekkers, *Circulation.* **102** (2000) 677-684.

[11] J. R. Cockcroft, P. J. Chowienczyk, S. E. Brett, *J. Pharmacol. Exp. Ther.* **274** (1995) 1067-1071.

[12] L. Cominacini, A. F. Pasini, U. Garbin, *J. Am. Coll. Cardiol.* **42** (2003) 1838-1844.

[13] M. Da es, S. E. Brett, P. J. Chowienczyk, T. G. Mant, J. M. Ritter, *Br. J. Clin. Pharmacol.* **48** (1999) 460-463.

[14] L. Kalinowski, L. W. Dobrucki, M. Szczepanska Konkel, *Circulation.* **107** (2003) 2747-2752.

[15] Y. S. Gao, T. Nagao, R. A. Bond, W. J. Janssens, P. M. Vanhoutte, *J. Cardiovasc. Pharmacol.* **17** (1991) 964-969.

[16] H. J. Garban, G. M. Buga, L. J. Ignarro, *J. Cardiovasc. Pharmacol.* **43** (2004) 638-644.

[17] M. Kakoki, Y. Hirata, H. Hayaka, *Hypertension.* **33** (1999) 467-471.

[18] A. J. Bowman, *Br. J. Clin. Pharmacol.* **38** (1994) 199-204.

[19] J. R. Cockcroft, *J. Pharmacol. Exp. Ther.* **274** (1995) 1067-1071.

[20] G. Cheymol, R. Woestenborghs, E. Snoeck, *Eur. J. Clin. Pharmacol.* **51** (1997) 493 -498.

[21] L. Van Neuten, *J. Human. Hypertens*. **11** (1997) 139-144.

[22] J. DeCree, *Angiology.* **43** (1992) 369-377.

[23] W. Mc Neely, K. Goa, *A review. Drugs.* **57** (1999) 633-651.

[24] Robertson JIS. Nebivolol: A Development Review. Menarini Pharmaceuticals 1.3.99.

[25] Nebilet SPC. A. Menarini Pharmaceuticals UK Ltd. February (1999).

[26] M. M. Kamila, N. Mondal, L. K. Ghosh, B. K. Gupta, *Pharmazie*. **62** (2007) 486-487.

[27] N. V. S. Ramkrishna, K. N. Vishwottam, M. Koteshwara, S. Manoj, M. Santosh, D. P. Varma, *J. Pharm. Biomed. Anal.* **39** (2005) 1006-1013.

[28] K. R. Rajeswari, G. G. Sankar, A. L. Rao, D. B. Raju and J. V. L. N. Seshagiri Rao, *Asian Journal of Chemistry.* **17** (2005) 1259-1263.

[29] T. S. Reddy and P. S. Devi, *J. Planar. Chrom*. **20** (2007) 149-152.

[30] P. S. Selvan, K. V. Gowda, U. Mandal, W. D. S. Solomon, T. K. Pal, *J. Chromatogr. B* **858** (2007) 143-150.

[31] L. J. Patel, B. N. Suhagia, P. B. Shah, *Indian J. Pharm. Sci.* **69** (2007) 594-596.

Part-[A]

(Section-II)

HPLC Method Development and Validation of combine dosage form of Aspirin and Clopidogrel

HPLC METHOD DEVELOPMENT AND VALIDATION OF COMBINE DOSAGE FORM OF ASPIRIN AND CLOPIDOGREL

1. Introduction to Drug

Platelet aggregation and thrombus formation play a critical role in the initiation and development of key complications of acute coronary syndromes (ACSs). Antiplatelet therapy and antithrombotic therapy have been demonstrated to favorably modify clinical outcome, and recent trials of revascularization in ACSs have demonstrated a reduction in the frequency of major cardiac events [1-13]. Antiplatelet and antithrombin therapy can have synergistic actions that reduce the risk of spontaneous or revascularization, especially percutaneous coronary intervention (PCI)-related events. Yet, all effective antithrombotic agents also increase the risk of bleeding, especially bleeding that results from vascular accessor associated with surgery, including coronary artery bypass grafting (CABG). The Clopidogrel in Unstable angina to prevent Recurrent ischemic Events (CURE) trial demonstrated that the combination of clopidogrel and aspirin was superior to aspirin alone for patients hospitalized with non-ST-elevation ACSs.

1.1 Aspirin

Aspirin is chemically acetylsalicylic acid (Figure 1) Its molecular formula is $C_9H_8O_4$ having molecular weight 180 g/mole [14]. It is slightly soluble in water, freely soluble in alcohol, soluble in chloroform and ether, sparingly soluble in absolute ether.

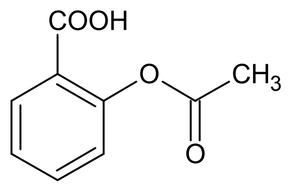


Figure 1: Chemical structure of aspirin

Aspirin, one of the first drugs to come into common usage, is still the most widely used drug in the world, is a non-steroidal anti-inflammatory drug that exhibits anti-inflammatory, analgesic and antipyretic activities. Aspirin is now accepted as an important weapon in the prevention of heart disease. A single dose of 300 mg is now recommended for patients in the acute stages of a heart attack followed by a daily dose of 75-100 mg. A similar low dose treatment regime is recommended for patients with angina, a history of heart problem or who have undergone coronary by-pass surgery. Major use of aspirin is as an anti-platelet aggregating agent.

1.1.1 Pharmacology

Mechanism of Action: Aspirin is an inhibitor of the enzyme cyclooxygenase, the reaction being considered to be due to an irreversible acetylation process. In blood platelets such enzyme inhibition prevents the synthesis of thromboxane $A_{2,}$ a compound that is a vasoconstrictor, causes platelet aggregation and is thus potentially thrombotic. Thus aspirin inhibits platelet inhibition. Aspirin is an effective antithrombotic at doses as low as 80 mg, but the rapid, acute effect probably requires 162.5 mg.

Combination: The combination of clopidogrel with aspirin provides enhanced prevention of atherothrombotic events by blocking the platelet aggregation by both ways. (ADP Pathway and collagen induced pathway) and it shows a synergistic antiplatelet action in controlling the ischemic events.

1.1.2 Pharmacokinetics

Absorption: Aspirin is absorbed rapidly from the gastrointestinal tract. Following oral administration, absorption of nonionised aspirin occurs in the stomach and intestine. Some aspirin is hydrolyzed to salicylate in the gut wall. After absorption is rapidly converted to salicylate but during the first 20 minutes following oral administration. Aspirin is the predominant from of the drug in the plasma.

Distribution: Aspirin is 80 to 90% bound to plasma proteins and is widely distributed its volume of distribution is reported to be 170 ml per kg body weight in adults. As plasma drug concentrations increase, the binding sites on the proteins become saturated and the volume of distribution increases. Both aspirin and salicylate have pharmacological activity; only aspirin has an antiplatelet effect. Salicylate is extensively bound to plasma proteins and is rapidly distributed to all body parts. Salicylate appears in breast milk and crosses the placenta.

***Metabolism and Excretion*:** Salicylate is mainly eliminated by hepatic metabolites include salicyluric acid, salicyl phenolic glucuronide, salicyclic acyl glucuronide, gentisic acid and gentisuric acid. Steady state plasma-salicylate concentrations increase disproportionately with dose. Following a 325 mg aspirin dose, elimination is a first order process and the plasma salicylate half-life is about 2 to 3 hours. At high aspirin doses, the half-life increases to 15 to 30 hours. Salicylate is also excreted unchanged in the urine; the amount excreated by this route increasing dose and also depends on urinary pH, about 30% of a dose being extracted in alkaline in urine compared with 2% of a dose in acidic urine. Renal excretion involves glomerular filtration, active renal tubular secretion, and passive tubular reabsorption. Salicylate is removed by heamodialysis.

1.1.3 Indications

It is indicated for the prevention of secondary events after the carotid stents. It is also indicated in the prevention of ischemic stroke and unstable angina.

1.1.4 Contraindications

- It should not be given to any patients with a history of sensitivity reactions to aspirin, which includes those in whom attacks of a asthma, angioedema, urticari, or rhinitis have been precipitated by aspirin.
- It should be avoided in severe renal or hepatic impairment.
- It should not be administered to patients with hemonhagic disorders or to patients with gout since low doses increase urate concentrations.

1.1.5 Drug Interactions

- Aspirin may enhance the activity of coumarin anticoagulants,sulphonylurea, hypoglycemic drugs, methotrexate, phenytoin, and valproic acid.
- Concurrent administration of aspirin with dypyridamole, metoprolol may increase peak plasma-salicylate concentrations.
- The risk of gastro intestinal bleeding and ulceration associated with aspirin is increased when used with corticosteroids.
- Antacids and adsorbents may increase the excretion of aspirin in alkaline urine.
- Aspirin diminishes the effect of uricosurics such as probenecid and sulphinpyrazone.

1.1.6 Adverse Reaction

The adverse reactions reported with aspirin are gastro intestinal disturbances such as nausea and vomiting, urticaria, skin eruptions like angioedema, rhinitis and severe paroxysmal bronchospasm and dyspnoea may be provoked in persons with asthma, chronic rhinitis and hepatotoxicity in patients with juvenile chronic arthritis or other connective disorders and blood disorders like thrombocytopenia.

1.2 Clopidogrel

Clopidogrel bisulfate, chemically it is [S- (a)(2-chlorophenyl)-6,7-dihydrothieno (3,2-C) pyridine-5 (4H) acetic acid methyl ester sulphate] (Figure 2). The empirical formula of clopidogrel bisulfate is $C_{16}H_{16}ClNO_2S{\bullet}H_2SO_4$ and its molecular weight is 419.9 g/mole [14]. It is a white to off-white powder. It is practically insoluble in water at neutral pH but freely soluble at pH 1. It also dissolves freely in methanol, dissolves sparingly in methylene chloride and is practically insoluble in ethyl ether. It has a specific optical rotation of about +56°. The structural formula is as follows:

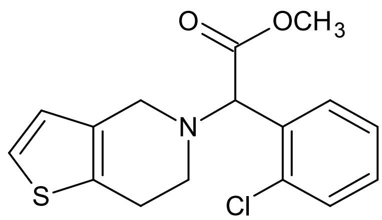


Figure 2: Chemical Structure of clopidogrel

1.2.1 Pharmacology

Mechanism of Action: Clopidogrel is an inhibitor of platelet aggregation. A variety of drugs that inhibit platelet function have been shown to decrease morbid events in people with established cardiovascular atherosclerotic disease as evidenced by stroke or transient ischemic attacks, myocardial infarction, unstable angina or the need for vascular by-pass or angioplasty. This indicates that platelets participate in the initiation and/or evolution of these events and that inhibiting them can reduce the event rate.

Clopidogrel selectively inhibits the binding of adenosine diphosphate (ADP) to its platelet receptor and the subsequent ADP-mediated activation of the glycoprotein

GPIIb/IIIa complex, thereby inhibiting platelet aggregation. Biotransformation of clopidogrel is necessary to produce inhibition of platelet aggregation, but an active metabolite responsible for the activity of the drug has not been isolated. Clopidogrel also inhibits platelet aggregation induced by agonists other than ADP by blocking the amplification of platelet activation by released ADP. Clopidogrel does not inhibit phosphodiesterase activity.

Clopidogrel acts by irreversibly modifying the platelet ADP receptor. Consequently, platelets exposed to clopidogrel are affected for the remainder of their lifespan. Dose dependent inhibition of platelet aggregation can be seen 2 hours after single oral doses of clopidogrel bisulfate. Repeated doses of 75 mg clopidogrel bisulfate per day inhibit ADP-induced platelet aggregation on the first day and inhibition reaches steady state between day 3 and day 7. At steady state, the average inhibition level observed with a dose of 75 mg clopidogrel bisulfate per day was between 40% and 60%. Platelet aggregation and bleeding time gradually return to baseline values after treatment is discontinued, generally in about 5 days.

1.2.2 Pharmacokinetics

After repeated 75 mg oral doses of clopidogrel (base), plasma concentrations of the parent compound, which has no platelet inhibiting effect, are very low and are generally below the quantification limit (0.00025 mg/L) beyond 2 hours after dosing. Clopidogrel is extensively metabolized by the liver. The main circulating metabolite is the carboxylic acid derivative, and it too has no effect on platelet aggregation. It represents about 85% of the circulating drug related compounds in plasma.

Following an oral dose of ^{14}C-labeled clopidogrel in humans, approximately 50% was excreted in the urine and approximately 46% in the feces in the 5 days after dosing. The elimination half-life of the main circulating metabolite was 8 hours after single and repeated administration. Covalent binding to platelets accounted for 2% of radiolabel with a half-life of 11 days.

Effect of food: Administration of clopidogrel bisulfate with meals did not significantly modify the bioavailability of clopidogrel as assessed by the pharmacokinetics of the main circulating metabolite.

Absorption and distribution: Clopidogrel is rapidly absorbed after oral administration of repeated doses of 75 mg clopidogrel (base), with peak plasma levels (3 mg/L) of the main circulating metabolite occurring approximately 1 hour after dosing. The pharmacokinetics of the main circulating metabolite are linear (plasma concentrations increased in proportion to dose) in the dose range of 50 to 150 mg of clopidogrel. Absorption is at least 50% based on urinary excretion of clopidogrel related metabolites.

Clopidogrel and the main circulating metabolite bind reversibly in vitro to human plasma proteins (98% and 94%, respectively). The binding is nonsaturable *in vitro* up to a concentration of 100 μg/ml.

Metabolism and elimination: *In vitro* and *in vivo*, clopidogrel undergoes rapid hydrolysis into its carboxylic acid derivative. In plasma and urine, the glucuronide of the carboxylic acid derivative is also observed.

1.2.3 Indications and Usage for Clopidogrel

Clopidogrel bisulfate is indicated for the reduction of atherothrombotic events as follows:

- Recent Myocardial Infarction (MI), Recent Stroke or Established Peripheral Arterial Disease:

  For patients with a history of recent MI, recent stroke or established peripheral arterial disease, clopidogrel bisulfate has been shown to reduce the rate of a combined endpoint of new ischemic stroke (fatal or not), new MI (fatal or not) and other vascular death.
- Acute Coronary Syndrome:

  For patients with acute coronary syndrome (unstable angina/non-Q-wave MI) including patients who are to be managed medically and those who are to be managed with percutaneous coronary intervention (with or without stent) or CABG, clopidogrel bisulfate has been shown to decrease the rate of a combined endpoint of cardiovascular death, MI or stroke as well as the rate of a combined endpoint of cardiovascular death, MI stroke or refractory ischemia.

1.2.4 Contraindications

The use of clopidogrel bisulfate is contraindicated following conditions:

- In the hypersensitivity to the drug substance or any component of the product.
- Active pathological bleeding such as peptic ulcer or intracranial hemorrhage.

1.2.5 Dosing

The recommended dose of the combination of clopidogrel 75 mg and aspirin 75 mg or combination of clopidogrel 75 mg and aspirin 150 mg is one tablet daily.

2. Literature Review

There are many reported method for the determination of either aspirin or clopidogrel alone or in combination with other drug in pharmaceutical dosage forms or individually in biological fluids are as under:

2.1 Literature Review for Aspirin

2.1.1 **J. T. Franeta, D. Agbaba, S. Eric, S. Pavkov, M. Aleksic, S. Vladimirov** have developed a method for the simultaneous determination of acetylsalicylic acid, paracetamol, caffeine and phenobarbital in tablets. Separation was achieved using C18 column (250 mm × 4.6 mm i.d, 5μm particle size). Mixture of acetonitrile-water (25:75, v/v) adjusted to pH 2.5 with phosphoric acid was used as a mobile phase at a flow rate of 2.0 ml/min UV detection was at 207 nm. The method could find application in routine quality control analysis of pharmaceutical formulations [15].

2.1.2 **A. Verstraeten, E. Roets, J. Hoogmartens** have developed a quantitative high-performance liquid chromatographic method for the determination of aspirin and releted substances in tablets using C 8 column. Methanol-water-1M phosphoric acid (59:36:5,v/v/v) as the mobile phase has been used for the analysis of several naturally aged batches of fourteen brands of acetylsalicylic acid tablets. Comparison is made with classical spectrophotometric method [16].

2.1.3 **J. Fogel, P. Epstein, P. Chen** have developed a reversed phase high-performance liquid chromatography method for the simultaneous assay of acetylsalicylic acid and salicylic acid in film coated aspirin tablet using a 5 μm C 18 column with water-acetonitrile-phosphoric acid (76:24:0.5, v/v/v) as the mobile phase enabled the chromatographic separation to be completed in 4 min [17].

2.1.4 **M. Gandhimathi**, **T. K. Ravi, A. Abraham, R. Thomas** have developed a simple reversed phase high-performance liquid chromatography method for the simultaneous estimation of aspirin and isosorbide 5-mononitrate in combined formulation. The method was carried out on a Thermo Quest C18 column using a mixture of water-methanol (water pH adjusted to 3.4 using dilute orthophosphoric acid) and detection was carried out at 215 nm using chlorzoxazone as internal standard. Propose method can be use in routine quality control analysis of pharmaceutical formulations [18].

2.1.5 **Y. Dong, Y. Z. Zhao, Y. N. Zhang** have developed determination method of aspirin and free salicylic acid in lysinipirine injection by high performance liquid chromatography. A Hypersil BDS C18 column was used with the mobile phase of methanol-water-acetic acid (35:65:3, v/v/v) and the detection wavelength of 280 nm. Method can be used for the qualitative analysis of aspirin and salicylic acid in lysinipirine injection formulation [19].

2.1.6 **M. Sawyer, V. Kumar** have developed a rapid reversed phase HPLC procedure and validated for the simultaneous quantitation of aspirin, salicylic acid and caffeine extracted from an effervescent tablet. The method uses a Hypersil C18 column (150 mm × 4.6 mm i.d.,5 μm particle size) for an isocratic elution in a water-methanol-acetic acid mobile phase at a wavelength of 275 nm. The method could find application in routine quality control analysis of pharmaceutical tablet formulations [20].

2.1.7 **R. Thomis, E. Roets, J. Hoogmartens** have reported analysis method of tablets containing aspirin, acetaminophen and ascorbic acid by high-performance liquid chromatography. Method enables the quantitation of the components and the main impurities of tablets containing aspirin, acetaminophen and ascorbic acid. A C 8 reverse phase column was used; the mobile phase was methanol-0.2 M phosphate buffer (pH 3.5)-water (20:10:70, v/v/v). Method was used for the stability sample analysis [21].

2.1.8 **A. W. Abu-Qare, M. B. Abou-Donia** have developed method for the separation and quantification of the pyridostigmine bromide, acetaminophen, aspirin and caffeine in rat plasma and urine. The compounds were extracted using C18 Sep-Pak(R) cartridges then analyzed by high-performance liquid chromatography (HPLC) with reversed phase C18 column and UV detection at 280 nm. The compounds were separated using gradient of 1-85 % acetonitrile in water (pH 3.0) at a flow rate ranging between 1 and 1.5 ml/min in a period of 14 min [22].

2.1.9 **C. Akay, B. Gümüsel, T. Degim, S. Tartilmis, S. Cevheroglu** have developed simple and accurate high-performance liquid chromatography method to measure the amount of acetaminophen, acetylsalicylic acid and ascorbic acid in tablet formulation. Three drugs, acetaminophen, aspirin and ascorbic acid, were analyzed simultaneously. A commercial pharmaceutical effervescent tablet was examined and the amount of each of these agents successfully determined [23].

2.1.10 **R. Pirola, S. R. Bareggi, G. De Benedittis** have reported determination of acetylsalicylic acid and salicylic acid in skin and plasma by high-performance liquid chromatography. Separation of acetylsalicylic acid and salicylic acid achieved on C18 column using a water-phosphate buffer (pH 2.5)-acetonitrile (35:40:25, v/v/v) as mobile phase at 1 ml/min flow rate [24].

2.1.11 **F. Kees, D. Jehnich, H. Grobecker** have reported simultaneous determination of acetylsalicylic acid and salicylic acid in human plasma by high-performance liquid chromatography. Aspirin and salicylic acid determined by reversed phase C18 column (150 mm × 4.0 mm i.d., 4 µm particle size) using mobile phase water-85% orthophosphoric acid-acetonitrile (740:0.9:180,v/v/v) and photometric detection (237 nm). 2-Methylbenzoic acid was used as internal standard [25].

2.1.12 **P. P. Ascione, G. P. Chrekian** have developed an automated high-pressure liquid chromatographic method for the separation and determination of aspirin, phenacetin and caffeine in pharmaceutical dosage forms. Separation of these compounds for quantitation was achieved on a controlled pore glass support, utilizing a mixture of acetic acid and chloroform as the mobile phase. The feasibility of determining free salicylic acid in analgesics also was established [26].

2.1.13 **G. Alibrandi, S. Coppolino, S. D'Aliberti, R. Ficarra, N. Micali, A. Villari** reported fast drug stability determination by LC variable parameter kinetic experiments. Variable parameter kinetic experiments were carried out using HPLC as analytical instrument. The hydrolysis of aspirin was followed both at variable temperature and at variable pH conditions [27].

2.1.14 **G. P. McMahon, S. J. O'Connor, D. J. Fitzgerald, S. le Roy, M. T. Kelly** have developed a high-performance liquid chromatographic method for the simultaneous determination of aspirin and salicylic acid in transdermal perfusates. The compounds were separated on a C8 Nucleosil column (250 mm × 4.6 mm i.d., 5 μm particle size) using a mobile phase containing a mixture of water-acetonitrile-orthophosphoric acid (650:350:2, v/v/v) and a flow rate of 1 ml/min. The method has been applied to the determination of aspirin and salicylic acid in phosphate-buffered saline following in vitro application of the compounds to mouse skin samples [28].

2.1.15 **Z. Kokot, K. Burda** have developed simple assay procedure for simultaneous analysis of aspirin and salicylic acid in aspirin delayed release tablet formulation by 'zero crossing' second derivative UV spectrophotometry [29].

2.1.16 **S. H. Hansen, M. E. Jensen, I. Bjørnsdottir** have reported the separation of aspirin and three of its metabolites salicylic acid, salicyluric acid and gentisic acid demonstrated in a non-aqueous capillary electrophoresis system with reversed electroosmotic flow. Solvent mixtures of methanol and acetonitrile were used for the electrophoresis media and different electrolytes have been investigated. The separation method was applied to the assay of aspirin and its major metabolites in plasma and urine [30].

2.1.17 **S. Torrado, S. Torrado, R. Cadórniga** have compared second derivative spectroscopy, colorimetry and fluorescence spectroscopy with a high-performance liquid chromatographic method for the assay of salicylic acid in preparations of aspirin [31].

2.1.18 **I. M. Jalal, S. I. Sa'sa'** has published the official compendial method for the determination of dextropropoxyphene napsylate, caffeine, aspirin and salicylic acid involves a lengthy extraction by gas chromatography and spectrophotometry. The analytical scheme reported here provides a fast, sensitive and stability-indicating reversed-phase HPLC assay for all these components concurrently [32].

2.1.19 **G. Santoni, L. Fabbri, P. Gratteri, G. Renzi and S. Pinzauti** has developed a reversed phase HPLC method for the simultaneous determination of aspirin, propyphenazone and codeine phosphate in an analgesic tablet formulation. The elution was isocratic using two C 8 columns and methanol-water (45:55, v/v) as mobile phase with 1.4% acetic acid and 5 mM tetramethylammonium bromide [33].

2.1.20 **S. L. Ali** has reported application of gas-liquid chromatography and high-performance liquid chromatography to the analysis of trace amounts of salicylic acid, acetylsalicylic anhydride and acetylsalicylsalicylic acid in aspirin samples and aspirin formulations [34].

2.1.21 **V. Kmetec** has reported simultaneous determination of acetylsalicylic, salicylic, ascorbic and dehydroascorbic acid by high performance liquid chromatography [35].

2.2. Literature Review for Clopidogrel

2.2.1 **H. Agrawal, N. Kaul, A. R. Paradkar, K. R. Mahadik** have developed and validated stability indicating high-performance thin layer chromatographic method of analysis of clopidogrel bisulphate both as a bulk drug and in formulations. The method employed TLC aluminium plates precoated with silica gel $60F_{254}$ as the stationary phase. The solvent system consisted of carbon tetrachloride-chloroform-acetone (6:4:0.15, v/v/v). Clopidogrel bisulphate was subjected to acid and alkali hydrolysis, oxidation, photo degradation and dry heat treatment. The method could be employed as a stability indicating one [36].

2.2.2 **E. Souri, H. Jalalizadeh, A. Kebriaee-Zadeh, M. Shekarchi, A. Dalvandi** have developed reproducible method for determination of carboxylic acid metabolite of clopidogrel in human plasma. After liquid-liquid extraction in acidic medium with chloroform, samples were quantified on a C8, 5 mm column using a mixture of 30 mm dipotassium hydrogen phosphate (pH 3)-tetra hydro furan-acetonitrile (79:2:19, v/v/v) as mobile phase with UV detection at 220 nm. The flow rate was set at 0.9 ml/min. Ticlopidine was used as internal standard and the total run time of analysis was about 12 min. The method was used to study the pharmacokinetics of clopidogrel [37].

2.2.3 **S. S. Singh, K. Sharma, D. Barot, P. R. Mohan, V. B. Lohray** have developed a high-performance liquid chromatographic method for the estimation of carboxylic acid metabolite of clopidogrel bisulfate in rat plasma using atorvastatin as internal standard. Plasma samples were extracted with a mixture of ethyl acetate-dichloro methane (80:20, v/v) followed by subsequent reconstitution in a mixture of water: methanol: acetonitrile (40:40:20, v/v/v). The chromatographic separation was achieved with gradient elution on Kromasil ODS (250 mm x 4.6 mm i.d., 5 µm particle size) analytical column maintained at 30 °C. Carboxylic acid metabolite of clopidogrel as well as the internal standard were detected at a wavelength of 220 nm. The method was applied to the pharmacokinetic study of the two different polymorphs of clopidogrel bisulfate in wister rat [38].

2.2.4 **A. Robinson, J. Hillis, C. Neal, A. C. Leary** have developed and validated LC-MS/MS bio-analytical method for the determination of unchanged clopidogrel in human plasma. Analysis was performed using a C 8 column (temperature controlled to 50 °C) by gradient elution at a flow rate of 0.9 ml/min over a 3 min run time. Detection was achieved using a Sciex API 4000, triple quadrupole mass spectrometer, in positive turboionspray (electrospray) ionization mode. This validated method was used to support a pharmacokinetic study in healthy volunteers [39].

2.2.5 **R. V. Nirogi, V. N. Kandikere, M. Shukla, K. Mudigonda, S. Maurya, R. Boosi** have reported high-performance liquid chromatography/positive electrospray ionization tandem mass spectrometry method for the quantification of clopidogrel in human plasma. The analytes were separated using an isocratic mobile phase on a reversed-phase column and analyzed by mass spectrometry in the multiple reaction-monitoring mode. The validated method has been used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies [40].

2.2.6 **H. Ksycinska, P. Rudzki, M. Bukowska-Kiliszek** have reported a method for determination of clopidogrel metabolite (SR26334) in human plasma. Samples were quantified using reversed phase high performance liquid chromatography with mass detection. The determination was performed on a Luna C18, (75 mm x 4.6 mm i.d., 3 µm particle size) column with an acetonitrile-water-formic acid mixture (60:40:0.1, v/v/v) as a mobile phase. The flow rate was set at 0.2 ml/min. The method has been used to study clopidogrel metabolite pharmacokinetics in healthy volunteers [41].

2.2.7 **A. Mitakos, I. Panderi** have documented a reversed phase HPLC method for the determination of clopidogrel in pharmaceutical dosage forms. The determination was performed on a semi-micro column BDS C8 (250 mm x 2.1 mm i.d., 5 µm particle size). The mobile phase consisted of a mixture of 0.010 M sodium dihydrogen phosphate (pH 3.0)-acetonitrile (35:65, v/v), pumped at a flow rate 0.3 ml/min. The UV detector was operated at 235 nm. The method was applied in the quality control of commercial tablets and content uniformity test [42].

2.2.8 **P. Lagorce, Y. Perez, J. Ortiz, J. Necciari, F. Bressolle** haved eveloped GC-MS method for the analysis of the carboxylic acid metabolite clopidogrel in plasma and serum. The analytical procedure involves a robotic liquid-liquid extraction with diethyl ether followed by a solid-liquid extraction on C18 cartridges. The derivatization process was performed using n-ethyl diisopropylethylamine and alpha-bromo-2,3,4,5,6-pentafluoro toluene. The method use for the pharmacokinetic studies [43].

2.3 Literature Review for Aspirin and Clopidogrel

Some method reported for the simultaneous determination of aspirin and clopidogrel in pharmaceutical formulation and biological fluids are as under:

2.3.1 **Y. Tomoko, N. Mihoko, W. Mitsuhiro, N. Kentchiro** have developed a semi-micro column HPLC-UV method for simultaneous determination of clopidogrel metabolite, aspirin and salicylic acid in rat plasma. These compounds in rat plasma simply extracted by liquid-liquid extraction with 10% n-hexane/ethyl acetate. The separation of these compounds was achieved within 22 min by a C18 column (250 mm x 1.5 mm) with a mixture of 10 mM phosphate buffer (pH 2.5) and acetonitrile as an eluant [44].

2.3.2 **P. Mishra, A. Dolly** have developed two simple spectrophotometric methods for the simultaneous determination of aspirin and clopidogrel in pharmaceutical formulation. First method was based on the aditivity of the absorbance. Second method was based on the determination of graphical absorbance ratio at two selected wavelengths, one being the isoabsorptive point for the two drugs (225 nm) and the other being the absorption maximum of hydrolyzed aspirin (235.7 nm). Method can be use for the analysis of pharmaceutical formulation [45].

2.3.3 **M. Gandhimathi, T. K. Ravi** have developed a HPLC to determine aspirin and clopidogrel in combined dosage form. The chromatographic resolution of aspirin and clopidogrel was obtained in a mobile phase consisting of 0.1%(v/v) triethylamine (pH 4.0)-acetonitrile in the ratio (25: 75, v/v) in an isocratic elution. A detection wavelength of 225 nm and flow rate of 1 ml/min was used. Method can be used for the simultaneous determination of aspirin and clopidogrel in pharmaceutical formulation [46].

3. Aim of Present Work

In recent times, there is increased tendency towards the development of stability-indicating assays [47-49], using the approach of stress testing as enshrined in the International Conference on Harmonization (ICH) guideline Q1A (R2) [50]. Even this approach is being extended to drug combinations [51,52] to allow accurate and precise quantitation of multiple drugs in presence of their degradation products and interaction product if any.

Various publications are available regarding determination method of aspirin and clopidogrel but most of the methods are applicable to alone aspirin or clopidogrel in pharmaceutical dosage form or in bilogical fluids. Only three method are reported for the simulteneous determination of apirin and clopidogrel. One is semi-micro column HPLC-UV method for simultaneous determination of clopidogrel metabolite, aspirin and salicylic acid in rat plasma. Second is a spectrophotometric method, which is able to determine aspirin and clopidogrel in combine dosage form and third is simple high performance liquid chromatography, which applicable to routine quality control sample analysis. The separation is performed by high performance liquid chromatography for reasons of robustness and familiarity of analysts with this technique. To our knowledge, no stability-indicating analytical method for the determination of aspirin and clopidogrel in combine dosage forms has been published. The previous published methods are not directly applicable for this issue and need more investigation for method development and validation.

Consequently, the focus in the present study was to develop a validated stability indicating HPLC method for the combination, by degrading the drugs together under various stress conditions like acid hydrolysis, base hydrolysis, oxidation, thermal and photolytic stress which is recommended by ICH guideline.

The aim and scope of the proposed work are as under

- To developed suitable HPLC method for simultaneous determination aspirin and clopidogrel in tablet formulation.
- Forced degradation study of aspirin and clopidogrel under stress condition.
- To resolve all major impurities generated during the force degradation studies of aspirin and clopidogrel.
- Perform the validation for the developed method.

4. Experimental

4.1 Materials

Pharmacopoeial grade standards of aspirin and clopidogrel bisulphate were provided by reputed pharma company. A tablet containing 150 mg aspirin and 75 mg clopidogrel was commercially available. HPLC grade acetonitrile, methanol and water were obtained from Spectrochem Pvt. Ltd., Mumbai (India). Analytical grade hydrochloric acid, sodium hydroxide pellets, orthophosphoric acid and hydrogen peroxide solution (30% v/v) were obtained from Ranbaxy Fine Chemical, New Delhi (India).

4.2 Instrumentation

LC-10AT*vp* HPLC system was used as describe as Part-[A] (5.A).

4.3 Mobile Phase Preparation

The mobile phase was consisted of 0.3% ortho phosphoric acid (v/v)- acetonitrile (65:35, v/v). Mobile phase was filtered through a 0.45 µm nylon membrane (Millipore Pvt. Ltd. Bangalore, India) and degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai).

4.4 Diluent Preparation

Mobile phase used as a diluent.

4.5 Standard Preparation

Standard solution containing aspirin (0.075 mg/ml) and clopidogrel (0.0375 mg/ml) were prepared by dissolving 37.5 mg aspirin and 24.46 mg clopidogrel bisulphate (equivalent to 18.5 mg clopidogrel) in 100 ml volumetric flask by mobile phase (stock standard solution). Pipette out 10 ml stock solution into 50 ml volumetric flask and dilute up to mark with mobile phase (standard solution).

4.6 Test Preparation

Twenty tablets were weighed and the average tablet weight was determined. Tablets were crushed by mortar and pastel. Tablet powder was weighed equivalent to five times of average weight and transfer in to 200 ml volumetric flask. About 170 ml mobile phase was added and sonicated for of 30 min time interval with intermittent shaking. Content was brought back to room temperature and dilute to volume with mobile phase (stock solution). The stock solution was filtered through 0.45 µm nylon syringe filter. Pipette out 2 ml filtered stock solution in to 100 ml volumetric flask and diluted with mobile phase (test solution). The concentration obtain was 0.075 mg/ml of aspirin and 0.0375 mg/ml of clopidogrel.

4.7 Chromatographic Conditions

Chromatographic analysis was performed on a Phenomenex Luna C8(2) (250 mm 4.6 mm i.d., 5 µm particle size) column. The mobile phase was consisted of 0.3% orthophosphoric acid (v/v) - acetonitrile (65:35, v/v). The flow rate of the mobile phase was adjusted to 1.0 ml/min and the injection volume was 20 µl. Detection was performed at 226 nm.

5. Result and Discussion

5.1 Development and Optimization of the HPLC Method

In the presence work, an analytical method based on LC using UV detection was developed and validated for assay determination of aspirin and clopidogrel in tablet formulation. The analytical conditions were selected, keeping in mind the different chemical nature of aspirin and clopidogrel. The development trials were taken by using the degraded sample of each component was done, by keeping them in various extreme conditions.

The column selection has been done on the basis of backpressure, resolution, peak shape, theoretical plates and day-to-day reproducibility of the retention time and resolution between aspirin and clopidogrel peak. After evaluating all these factors, C8 (2) (250 mm 4.6 mm i.d., 5 µm particle size) column was found to be giving satisfactory results. The selection of buffer based on chemical structure of both the drugs. The acidic pH range was found suitable for solubility, resolution, stability, theoretical plates and peak shape of both components. Best results were obtained with 0.3% orthophosphoric acid solution improved the peak shape of aspirin and clopidogrel. Finally, by fixing 0.3% orthophosphoric acid (v/v) and mobile phase composition consisting of a mixture of 0.3% orthophosphoric acid (v/v)-acetonitrile (65:35, v/v). Optimized mobile phase proportion was provide good resolution between aspirin and clopidogrel and also for degradation product which is generated during force degradation study. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. Figure 3 and Figure 4 represent the chromatograms of standard and test preparation respectively.

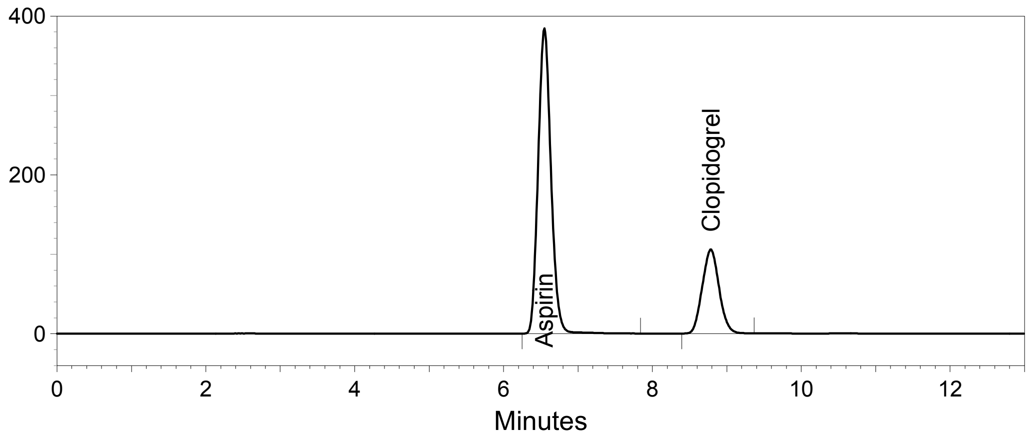


Figure 3: Chromatogram of standard preparation

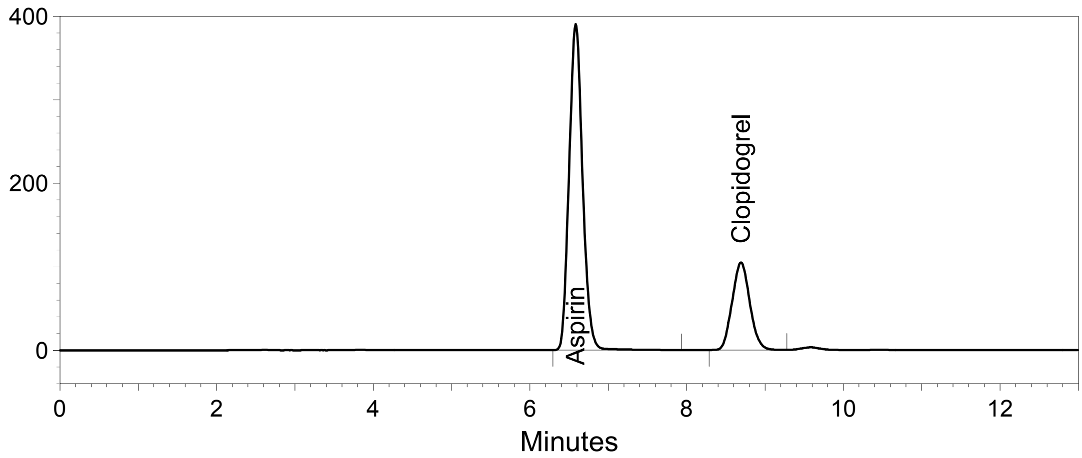


Figure 4: Chromatogram of test preparation

5.2 Degradation Study

In order to determine whether the analytical method or assay were stability-indicating, aspirin and clopidogrel combine tablets were stressed under various conditions to conduct forced degradation studies. Regulatory guidance in ICH Q2A, Q2B, Q3B and FDA 21 CFR section 211 all require the development and validation of stability-indicating potency assays. Unfortunately, the current guidance documents do not indicate detailed degradation conditions in stress testing. However, the used forced degradation conditions, stress agent concentration and time of stress, were found to effect a degradation and not complete degradation of active materials. The discovery of such conditions was based on development trial.

The degradation samples were prepared by transferring powdered tablets, equivalent to 150 mg aspirin and 75 mg clopidogrel into a 250 ml round bottom flask. Then prepared samples were employed for acidic, alkaline and oxidant media and also for thermal and photolytic stress conditions. After the degradation treatments were completed, the stress content solutions were allowed to equilibrate to room temperature and diluted with mobile phase to attain 0.075 mg/ml of aspirin and 0.0375 mg/ml of clopidogrel concentration. Specific degradation conditions were described as follows.

5.2.1 ***Acidic condition:*** Acidic degradation study was performed by heating the drug content in 1 N HCl (50 ml)at 80 ^{0}C for 1 h and mixture was neutralized with 1 N NaOH solution. The drug content was found to be degrading up to 16.93% in acidic condition (Figure 5).

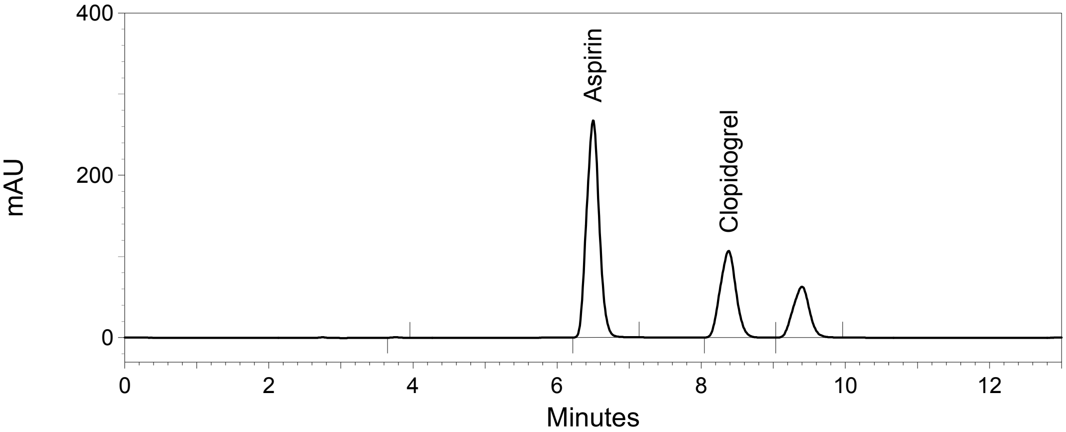


Figure 5: Chromatogram of acidic forced degradation study

5.2.2 Alkaline condition: Alkaline degradation study was performed by heating the drug content in 1 N NaOH (50 ml) at 80 ^{0}C for 1 h and mixture was neutralized with 1 N HCl solutions. In alkali degradation, it was found that around 22.00% of the drug degraded (Figure 6).

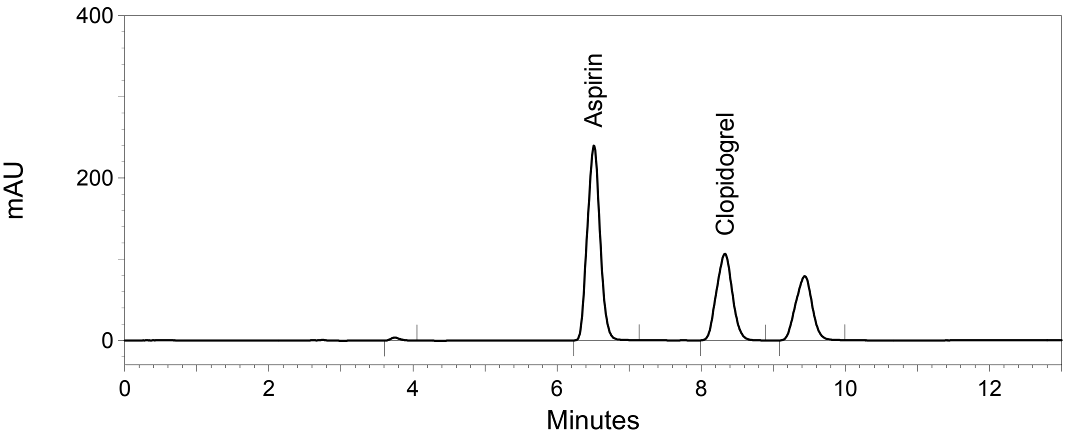


Figure 6: Chromatogram of alkali forced degradation study

5.2.3 Oxidative condition: Oxidation degradation study was performed by heating the drug content in 3% v/v H_2O_2(50 ml) at 80 ^0C for 30 min. In oxidative degradation, it was found that around 15.84% of the drug degraded (Figure 7).

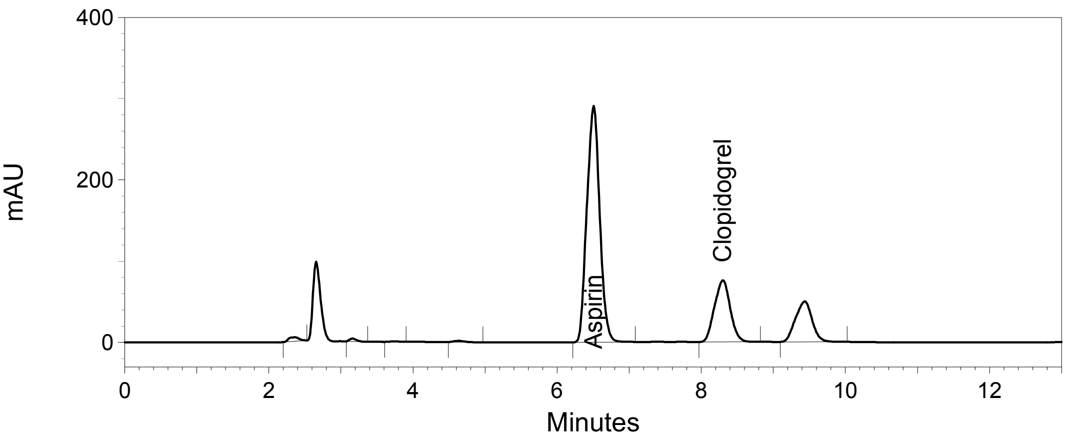


Figure 7: Chromatogram of oxidative forced degradation study

5.2.4 Thermal condition: Thermal degradation was performed by exposing solid drug at 80 ^0C for 72 h. Resultant chromatogram of thermal degradation study (Figure 8) indicate that nebivolol is found to be slightly stable under thermal degradation condition. Only 7.0% drug content were degraded.

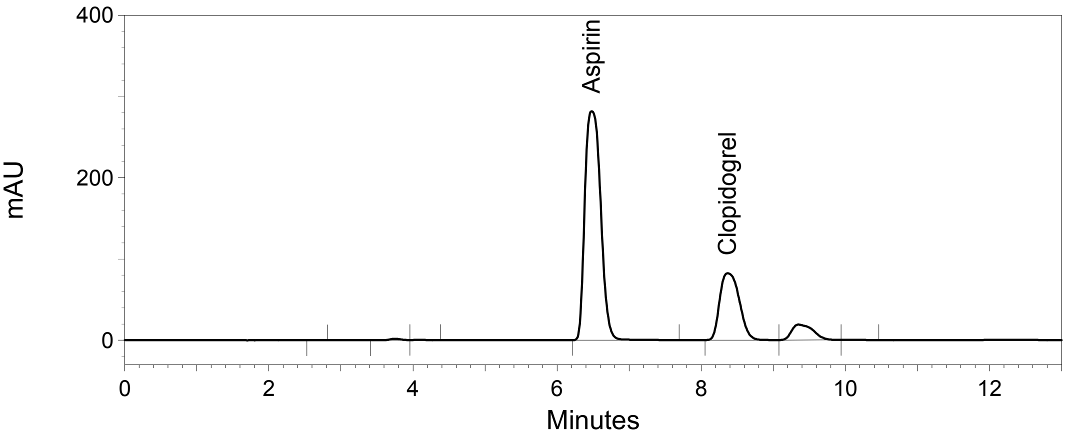


Figure 8: Chromatogram of thermal degradation study

5.2.5 Photolytic condition: Photolytic degradation study was performed by exposing the drug content in sun-light for 72 h. There is 5.0% degradation observed in above specific photolitic condition. Drug content was found to be more stable than other stress condition stable in UV-light (Figure 9).

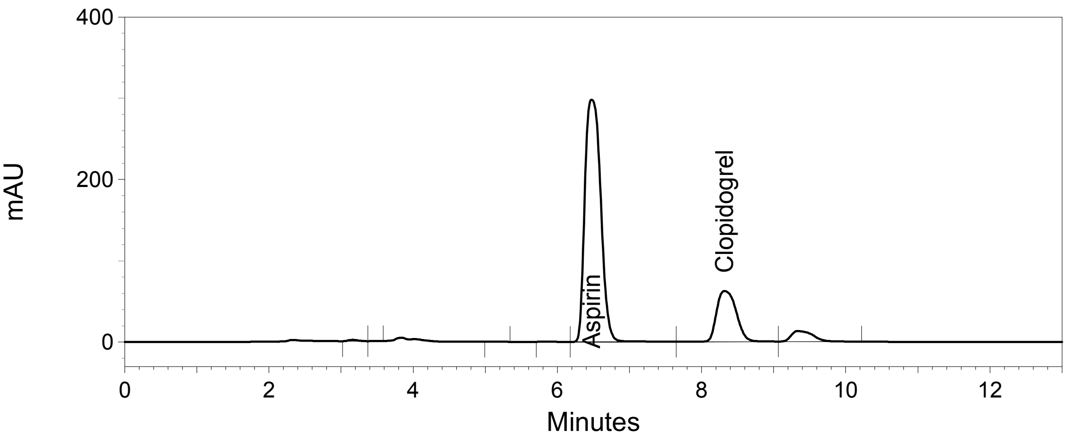


Figure 9: Chromatogram of UV-light degradation study

5.3 Method Validation

5.3.1 Specificity: The specificity of the method was determined by checking the interference of placebo with analyte and the proposed method were eluted by checking the peak purity of aspirin and clopidogrel during the force degradation study. The peak purity of the aspirin and clopidogrel were found satisfactory under different stress condition. There was no interference of any peak of degradation product with drug peak.

5.3.2 Linearity: For linearity seven points calibration curve were obtained in a concentration range from 0.030-0.120 mg/ml for aspirin and 0.015-0.060 mg/ml for clopidogrel. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation for aspirin was y= 60026378.57x + 51410.11 with correlation coefficient 0.9999(Figure 10) and for clopidogrel was y = 44544414.03x - 1890.29 with correlation coefficient 0.9999 (Figure 11). Where x is the concentration in mg/ml and y is the peak area in absorbance unit. Chromatogram obtain during linearity study were shown in Figure 12-18.

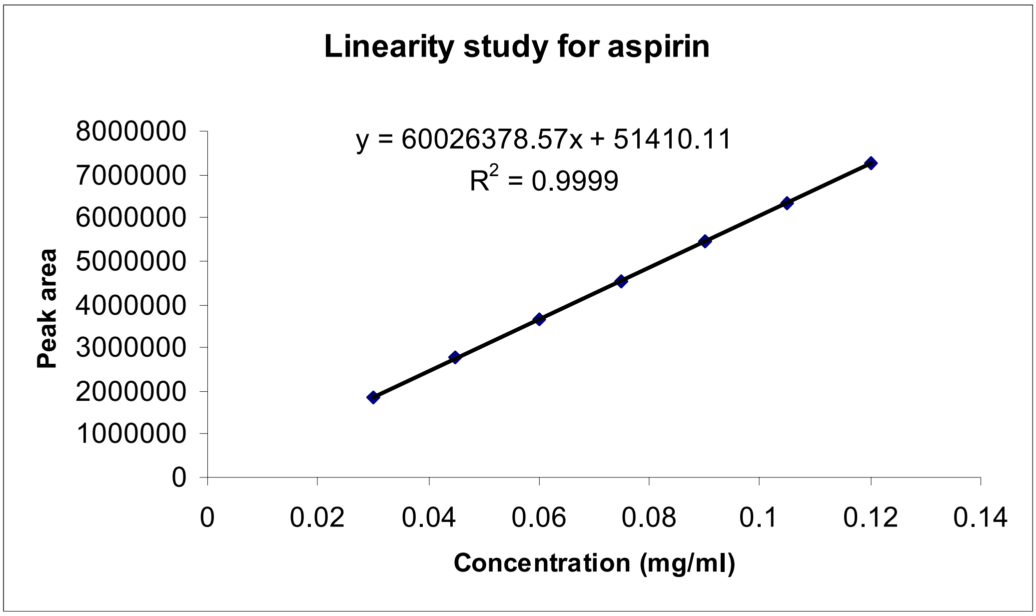


Figure 10: Linearity curve for aspirin

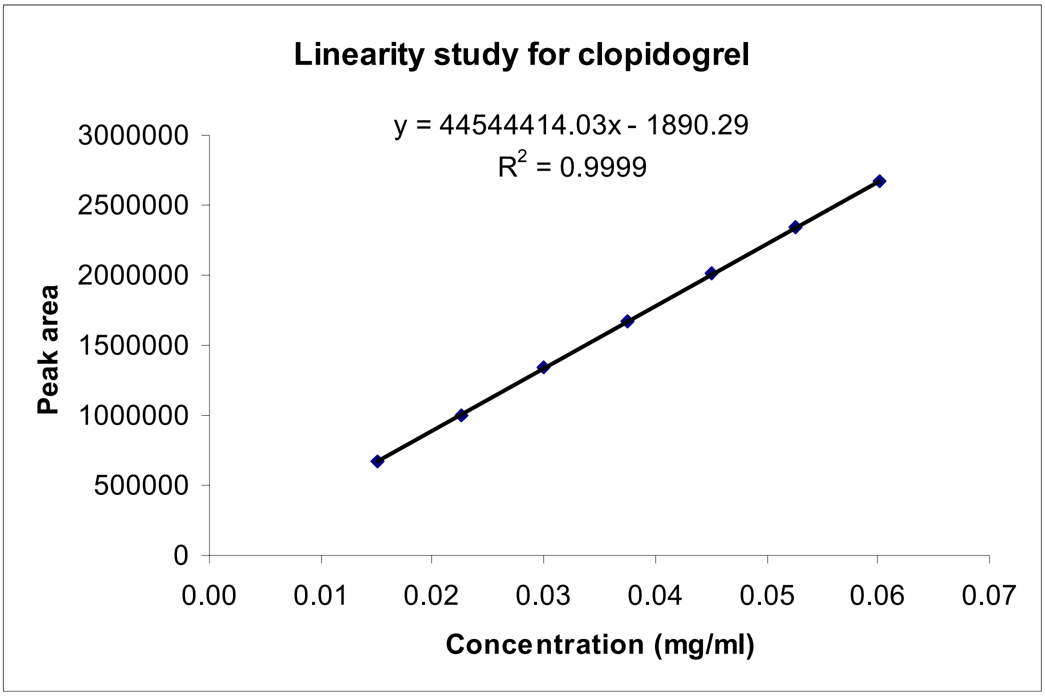


Figure 11: Linearity curve for clopidogrel

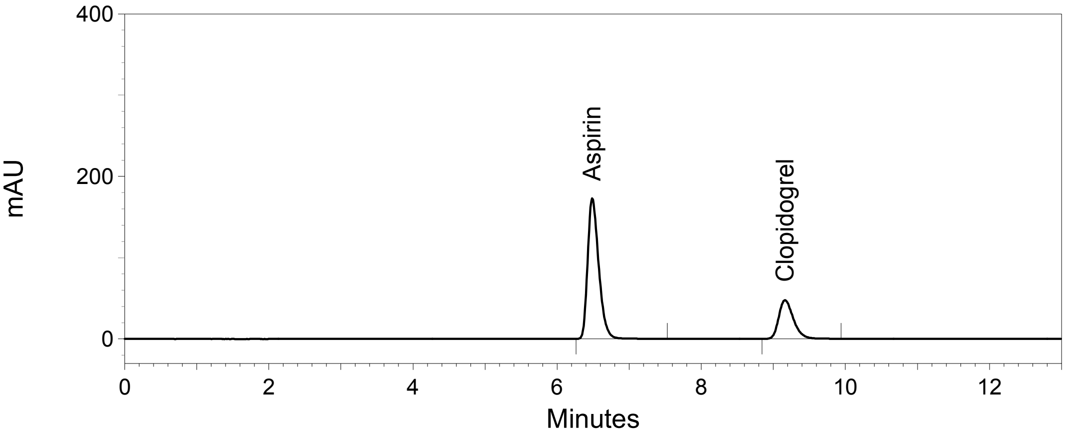


Figure 12: Linearity study chromatogram of level-1 (40%)

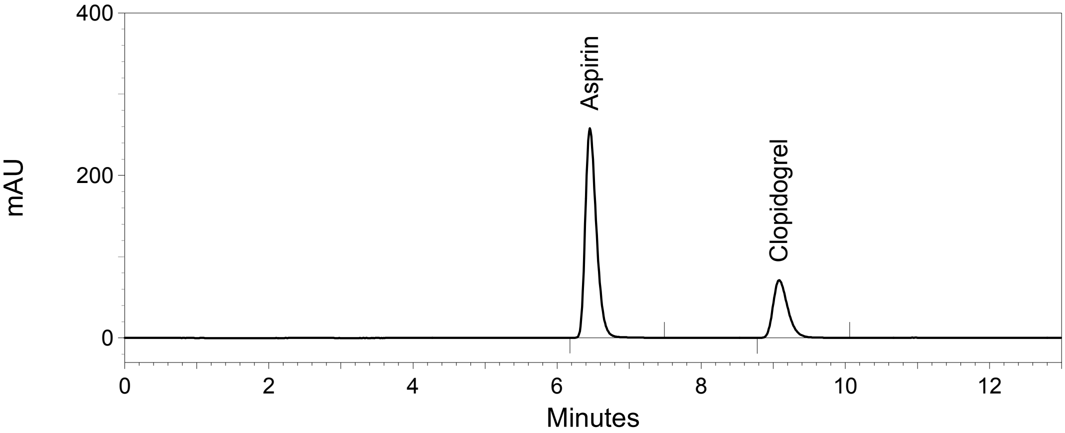


Figure13: Linearity study chromatogram of level-2 (60%)

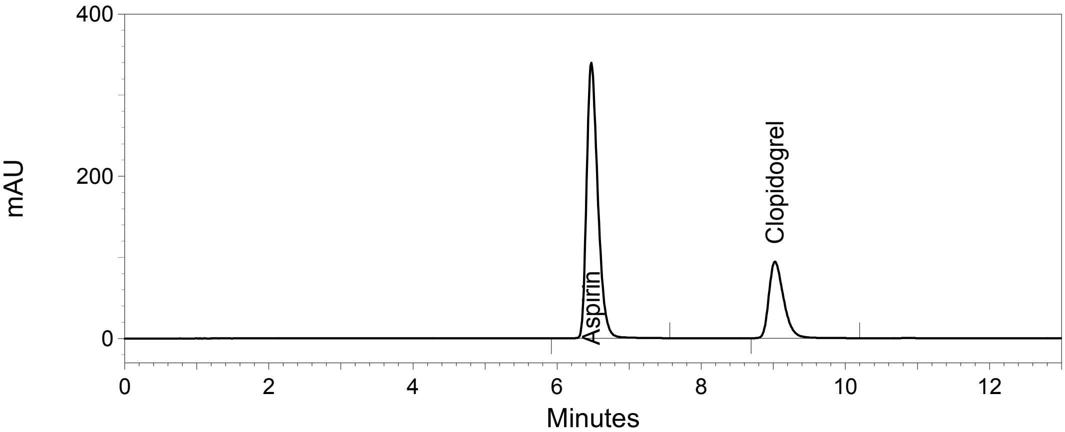


Figure14: Linearity study chromatogram of level-3 (80%)

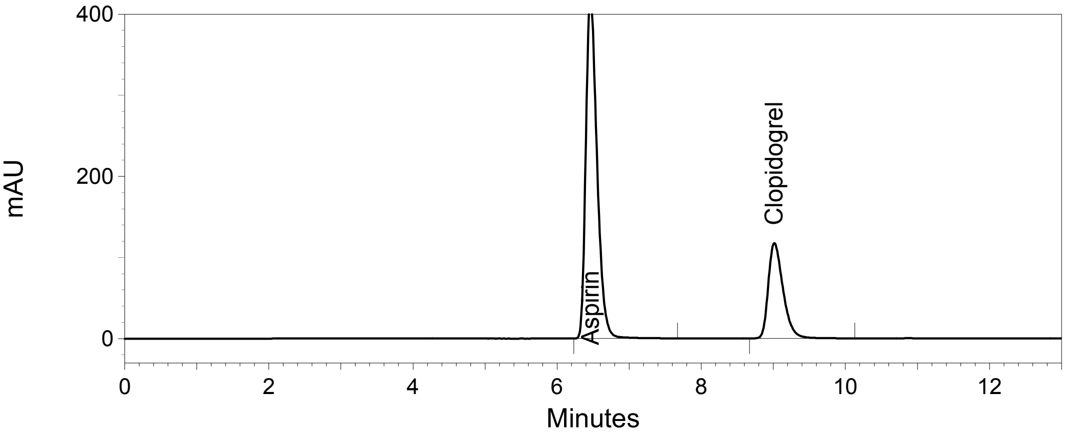


Figure 15: Linearity study chromatogram of level-4 (100%)

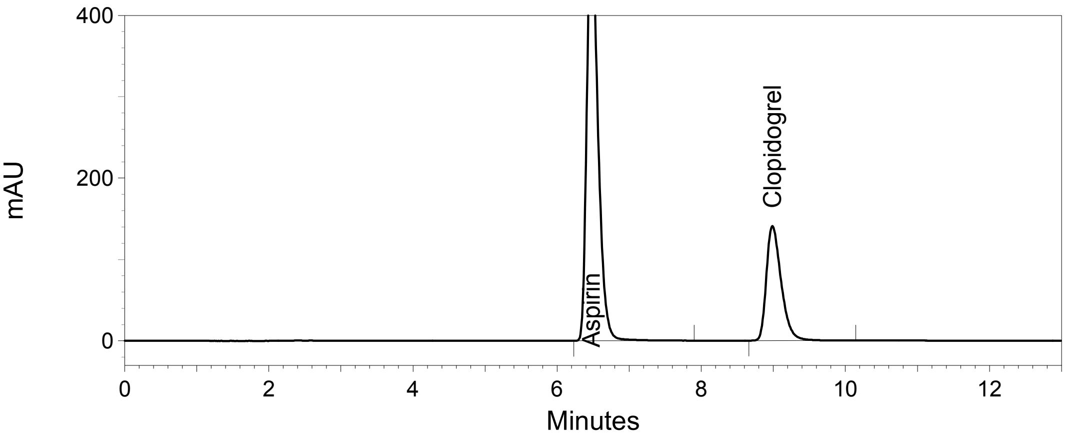


Figure 16: Linearity study chromatogram of level-5 (120%)

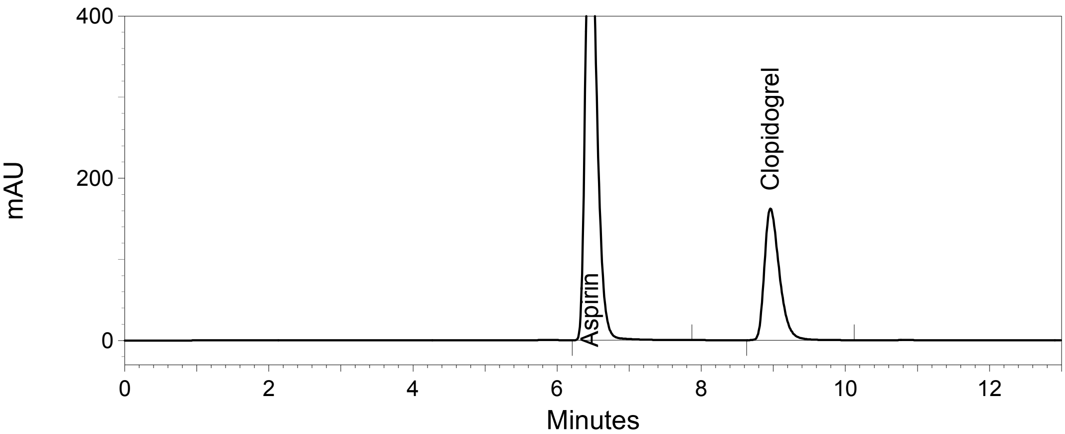


Figure 17: Linearity study chromatogram of level-6 (140%)

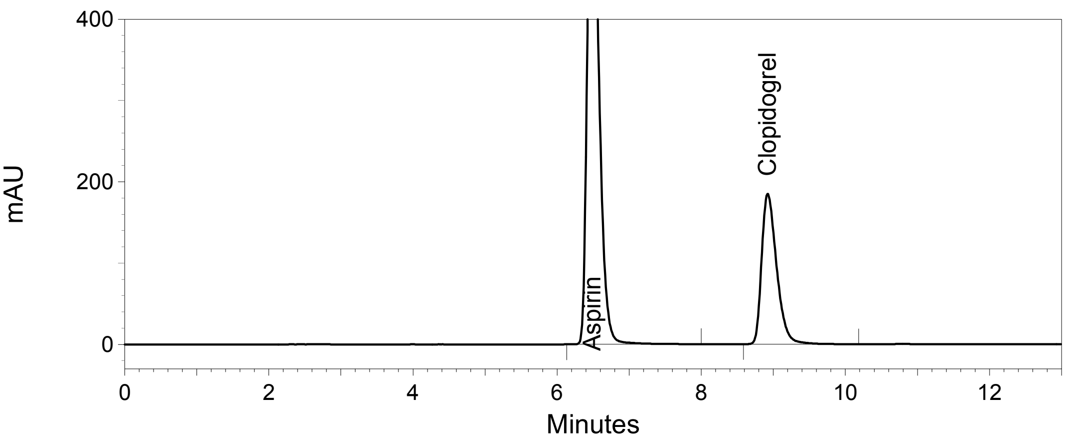


Figure 18: Linearity study chromatogram of level-7 (160%)

5.3.3 LOD and LOQ: The limit of detection and limit of quantification were evaluated by serial dilutions of aspirin and clopidogrel stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 fro LOQ. The LOD value for aspirin and clopidogrel were found to be 0.05 ppm and 0.15 ppm, respectively and the LOQ value 0.2 ppm and 0.3 ppm, respectively. Chromatogram of LOD and LOQ study were shown in Figure 19-22.

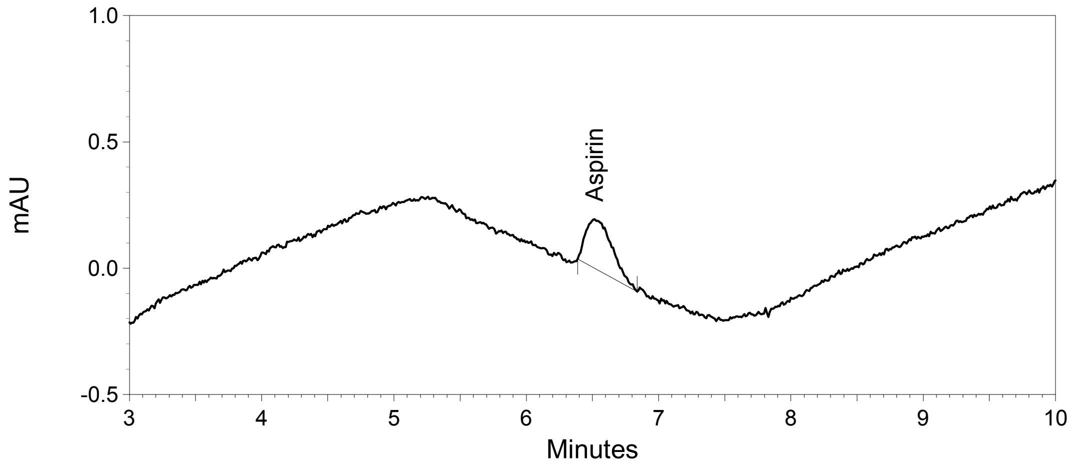


Figure 19: Chromatogram of LOD study of aspirin

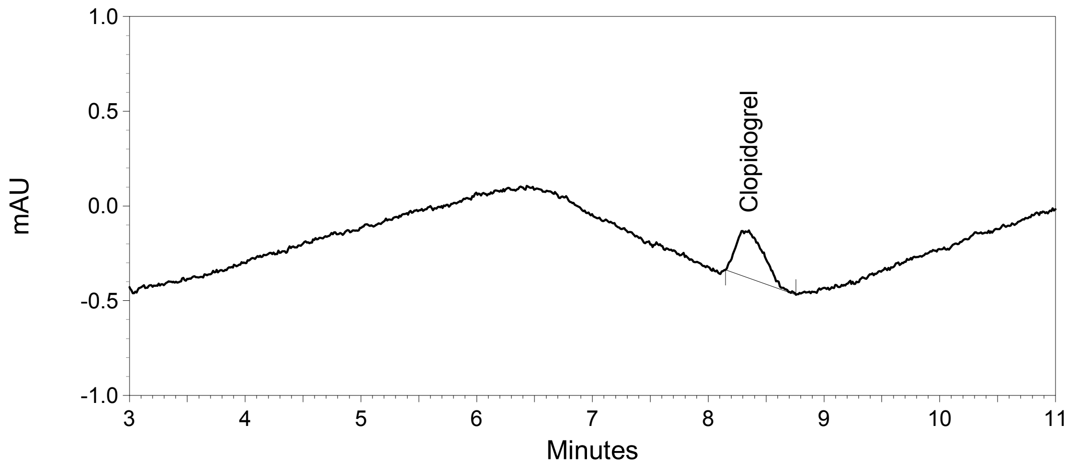


Figure 20: Chromatogram of LOD study of clopidogrel

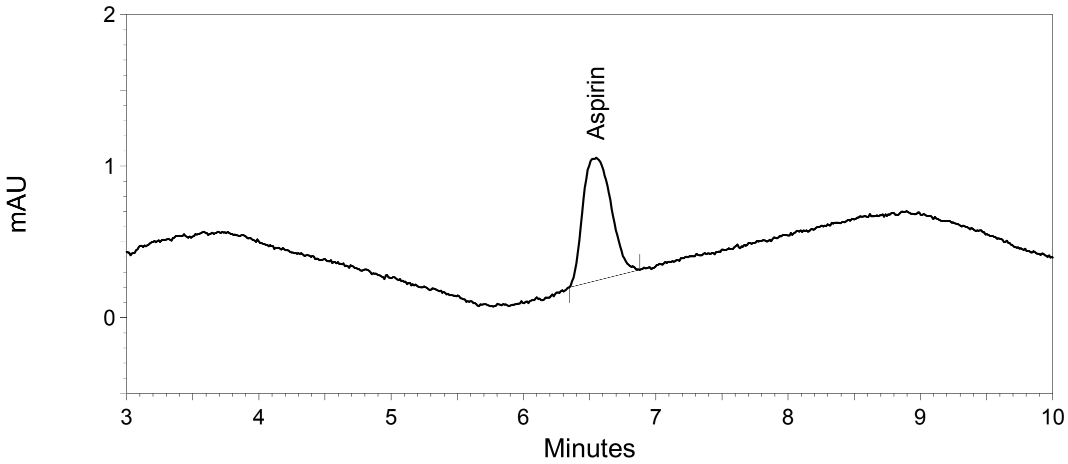


Figure 21: Chromatogram of LOQ study of aspirin

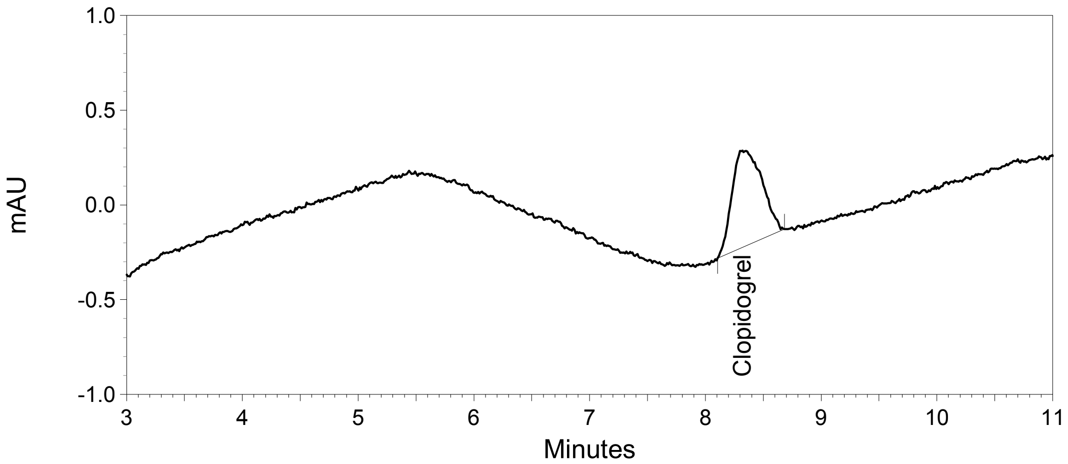


Figure 22: Chromatogram of LOQ study of clopidogrel

5.3.4 Precision: Data obtain from precision experiments are given in Table 1 for intraday and interday precision study for both aspirin and clopidogrel. The RSD values for intra day precision study and interday precision study was < 2.0% for aspirin and clopidogrel. Which confirm that the method was precise.

Table 1: Results of precision study

Set	Aspirin (%Assay)		Clopidogrel (%Assay)	
	Intraday (n = 6)	Interday (n = 6)	Intraday (n = 6)	Intraday (n = 6)
1	99.1	100.2	99.3	99.6
2	100.0	99.9	98.7	99.6
3	99.6	100.5	98.6	100.1
4	99.5	100.3	99.0	100.1
5	100.3	101.0	100.0	100.6
6	99.1	100.8	99.5	100.7
Mean	99.6	100.5	99.2	100.1
Standard deviation	0.48	0.40	0.53	0.47
% RSD	0.48	0.40	0.53	0.47

5.3.5 Accuracy: Recovery of aspirin and clopidogrel were determined at three different concentration levels. The mean recovery for aspirin was 99.12-99.83 % and 98.20-100.35 % for clopidogrel (Table 2). The result indicating that the method was accurate. Chromatogram obtain during accuracy study were shown in Figure 23-25.

Table 2: Results of accuracy study

	Level (%)	Amount Added Concentration[a] (mg/ml)	Amount Found Concentration[a] (mg/ml)	% Recovery[a]	% RSD[a]
Aspirin	50	0.03751	0.03721	99.22	0.07
	100	0.07497	0.07432	98.12	0.23
	150	0.11250	0.11232	99.83	0.05
Clopidogrel	50	0.01874	0.01840	98.20	0.19
	100	0.03748	0.03695	98.59	0.14
	150	0.05627	0.05647	100.35	0.24

[a] Each value corresponds to the mean of three determinations.

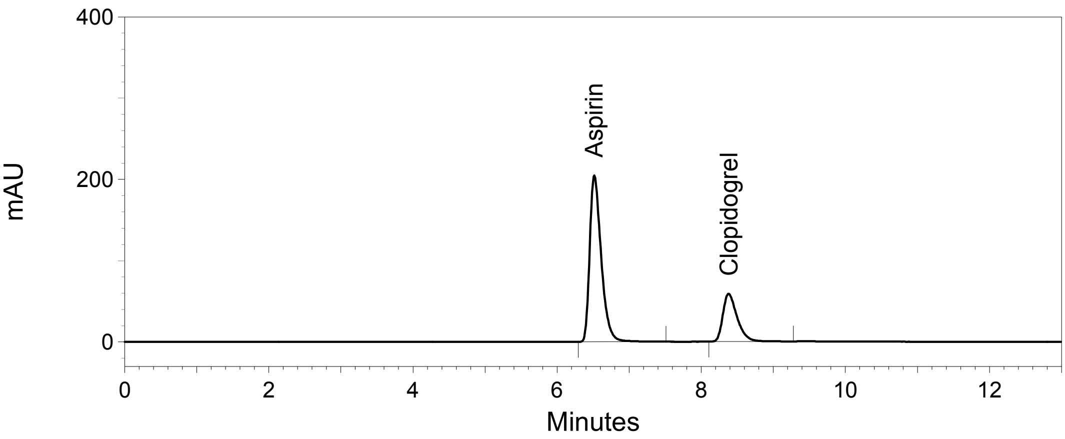


Figure 23: Accuracy study chromatogram of level-1 (50%)

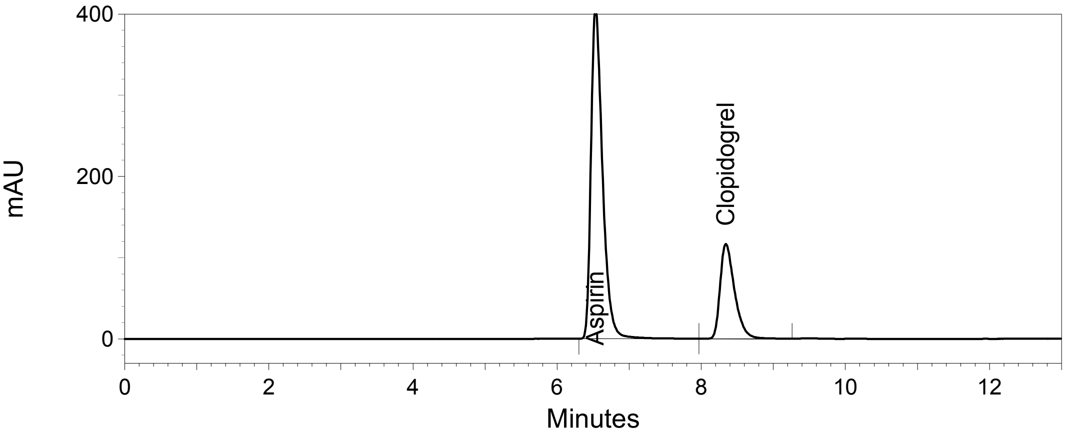


Figure 24: Accuracy study chromatogram of level-2 (100%)

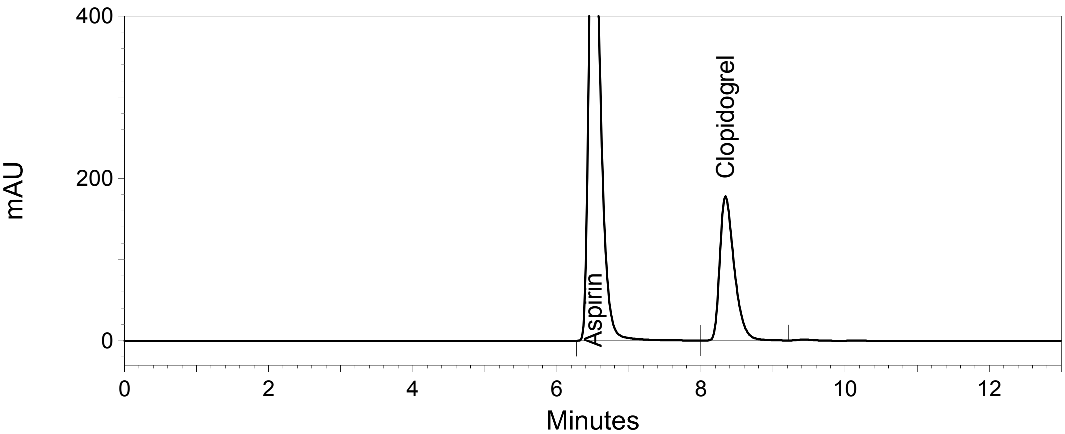


Figure 25: Accuracy study chromatogram of level-3 (150%)

5.3.6 Solution stability study: Table 3 shows the results obtain in the solution stability study at different time intervals for test preparation. It was concluded that the test preparation solution was found stable up to 48 h at 2-5 ^{0}C and 36 h at ambient temperature with the consideration of < 2.0% in %assay value difference of inteval value against initial value.

Table 3: Evaluation data of solution stability study

Intervals	% Assay for Test Solution Stored at 2 –5 ^{0}C		% Assay for Test Solution Stored at Ambient Temperature	
	Aspirin	Clopidogrel	Aspirin	Clopidogrel
Initial	101.3	100.0	101.3	100.0
12 h	100.9	99.3	100.1	99.5
24 h	100.3	99.6	99.8	99.0
36 h	100.1	99.0	99.8	98.6
48 h	99.9	98.7	98.1	98.7

5.3.7 Robustness: The result of robustness study of the developed assay method was established in Table 4 and Table 5. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory, hence the analytical method would be concluded as robust. Chromatogram obtain during robustness study were shown in Figure 26-32.

Table 4: Evaluation data of robustness study of aspirin

Robust Conditions	% Assay	System Suitability Parameters		
		Theoretical Plates	Asymmetry	% RSD
Flow 0.9 ml/min	100.5	6460	1.05	0.22
Flow 1.1 ml/min	100.3	5661	1.05	0.09
0.28 % H_3PO_4-ACN (65:35, v/v)	100.0	6117	1.00	0.40
0.32 % H_3PO_4-ACN (65:35, v/v)	99.7	5588	1.02	0.30
0.3% H_3PO_4-ACN (63:37,v/v)	100.2	5475	1.12	0.19
0.3% H_3PO_4-ACN (67:33,v/v)	100.1	5838	1.04	0.20
Column change	100.4	5425	1.05	0.34

Table 5: Evaluation data of robustness study for clopidogrel

Robust Conditions	% Assay	System Suitability Parameters			
		Theoretical Plates	Asymmetry	% RSD	Resolution
Flow 0.9 ml/min	98.5	6975	1.07	0.67	5.75
Flow 1.1 ml/min	100.0	5992	1.06	0.39	5.14
0.28 % H_3PO_4-ACN(65:35,v/v)	98.8	6899	1.03	1.03	6.35
0.32 % H_3PO_4-ACN(65:35,v/v)	99.2	6113	1.03	0.69	5.62
0.3% H_3PO_4-ACN(63:37,v/v)	99.5	5850	1.11	0.70	4.31
0.3% H_3PO_4-ACN (67:33,v/v)	99.0	6185	1.04	0.30	5.71
Column change	100.0	5996	1.04	0.25	4.97

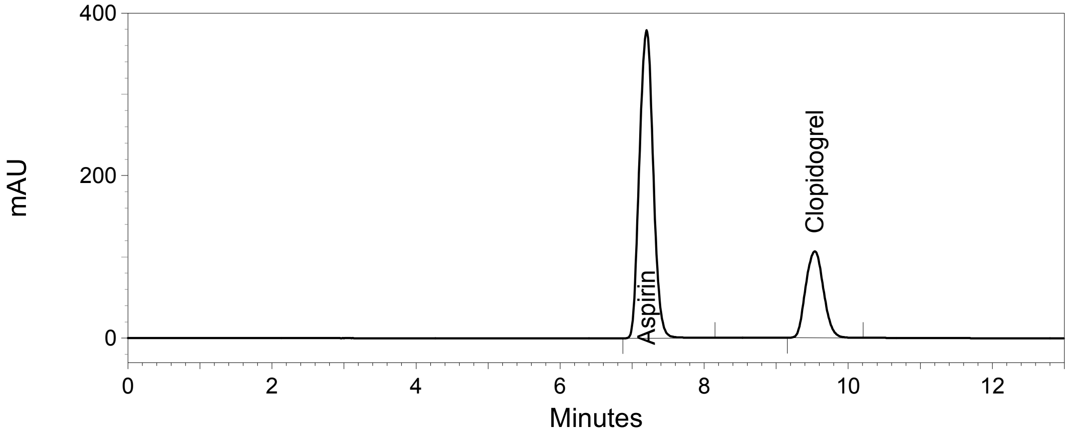


Figure 26: Standard chromatogram (0.9 ml/min flow rate)

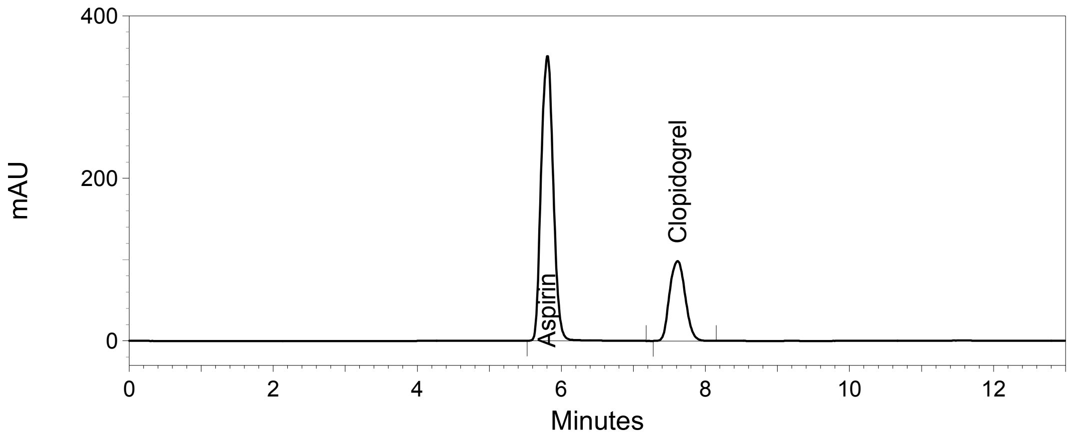


Figure 27: Standard chromatogram (1.1 ml/min flow rate)

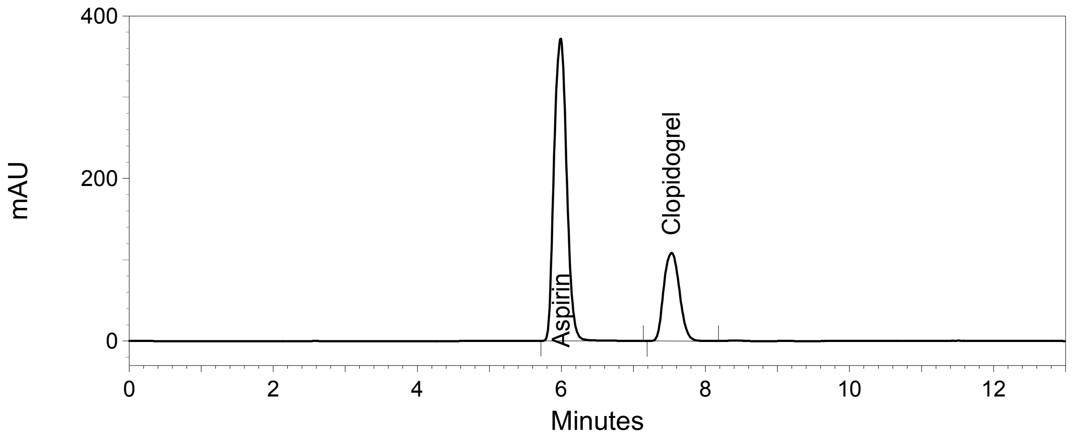


Figure 28: Standard chromatogram [0.3% H_3PO_4-ACN (63:37,v/v)]

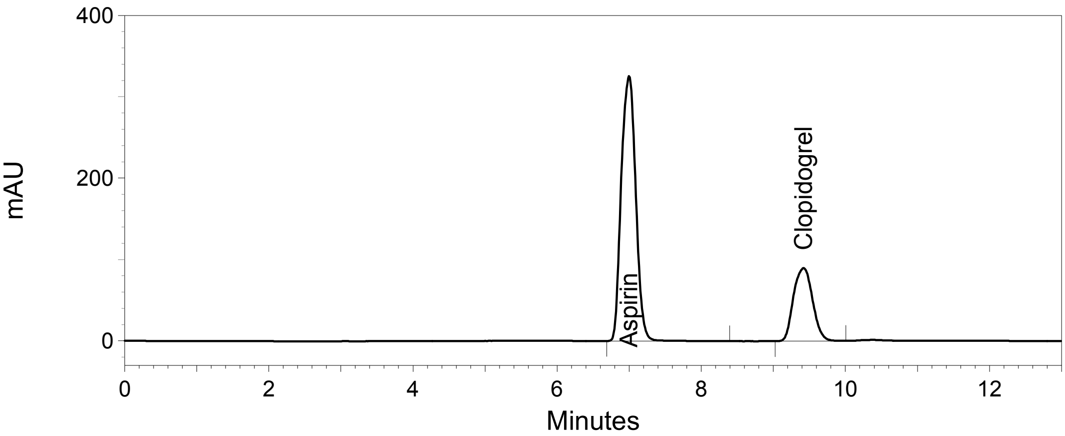


Figure 29: Standard chromatogram [0.3% H_3PO_4-ACN (67:33,v/v)]

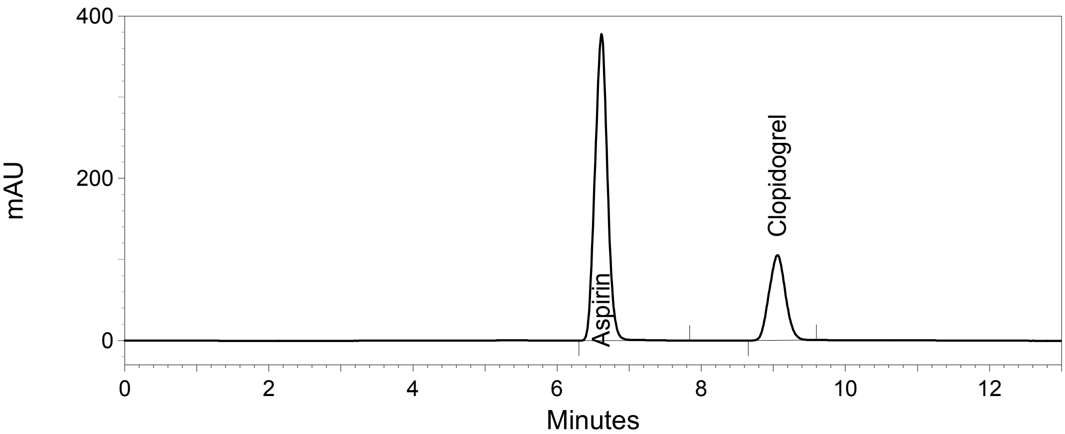


Figure 30: Standard chromatogram [0.28% H_3PO_4-ACN (65:35, v/v)]

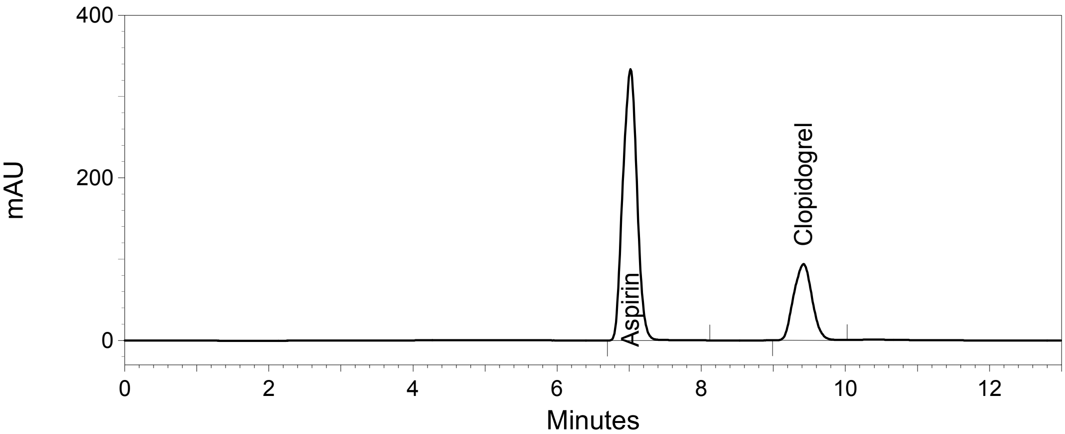


Figure 31: Standard chromatogram [0.32% H_3PO_4-ACN (65:35, v/v)]

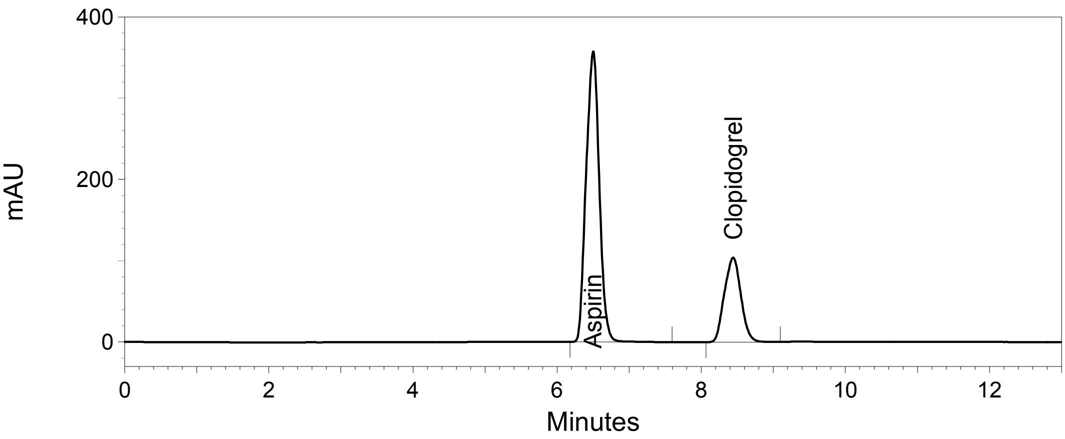


Figure 32: Standard chromatogram (Column change)

5.3.8 System suitability: A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate, resolution and % RSD of peak area were determined for same. Acceptance criteria for system suitability, asymmetry should not be more than 2.0, theoretical plate should not be less then 4000 and %RSD of peak area should not be more then 2.0, were full fill during all validation parameters.

6. Calculation and Data

Calculation formula used:

1. Calculation formula for aspirin

$$\% \text{ Assay} = \frac{\text{Mean Test Area}}{\text{Mean Standard Area}} \times \frac{\text{Standard Weight}}{100} \times \frac{10}{50} \times \frac{200}{\text{Test Weight}}$$

$$\times \frac{100}{2} \times \frac{\text{Average Weight}}{\text{Lable Claim}} \times \text{Potency of Standard}$$

2.Calculation formula for clopidogrel

$$\% \text{ Assay} = \frac{\text{Mean Test Area}}{\text{Mean Standard Area}} \times \frac{\text{Standard Weight}}{100} \times \frac{10}{50} \times \frac{321.83}{419.83}$$

$$\times \frac{200}{\text{Test Weight}} \times \frac{100}{2} \times \frac{\text{Average Weight}}{\text{Lable Claim}} \times \text{Potency of Standard}$$

3. Relative standard deviation

$$\% \text{ RSD} = \frac{\text{Standard Deviation of Measurements}}{\text{Mean Value of Measurements}} \times 100$$

4. Recovery

$$\% \text{ Recovery} = \frac{\text{Amount Found}}{\text{Amount Added}} \times 100$$

5. Amount Found

$$\text{Amount Found (mg/ml)} = \frac{\text{Mean Test Area}}{\text{Mean Standard Area}} \times \text{Standard Concentration}$$

6. Amount added

$$\text{Amount Added (mg/ml)} = \frac{\text{Weight}}{\text{Volume}}$$

Specificity Study for Analytical Method Validation of Aspirin Clopidogrel Tablets

- **For Aspirin**

Standard Weight (mg)	37.5		
Standard Dilution	100	10	50
Standard Potency	99.97 %		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0750		

Replicate	1	2	3	4	5
Standard Area	4600786	4604792	4601538	4598274	4603409
Mean Standard Area	4601760				
Stdev	2238.86				
% RSD	0.05				

Replicate	**Test Area**
1 2	4666587 4662838
Mean Test Area	4664713
Test Weight (mg)	2007.5
Label Claim (mg)	150
Average Test Weight (mg)	400.1
% Assay	101.0 %

Calculation:

Prototype calculation for one set:

$$\% \text{ Assay} = \frac{4664713}{4601760} \times \frac{37.5}{100} \times \frac{10}{50} \times \frac{200}{2007.5} \times \frac{100}{2} \times \frac{400.1}{150} \times 99.97$$

= 101.0 %

- **For Clopidogrel**

Standard Weight (mg)	25.2		
Standard Dilution	100	10	50
Standard Potency	99.38 %		
Factor 1	321.83		
Factor 2	419.83		
Standard Concentration (mg/ml)	0.0386		

Replicate	1	2	3	4	5
Standard Area	1655001	1660845	1662939	1662004	1666323
Mean Standard Area	1661422				
Stdev.	3693.83				
% RSD	0.22				

Replicate	**Test Area**
1 2	1638562 1638424
Mean Test Area	1638493
Test Weight (mg)	2007.5
Label Claim (mg)	75
Mean Test weight (mg)	400.1
% Assay	100.6 %

Calculation:

Prototype calculation for one set:

$$\% \text{ Assay} = \frac{1638493}{1661422} \times \frac{25.2}{100} \times \frac{10}{50} \times \frac{321.83}{419.83} \times \frac{200}{2007.5} \times \frac{100}{2} \times \frac{400.1}{75} \times 99.38$$

$$= 100.6\ \%$$

Linearity Study for Analytical Method Validation of Aspirin Clopidogrel Tablets

- **For Aspirin**

Standard Weight (mg)	37.5		
Standard Dilution	100	10	50
Standard Potency	99.97 %		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0750		
Concentration of Linearity Stock Solution (mg/ml)	0.3750		

Replicate	1	2	3	4	5
Standard Area	4651479	4643757	4621922	4607507	4582449
Mean Standard Area	4621423				
Stdev	27906.30				
% RSD	0.60				

Concentration Level (%)	Volume of Linearity stock solution taken (ml)	Diluted to (ml)	F Conce (m
40	4.0	50	0.0
60	6.0	50	0.0
80	8.0	50	0.0
100	10.0	50	0.0
120	12.0	50	0.0
140	14.0	50	0.1
160	16.0	50	0.1
		Correlation co-effic	
		Slope	
		Intercept	

➢ **For Clopidogrel**

Standard Weight (mg)	24.5		
Standard Dilution	100	10	50
Standard Potency	99.38 %		
Factor 1	321.83		
Factor 2	419.83		
Standard Concentration (mg/ml)	0.0375		
Concentration of Linearity Stock Solution (mg/ml)	0.1875		

Replicate	1	2	3	4	5
Standard Area	1693789	1688587	1683886	1683619	1679066
Mean Standard Area	1685789				
Stdev	5598.17				
% RSD	0.33				

Concentration Level (%)	**Volume of Linearity stock solution taken (ml)**	**Diluted to (ml)**	**F Conc (m**
40	4.0	50	0.
60	6.0	50	0.
80	8.0	50	0.
100	10.0	50	0.
120	12.0	50	0.
140	14.0	50	0.
160	16.0	50	0.
		Correlation co-ef	
		Slope	
		Intercept	

Precision Study for Analytical Method Validation of Aspirin Clopidogrel Tablets

- **For Aspirin**

Standard Weight (mg)	37.5		
Standard Dilution	100	10	50
Standard Potency	99.97 %		
Label Claim (mg)	150		
Mean Test Weight (mg)	400.1		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0750		

Replicate	1	2	3	4	5
Standard Area	4570460	4556977	4527249	4537188	4529478
Mean Standard Area	4544270				
Stdev	18748.71				
% RSD	0.41				

Description	Mean Area	Test Weight (mg)	% Assay
Set 1	4505006	2000.2	99.1
Set 2	4541669	1998.2	100.0
Set 3	4529604	2000.9	99.6
Set 4	4532550	2004.1	99.5
Set 5	4614090	2025.5	100.3
Set 6	4499658	1998.6	99.1
		Mean	99.60
		Stdev	0.48
		% RSD	0.48
		Confidence Level (95.0%)	0.51

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{4505006}{4544270} \times \frac{37.5}{100} \times \frac{10}{50} \times \frac{200}{2000.2} \times \frac{100}{2} \times \frac{400.1}{150} \times 99.97$$

= 99.1 %

➢ **For Clopidogrel**

Standard Weight (mg)	25.3		
Standard Dilution	100	10	50
Standard Potency	99.38 %		
Label claim (mg)	75		
Mean Test Weight (mg)	400.1		
Factor 1	321.83		
Factor 2	419.83		
Standard Concentration (mg/ml)	0.0388		

Replicate	1	2	3	4	5
Standard Area	1645676	1642399	1625264	1611740	1627881
Mean Standard Area	1630592				
Stdev	13766.51				
% RSD	0.84				

Description	Mean Area	Test weight (mg)	% Assay
Set 1	1574146	2000.2	99.3
Set 2	1563472	1998.2	98.7
Set 3	1564660	2000.9	98.6
Set 4	1573421	2004.1	99.0
Set 5	1605971	2025.5	100.0
Set 6	1576470	1998.6	99.5
		Mean	99.2
		Stdev	0.53
		% RSD	0.53
		Confidence Level (95.0%)	0.55

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{1574146}{1630592} \times \frac{25.3}{100} \times \frac{10}{50} \times \frac{321.83}{419.83} \times \frac{200}{2000.2} \times \frac{100}{2} \times \frac{400.1}{75} \times 99.38$$

$$= 99.3\ \%$$

Intermediate Precision Study for Analytical Method Validation of Aspirin Clopidogrel Tablet

➢ **For Aspirin**

Standard Weight (mg)	37.5		
Standard Dilution	100	10	50
Standard Potency	99.97 %		
Label Claim (mg)	150		
Mean Test Weight (mg)	400.1		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0750		

Replicate	1	2	3	4	5
Standard Area	4581410	4597915	4604993	4610703	4609156
Mean Standard Area	4600835				
Stdev	11934.85				
% RSD	0.26				

Description	Mean Area	Test Weight (mg)	% Assay
Set 1	4656542	2020.2	100.2
Set 2	4631669	2015.3	99.9
Set 3	4634482	2003.9	100.5
Set 4	4627280	2004.4	100.3
Set 5	4646497	1999.4	101.0
Set 6	4631718	1998.2	100.8
		Mean	100.5
		Stdev	0.40
		% RSD	0.40
		Confidence Level (95.0%)	0.42

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{4656542}{4600835} \times \frac{37.5}{100} \times \frac{10}{50} \times \frac{200}{2020.2} \times \frac{100}{2} \times \frac{400.1}{150} \times 99.97$$

= 100.2 %

➢ **For Clopidogrel**

Standard Weight (mg)	25.2		
Standard Dilution	100	10	50
Standard Potency	99.38 %		
Label Claim (mg)	75		
Mean Test Weight (mg)	400.1		
Factor 1	321.83		
Factor 2	419.83		
Standard Concentration (mg/ml)	0.0386		

Replicate	1	2	3	4	5
Standard Area	1666514	1664669	1660945	1662983	1664353
Mean Standard Area	1663893				
Stdev	2073.78				
% RSD	0.12				

Description	Mean Area	Test weight (mg)	% Assay
Set 1	1633699	2020.2	99.6
Set 2	1631190	2015.3	99.6
Set 3	1628789	2003.9	100.1
Set 4	1630551	2004.4	100.1
Set 5	1633870	1999.4	100.6
Set 6	1633828	1998.2	100.7
		Mean	100.1
		Stdev	0.47
		% RSD	0.47
		Confidence Level (95.0%)	0.49

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{1633699}{1683893} \times \frac{25.2}{100} \times \frac{10}{50} \times \frac{321.83}{419.83} \times \frac{200}{2020.2} \times \frac{100}{2} \times \frac{400.1}{75} \times 99.38$$

$$= 99.6\ \%$$

Comparison for Precision and Intermediate Precision Study for Analytical Method Validation for Aspirin Clopidogrel Tablets

➢ **For Aspirin**

	Set	%Assay
Precision study	1	99.1
	2	100.3
	3	99.6
	4	99.5
	5	101.0
	6	99.1
Intermediate precision study	1	101.2
	2	100.8
	3	100.7
	4	100.4
	5	101.1
	6	100.7
	Mean	100.3
	Stdev	0.77
	% RSD	0.77

➢ **For Clopidogrel**

	Set	% Assay
Precision study	1	99.3
	2	99.0
	3	98.6
	4	99.0
	5	100.7
	6	99.5
Intermediate precision study	1	100.5
	2	100.5
	3	100.2
	4	100.2
	5	100.7
	6	100.6
	Mean	99.9
	Stdev	0.77
	% RSD	0.77

Accuracy Study for Analytical Method Validation of Aspirin Clopidogrel Tablets

- **For Aspirin**

Standard Weight (mg)	37.7		
Standard Dilution	100	10	50
Standard Potency	99.97 %		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0754		

Replicate	1	2	3	4	5
Standard Area	4631817	4595351	4573625	4606829	4619422
Mean Standard Area	4605409				
Stdev	22400.61				
% RSD	0.49				

Recovery Level	Set No.	Mean Area	Weight (mg)	Volume-I (ml)	Volume-II (ml)	Volume-III (ml)	*Amount Added* Concentration (mg/ml)	*Amount Found* Concentration (mg/ml)	% Recovery	Mean % Recovery	Stdev	% RSD
50%	Set 1	2271481	375.1	200	2	100	0.03751	0.03719	99.15	99.22	0.07	0.07
	Set 2	2273400	374.9	200	2	100	0.03749	0.03722	99.28			
	Set 3	2274191	375.2	200	2	100	0.03752	0.03723	99.23			
100%	Set 1	4528928	750.0	200	2	100	0.07500	0.07415	98.87	98.12	0.22	0.23
	Set 2	4543714	749.7	200	2	100	0.07497	0.07439	99.23			
	Set 3	4544790	749.5	200	2	100	0.07495	0.07441	99.28			
150%	Set 1	6857099	1125.1	200	2	100	0.11251	0.11226	99.78	99.83	0.05	0.05
	Set 2	6860021	1124.9	200	2	100	0.11249	0.11231	99.84			
	Set 3	6864199	1125.1	200	2	100	0.11251	0.11238	99.88			

Calculation:

Prototype calculation for one set:

$$\%\ \text{Recovery} = \frac{0.03719}{0.03751} \times 100 = 99.15\ \%$$

$$\text{Amount Found (mg/ml)} = \frac{2271481}{4605409} \times 0.0754 = 0.03719\ \text{mg/ml}$$

$$\text{Amount Added (mg/ml)} = \frac{375.1}{200} \times \frac{2}{100} = 0.03751\ \text{mg/ml}$$

- **For Clopidogrel**

Standard Weight (mg)	24.9		
Standard Dilution	100	10	50
Standard Potency	99.38 %		
Factor 1	321.83		
Factor 2	419.83		
Standard Concentration (mg/ml)	0.0382		

Replicate	1	2	3	4	5
Standard Area	1696020	1683394	1678190	1682439	1693078
Mean Standard Area	1686624				
Stdev	7566.68				
% RSD	0.45				

Recovery Level	Set No.	Mean Area	Weight (mg)	Volume-I (ml)	Volume-II (ml)	Volume-III (ml)	*Amount Added* Concentration (mg/ml)	*Amount Found* Concentration (mg/ml)	% Recovery	Mean % Recovery	Stdev	% RSD
50%	Set 1	812320	187.4	200	2	100	0.01874	0.01840	98.19	98.20	0.19	0.19
	Set 2	814621	187.5	200	2	100	0.01875	0.01845	98.40			
	Set 3	810857	187.3	200	2	100	0.01873	0.01836	98.02			
100%	Set 1	1627220	374.4	200	2	100	0.03744	0.03685	98.42	98.59	0.14	0.14
	Set 2	1632699	374.8	200	2	100	0.03748	0.03698	98.67			
	Set 3	1634070	375.1	200	2	100	0.03751	0.03701	98.67			
150%	Set 1	2489276	562.7	200	2	100	0.05627	0.05638	100.20	100.35	0.27	0.27
	Set 2	2488239	562.5	200	2	100	0.05625	0.05636	100.20			
	Set 3	2501814	562.9	200	2	100	0.05629	0.05666	100.66			

Calculation:

Prototype calculation for one set:

$$\% \ \text{Recovery} = \frac{0.01840}{0.01874} \times 100 = 98.19\ \%$$

$$\text{Amount Found (mg/ml)} = \frac{812320}{1686624} \times 0.0382 = 0.01840 \text{ mg/ml}$$

$$\text{Amount Added (mg/ml)} = \frac{187.4}{200} \times \frac{2}{100} = 0.01874 \text{ mg/ml}$$

Solution Stability Study for Analytical Method Validation of Aspirin Clopidogrel Tablets

➢ **For Aspirin**

System suitability of standard preparation for solution stability					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	4590829	4595107	4578851	4586051	4604645
2	4568423	4592801	4577832	4593989	4624750
3	4568097	4577366	4582843	4612737	4625372
4	4592898	4591664	4555542	4597328	4615701
5	4568068	4600193	4571090	4593089	4622415
Mean	4577663	4591426	4573232	4596639	4618577
Stdev	12984.59	8515.29	10754.40	9891.30	8680.15
%RSD	0.28	0.19	0.24	0.22	0.19

Solution stability for standard preparation at 2-8 °C				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	4590829	4590829	4590829	4590829
2	4568423	4568423	4568423	4568423
3	4568097	4568097	4568097	4568097
4	4592898	4592898	4592898	4592898
5	4568068	4568068	4568068	4568068
1	4580963	4552862	4578125	4568463
2	4560334	4559105	4556889	4580056
Mean	4575659	4571469	4574761	4576691
Stdev	12632.41	15084.85	13211.31	11240.89
%RSD	0.28	0.33	0.29	0.25

Solution stability for standard preparation at room temperature				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	4590829	4590829	4590829	4590829
2	4568423	4568423	4568423	4568423
3	4568097	4568097	4568097	4568097
4	4592898	4592898	4592898	4592898
5	4568068	4568068	4568068	4568068
1	4529151	4503255	4465031	4441407
2	4515593	4493929	4466973	4427586
Mean	4561866	4555071	4545760	4536758
Stdev	29250.60	40103.27	55509.71	70770.56
%RSD	0.64	0.88	1.22	1.56

Solution stability for test preparation at			
	Initial	***After 12 hours***	***After 24 hours***
	Standard	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area
1	4590829	4595107	4578851
2	4568423	4592801	4577832
3	4568097	4577366	4582843
4	4592898	4591664	4555542
5	4568068	4600193	4571090
Replicate	Test Area	Test Area	Test Area
1	4633289	4614247	4589238
2	4613030	4599953	4585954
Mean	4623160	4607100	4587596
% Assay	101.3	100.9	100.3
Standard weight (mg)	37.6	37.7	37.5
Test weight (mg)	2000.1	2000.1	2000.1
%Absolute difference compare to that of initial		0.4	1.0

Solution stability for test preparation at room temperature					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	4590829	4595107	4578851	4586051	4604645
2	4568423	4592801	4577832	4593989	4624750
3	4568097	4577366	4582843	4612737	4625372
4	4592898	4591664	4555542	4597328	4615701
5	4568068	4600193	4571090	4593089	4622415
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	4633289	4564891	4542451	4487609	4455559
2	4613030	4564260	4527236	4482632	4449981
Mean	4623160	4564576	4534844	4485121	4452770
% Assay	101.3	100.1	99.8	99.8	98.1
Standard weight (mg)	37.6	37.7	37.7	38.2	37.9
Test weight (mg)	2000.1	2000.1	2000.1	2000.1	2000.1
% Absolute difference compare to that of initial		1.2	1.4	1.4	3.2

Calculation:

Prototype calculation for one set:

$$\% \text{ Assay} = \frac{4623160}{4577663} \times \frac{37.6}{100} \times \frac{10}{50} \times \frac{200}{2000.1} \times \frac{100}{2} \times \frac{400.1}{150} \times 99.97$$

= 101.3 %

➢ **For Clopidogrel**

System suitability of standard preparation for solution stability					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	1638380	1659679	1670899	1664590	1667806
2	1637594	1660579	1660183	1663458	1677669
3	1640913	1662667	1658794	1663701	1673047
4	1646765	1663436	1655880	1661151	1669403
5	1638080	1662128	1655210	1672996	1670406
Mean	1640346	1661698	1660193	1665179	1671666
Stdev	3811.12	1538.91	6324.71	4550.37	3858.48
%RSD	0.23	0.09	0.38	0.27	0.23

Solution stability for standard preparation at 2 -8 °C				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	1638380	1638380	1638380	1638380
2	1637594	1637594	1637594	1637594
3	1640913	1640913	1640913	1640913
4	1646765	1646765	1646765	1646765
5	1638080	1638080	1638080	1638080
1	1655363	1652956	1654426	1653554
2	1652007	1650203	1646895	1657608
Mean	1644157	1643556	1643293	1644699
Stdev	7278.95	6352.80	6303.82	8143.27
%RSD	0.44	0.39	0.38	0.50

Solution stability for standard preparation at room temperature				
	After 12 hours	*After 24 hours*	*After 36 hours*	*After 48 hours*
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	1638380	1638380	1638380	1638380
2	1637594	1637594	1637594	1637594
3	1640913	1640913	1640913	1640913
4	1646765	1646765	1646765	1646765
5	1638080	1638080	1638080	1638080
1	1653617	1647434	1646010	1649559
2	1656717	1643628	1646155	1647690
Mean	1644581	1641828	1641985	1642712
Stdev	7923.48	4158.15	4185.55	5127.39
%RSD	0.48	0.25	0.25	0.31

Solution stability for test preparation a			
	Initial	*After 12 hours*	*After 24 hours*
	Standard	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak are
1	1638380	1659679	1670899
2	1637594	1660579	1660183
3	1640913	1662667	1658794
4	1646765	1663436	1655880
5	1638080	1662128	1655210
Replicate	Test Area	Test Area	Test Are
1	1608749	1610497	1619269
2	1606925	1612528	1609494
Mean	1607837	1611513	1614382
% Assay	100.0	99.3	99.6
Standard weight (mg)	25.1	25.2	25.2
Test weight (mg)	2000.1	2000.1	2000.1
% Absolute difference compare to that of initial		0.7	0.4

Solution stability for test preparation at room temperature					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	1638380	1659679	1670899	1664590	1667806
2	1637594	1660579	1660183	1663458	1677669
3	1640913	1662667	1658794	1663701	1673047
4	1646765	1663436	1655880	1661151	1669403
5	1638080	1662128	1655210	1672996	1670406
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	1608749	1618655	1606974	1603043	1602744
2	1606925	1609690	1603593	1602378	1606073
Mean	1607837	1614173	1605284	1602711	1604409
% Assay	100.0	99.5	99.0	98.6	98.7
Standard weight (mg)	25.1	25.2	25.2	25.2	25.3
Test weight (mg)	2000.1	2000.1	2000.1	2000.1	2000.1
% Absolute difference compare to that of initial		0.5	1.0	1.4	1.3

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{1607837}{1640346} \times \frac{25.1}{100} \times \frac{10}{50} \times \frac{321.83}{419.83} \times \frac{200}{2000.1} \times \frac{100}{2} \times \frac{400.1}{75} \times 99.38$$

= 100.0 %

Robustness Study for Analytical Method Validation of Aspirin Clopidogrel Tablets (Aspirin)

	Flow Rate at 0.9ml/min	Flow Rate at 1.1ml/min	Buffer-ACN 63: 37	Buffer-ACN 67: 33	Buffer (0.28%H_3PO_4)	Buffer (0.32%H_3PO_4)	Column Change
Replicate	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area
1	5081911	4077514	4572932	4562436	4563846	4583853	4543345
2	5060636	4083093	4552223	4546669	4557424	4547268	4538676
3	5066970	4083408	4552636	4549869	4521787	4567073	4551357
4	5057257	4087618	4555136	4568355	4564188	4562101	4540532
5	5079572	4085878	4554982	4559577	4561053	4556899	4576198
Mean	5069269	4083502	4557582	4557381	4553660	4563439	4550022
Stdev	11068.81	3828.16	8682.56	8971.92	18022.94	13564.66	15413.79
% RSD	0.22	0.09	0.19	0.20	0.40	0.30	0.34
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area
1	5176439	4140295	4624996	4613674	4649426	4614769	4626249
2	5140665	4149367	4616129	4609881	4624363	4618536	4597002
Mean	5158552	4144831	4620563	4611778	4636895	4616653	4611626
Standard weight (mg)	37.5	37.5	37.6	37.6	37.4	37.4	37.8
Test weight (mg)	2024.1	2024.1	2028.1	2028.1	2030.9	2024.9	2034.4
Label claim (mg)	150	150	150	150	150	150	150
Average test weight (mg)	400.1	400.1	400.1	400.1	400.1	400.1	400.1
% Assay	100.5	100.3	100.2	100.1	100.0	99.7	100.4

Calculation:

Prototype calculation for one set: $\%\ \text{Assay} = \frac{5158552}{5069269} \times \frac{37.5}{100} \times \frac{10}{50} \times \frac{200}{2024.1} \times \frac{100}{2} \times \frac{400.1}{150} \times 99.97 = 100.5\ \%$

Robustness Study for Analytical Method Validation of Aspirin Clopidogrel Tablets (Clopidogrel)

	Flow Rate at 0.9ml/min	Flow Rate at 1.1ml/min	Buffer-ACN 63: 37	Buffer-ACN 67: 33	Buffer (0.28%H_3PO_4)	Buffer (0.32%H_3PO_4)	Column Change
Replicate	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area
1	1833662	1474547	1678060	1648203	1674275	1659486	1669369
2	1833196	1478012	1650745	1651489	1650730	1672102	1663420
3	1856688	1478063	1650788	1661343	1664746	1656494	1659517
4	1840583	1482845	1652717	1655273	1691887	1665541	1664956
5	1858706	1489549	1656769	1651997	1651258	1685395	1669169
Mean	1844567	1480603	1657816	1653661	1666579	1667804	1665286
Stdev	12358.41	5807.27	11579.16	4972.12	17240.99	11512.41	4141.88
% RSD	0.67	0.39	0.70	0.30	1.03	0.69	0.25
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area
1	1779105	1428881	1630128	1618301	1618242	1635590	1665104
2	1770561	1462142	1611481	1596546	1633854	1623203	1628714
Mean	1774833	1445512	1620805	1607424	1626048	1629397	1646909
Standard weight (mg)	25.5	25.5	25.4	25.4	25.3	25.3	25.3
Test weight (mg)	2024.1	2024.1	2028.1	2028.1	2030.9	2024.9	2034.4
Label claim (mg)	75	75	75	75	75	75	75
Average test weight (mg)	400.1	400.1	400.1	400.1	400.1	400.1	400.1
% Assay	98.5	100.0	99.5	99.0	98.8	99.2	100.0

Calculation:

Prototype calculation for one set: $\% \ Assay = \frac{1774833}{1844567} \times \frac{25.5}{100} \times \frac{10}{50} \times \frac{200}{2024.1} \times \frac{100}{2} \times \frac{321.83}{419.83} \times \frac{400.1}{75} \times 99.38 = 98.5\ \%$

REFERANCES

[1] Collaborative meta-analysis of randomized trials of anti-platelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients: 'Anti-platelet Trialists' Collaboration. *BMJ*. **324** (2002) 71-86.

[2] D. F. Kong, R. M. Califf, D. P. Miller, *Circulation*. **98** (1998) 2829-2835.

[3] E. Boersma, R. A. Harrington, D. J. Moliterno, *Lancet*. **359**(2002) 189-198.

[4] Clopidogrel in Unstable Angina to Prevent Recurrent Events Trial Investigators. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N. Engl. J. Med*.**345** (2001) 494-502.

[5] S. Mehta, S. Yusuf, R. J. G. Peters, *Lancet*. **358** (2001) 527-533.

[6] PURSUIT Trial Investigators. Inhibition of platelet glycoprotein IIb/IIIa with eptifibatide in patients with acute coronary syndromes. *N. Engl. J. Med*. **339** (1998) 436-443.

[7] PRISM Study Investigators. A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. *N. Engl. J. Med*. **338** (1998) 1498-1505.

[8] GUSTO-IV ACS Investigators. Effect of glycoprotein IIb/IIIa receptor blocker abciximab on outcome in patients with acute coronary syndromes without early coronary revascularization: the GUSTO IV-ACS ran-domised trial. *Lancet*. **357** (2001) 1915-1924.

[9] Invasive compared with non-invasive treatment in unstable coronary-artery disease: FRISC II prospective randomized multicentre study: Fragmin and FastRevascularization During Instability in Coronary Artery Disease (FRISC II) Investigators. *Lancet*. **354** (1999) 708-715.

[10] Outcome at 1 year after an invasive compared with a non-invasive strategy in unstable coronary-artery disease: the FRISC II invasive ran-domised trial: FRISC II Investigators: Fast Revascularisation During Instability in Coronary Artery Disease. *Lancet*. **356** (2000) 9-16.

[11] C. P. Cannon, W. S. Weintraub, L. A. Demopoulos, *N. Engl. J. Med*. **344** (2001) 1879-1887.

[12] S. D. Berkowitz, C. B. Granger, K. S. Pieper, *Circulation*. **95** (1997) 2508-2516.

[13] K. A. A. Fox, P. A. Poole-Wilson, R. A. Henderson, *Lancet.* **360** (2002) 743-751.

[14] S. Bodavari, Eds. In., The Merck Index, 14th Edn., Merck & Co., Inc.,Whitehouse Station, NJ. (2006) 851 and 2396.

[15] J. T. Franeta, D. Agbaba, S. Eric, S. Pavkov, M. Aleksic, S. Vladimirov,*Farmaco.* **57** (2002) 709-713.

[16] A. Verstraeten, E. Roets, J. Hoogmartens, *J. Chromatogr. A* **388** (1987) 201-216.

[17] J. Fogel, P. Epstein, P. Chen, *J Chromatogr*. **317** (1984) 507-511.

[18] M. Gandhimathi, T. K. Ravi, A. Abraham, R. Thomas, *J. Pharm. Biorned.Anal.* **32** (2003) 1145-1148.

[19] Y. Dong, Y. Z. Zhao, Y. N. Zhang, *Se Pu*. **20** (2002) 277-278.

[20] M. Sawyer, V. Kumar, *Journal of Chromatographic Science*. **41** (2003) 393-397.

[21] R. Thomis, E. Roets, J. Hoogmartens, *J. Pharm. Sci*. **73** (1984) 1830-1833.

[22] A. W. Abu-Qare, M. B. Abou-Donia, *J. Pharm. Biomed. Anal*. **26** (2001) 939-947.

[23] C. Akay, B. Gümüsel, T. Degim, S. Tartilmis, S. Cevheroglu, *Drug Metabol. Drug Interact*. **15** (1999) 197-205.

[24] R. Pirola, S. R. Bareggi, G. De Benedittis, *J. Chromatogr. B Biomed. Sci. Appl.* **705** (1998) 309-315.

[25] F. Kees, D. Jehnich, H. Grobecker, *J. Chromatogr. B Biomed. Appl*. **677** (1996) 172-177.

[26] P. P. Ascione, G. P. Chrekian, *J. Pharm. Sci*. **64** (1975) 1029-1033.

[27] G. Alibrandi, S. Coppolino, S. D'Aliberti, R. Ficarra, N. Micali, A. Villari, *J. Pharm. Biomed. Anal.* **32** (2003) 1073-1079.

[28] G. P. McMahon, S. J. O'Connor, D. J. Fitzgerald, S. le Roy, M. T. Kelly, *J. Chromatogr. B Biomed. Sci. Appl*. **707** (1998) 322-327.

[29] Z. Kokot, K. Burda, *J. Pharm. Biomed. Anal*. **18** (1998) 871-875.

[30] S. H. Hansen, M. E. Jensen, I. Bjørnsdottir, *J. Pharm. Biomed. Anal*. **17** (1998) 1155-1160.

[31] S. Torrado, S. Torrado, R. Cadórniga, *J. Pharm. Biomed. Anal*. **12** (1994) 383-387.

[32] I. M. Jalal, S. I. Sasa, *Talanta*. **31** (1984) 1015-1017.

[33] G. Santoni, L. Fabbri, P. Gratteri, G. Renzi, S. Pinzauti, *International Journal of Pharmaceutics*. **80** (1992) 263-266.

[34] S. L. Ali, *J. Chromatogr*. A **126** (1976) 651-663.

[35] V. Kmetec, *J. Pharm. Biomed. Anal*.**10** (1992) 1073-1076

[36] H. Agrawal, N. Kaul, A. R. Paradkar, K. R. Mahadik, *Talanta*. **61** (2003) 581-589

[37] E. Souri, H. Jalalizadeh, A. Kebriaee-Zadeh, M. Shekarchi, A. Dalvandi, *Biomed. Chromatogr*. **20** (2006) 1309 – 1314.

[38] S. S. Singh, K. Sharma, D. Barot, P. R. Mohan, V. B. Lohray, *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci*. **821** (2005) 173-180.

[39] A. Robinson, J. Hillis, C. Neal, A. C. Leary, *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci*. **848** (2007) 344-354.

[40] R. V. Nirogi, V. N. Kandikere, M. Shukla, K. Mudigonda, S. Maurya, R. Boosi, *Rapid. Commun. Mass Spectrom*. **20** (2006) 1695-700.

[41] H. Ksycinska, P. Rudzki, M. Bukowska-Kiliszek, *J. Pharm. Biomed. Anal*.**41** (2006) 533-539.

[42] A. Mitakos, I. Panderi, *J. Pharm. Biomed. Anal*. **28** (2002) 431-438.

[43] P. Lagorce, Y. Perez, J. Ortiz, J. Necciari, F. Bressolle, *J. Chromatogr. B Biomed. Sci. Appl.* **720** (1998) 107-117.

[44] Y. Tomoko, N. Mihoko, W. Mitsuhiro, N. Kentchiro, *Chromatography*. **27**(2006) 71-72**.**

[45] P. Mishra, A. Dolly, *Indian J. Pharm. Sci*., **68**(2006) 365-368.

[46] M Gandhimathi, T. K. Ravi, *Indian J. Pharm. Sci*., **69**(2007)123-125.

[47] S. Singh, B. Singh, R. Bahuguna, L. Wadhwa, R. Saxena, *J. Pharm. Biomed. Anal.* **41** (2006) 1037-1040.

[48] A. Mohammadi, I. Haririan, N. Rezanour, L. Ghiasi, R. B. Walker, *J.Chromatogr. A* **1116** (2006) 153-157.

[49] I. Ivana, Z. Ljiljana, Z. Mira, *J. Chromatogr. A* **1119** (2006) 209-215.

[50] ICH, Q1A (R2) Stability testing of new drug substances and products, in: International Conference on Harmonization, IFPMA, Geneva, (2003).

[51] G. Grosa, E. D. Grosso, R. Russo, G. Allegrone, *J. Pharm. Biomed. Anal.* **41** (2006) 798-803.

[52] A. Mishal, D. Sober, *J. Pharm. Biomed. Anal.* **39** (2005) 819-823.

Part-[A]

(Section-III)

HPLC Method Development and Validation of combine dosage form of Tramadol hydrochloride and Aceclofenac

HPLC METHOD DEVELOPMENT AND VALIDATION OF COMBINE DOSAGE FORM OF TRAMADOL HYDROCHLORIDE AND ACECLOFENAC

1. Introduction to Drug

1.1 Tramadol Hydrochloride

Tramadol hydrochloride, *(1,2)*–2-[(dimethylamino)-methyl]–1-(3-methoxy-phenyl) cyclohexanol hydrochloride (Figure 1) Its molecular formula is $C_{16}H_{25}NO_2HCl$ having molecular weight 299.24 g/mole. It white crystalline powder and soluble in water [1].

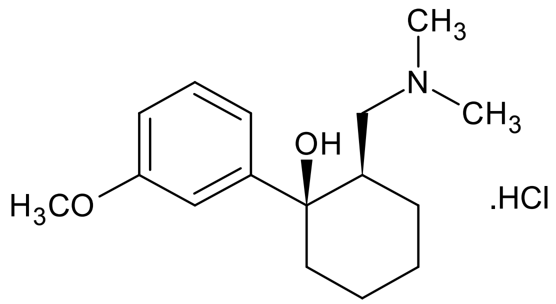


Figure 1: Chemical structure of tramadol hydrochloride

Tramadol is a centrally acting analgesic agent, which has been shown to be a synthetic analogue of codeine [2]. It is metabolized by the cytochrome P450 enzyme system in the liver to form 11 metabolites of which M1 (O-desmethyltramadol) predominates and possesses analgesic properties [3]. It has been used since 1977 for the relief of strong physical pain and has been the most widely sold opioid analgesic drug in the world [4]. Tramadol was developed by the German pharmaceutical company Grünenthal GmbH and marketed under the trade name Tramal. Grünenthal has also cross licensed the drug to many other pharmaceutical companies that market it under various names.Tramadol is usually marketed as the hydrochloride salt (tramadol hydrochloride) and is available in both injectable (intravenous and/or intramuscular) and oral preparations. It is also available in conjunction with paracetamol (acetaminophen) and aceclofenac.

1.1.1 Pharmacodynamics

Tramadol hydrochloride is a centrally acting synthetic opioid analgesic in an orally disintegrating tablet form. Although its mode of action is not completely understood, from animal tests, at least two complementary mechanisms appear applicable. Binding of parent and M1 metabolite to μ-opioid receptors and weak inhibition of re-uptake of norepinephrine and serotonin.

Opioid activity is due to both low affinity binding of the parent compound and higher affinity binding of the O-demethylated metabolite M1 to μ-opioid receptors. In animal models, M1 is up to 6 times more potent than tramadol in producing analgesia and 200 times more potent in μ-opioid binding. Tramadol induced analgesia is only partially antagonized by the opiate antagonist naloxone in several animal tests. The relative contribution of both tramadol and M1 to human analgesia is dependent upon the plasma concentrations of each compound.

Tramadol has been shown to inhibit re-uptake of norepinephrine and serotonin *in vitro*, as have some other opioid analgesics. These mechanisms may contribute independently to the overall analgesic profile of tramadol. Analgesia in human begins approximately within one hour after administration and reaches a peak in approximately two to three hours.

Apart from analgesia, tramadol administration may produce a constellation of symptoms (including dizziness, somnolence, nausea, constipation, sweating and pruritus) similar to that of other opioids. In contrast to morphine, tramadol has not been shown to cause histamine release. At therapeutic doses, tramadol has no effect on heart rate, left-ventricular function or cardiac index. Orthostatic hypotension has been observed.

1.1.2 Pharmacokinetics

The analgesic activity of tramadol is due to both parent drug and the M1 metabolite. Tramadol is administered as a racemate and both the [-] and [+] forms of both tramadol and M1 are detected in the circulation. Tramadol is well absorbed orally with an absolute bioavailability of 75%. Tramadol has a volume of distribution of approximately 2.7 L/kg and is 20% bound to plasma proteins. Tramadol is extensively metabolized by a number of pathways, including CYP2D6 and CYP3A4, as well as by conjugation of parent and metabolites. One metabolite, M1, is pharmacologically active in animal models. The formation of M1 is dependent upon CYP2D6 and as such is subject to inhibition, which may affect the therapeutic response. Tramadol and its metabolites are excreted primarily in the urine with observed plasma half-lives of 6.3 and 7.4 hours for tramadol and M1, respectively. Linear pharmacokinetics have been observed following multiple doses of 50 and 100 mg to steady state.

No difference has been identified in systemic exposure (AUC), peak exposure (C_{max}), time to peak exposure (T_{max}) and apparent elimination half-life ($t_{Â½}$) of tramadol and metabolites M1 and M5 between administration of tramadol hydrochloride with and without water and tablet.

Absorption: Racemic tramadol is rapidly and almost completely absorbed after oral administration. The mean absolute bioavailability of a 100 mg oral dose is approximately 75%. The mean peak plasma concentration of racemic tramadol and M1 occurs at two and three hours, respectively, after administration in healthy adults. In general, both enantiomers of tramadol and M1 follow a parallel time course in the body following single and multiple doses although small differences (~ 10%) exist in the absolute amount of each enantiomer present.

Food Effects: Oral administration of tramadol hydrochloride with food does not significantly affect its extent of absorption, however, food does delay t_{max} by about 30 minutes compared to fasting conditions. The clinical significance of this delay is not known.

Distribution: The volume of distribution of tramadol was 2.6 and 2.9 L/kg in male and female subjects, respectively, following a 100 mg intravenous dose. The binding of tramadol to human plasma proteins is approximately 20% and binding also appears to be independent of concentration up to 10 µg/ml. Saturation of plasma protein binding occurs only at concentrations outside the clinically relevant range.

Metabolism: Tramadol is extensively metabolized after oral administration. Approximately 30% of the dose is excreted in the urine as unchanged drug, whereas 60% of the dose is excreted as metabolites. The remainder is excreted either as unidentified or as unextractable metabolites. The major metabolic pathways appear to be N - and O - demethylation and glucuronidation or sulfation in the liver. One metabolite (O -desmethyltramadol, denoted M1) is pharmacologically active in animal models. Formation of M1 is dependent on CYP2D6 and as such is subject to inhibition, which may affect the therapeutic response.

Approximately 7% of the population has reduced activity of the CYP2D6 isoenzyme of cytochrome P-450. These individuals are “poor metabolizers” of debrisoquine, dextromethorphan, tricyclic antidepressants, among other drugs. Based on a population PK analysis of phase-I studies in healthy subjects, concentrations of tramadol were approximately 20 % higher in “poor metabolizers” versus “extensive metabolizers,” while M1 concentrations were 40% lower. Concomitant therapy with inhibitors of CYP2D6 such as fluoxetine, paroxetine and quinidine could result in significant drug interactions. *In vitro* drug interaction studies in human liver microsomes indicate that inhibitors of CYP2D6 such as fluoxetine and its metabolite norfluoxetine, amitriptyline and quinidine inhibit the metabolism of tramadol to various degrees, suggesting that concomitant administration of these compounds could result in increases in tramadol concentrations and decreased concentrations of M1. The full pharmacological impact of these alterations in terms of either efficacy or safety is unknown. Concomitant use of SEROTONIN re-uptake INHIBITORS and MAO INHIBITORS may enhance the risk of adverse events, including seizure and serotonin syndrome.

Elimination: Tramadol is eliminated primarily through metabolism by the liver and the metabolites are eliminated primarily by the kidneys. The mean terminal plasma elimination half-lives of racemic tramadol and racemic M1 are 6.3 ± 1.4 and 7.4 ± 1.4 hours, respectively. The plasma elimination half-life of racemic tramadol increased from approximately six hours to seven hours upon multiple dosing.

1.2 Aceclofenac

Aceclofenac, 2-[(2,6-dichlorophenyl) amino]-phenylacetoxyacetic acid (Figure 2). The molecular formula of aceclofenac is $C_{16}H_{13}C_{12}NO_4$ and its molecular weight is 354.19 g/mole [1]. It is a white or almost white powder. It is practically insoluble in water, freely soluble in acetone and soluble in alcohol.

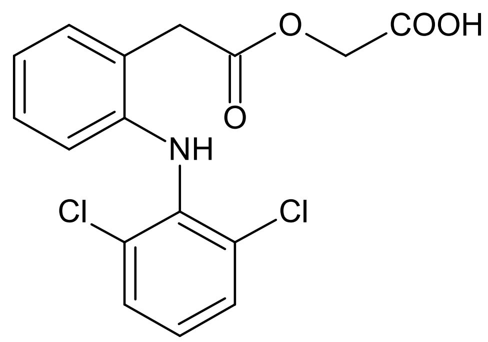


Figure 2: Chemical structure of aceclofenac

That shows analgesic properties and good tolerability profile in a variety of painful conditions [5, 6]. It is used in the treatment of rheumatic disorders and soft tissue injuries. Aceclofenac inhibits the cyclooxygenase enzyme and thus exerts its anti-inflammatory activity by inhibition of prostaglandin synthesis. This effect seems to be correlated to the appearance of acute protocolitis associated with nonsteroidal anti-inflammatory drug therapy [7-9].

1.2.1 Pharmacology

Mechanism of Action: The mode of action of aceclofenac is largely based on the inhibition of prostaglandin synthesis. Aceclofenac is potent inhibiter of the enzyme cyclo-oxygenase, which is involved in the production of prostaglandins. Aceclofenac has been shown to exert effects on a variety of mediators of inflammation. The drug inhibits synthesis of the inflammatory cytokines interleukin (IL)-1â and tumour necrosis factor and inhibits prostaglandin E_2 (PGE_2) production. Effects on cell adhesion molecules from neutrophils have also been noted. *In vitro* data indicate inhibition of cyclo-oxygenase (COX)-1 and 2 by aceclofenac in whole blood assays, with selectivity of COX-2 being evident.

In contrast to some other NSAIDs, aceclofenac has shown stimulatory effects on cartilage matrix synthesis, which may be linked to the ability of the drug to inhibit IL-1â activity. *In vitro* data indicates stimulation by the drug of synthesis of glycosaminoglycan in osteoarthritic cartilage. There is also evidence that aceclofenac stimulates the synthesis of IL-1 receptor antagonist in human articular chondrocytes subjected to inflammatory stimuli and that 4'-hydroxyaceclofenac has chondroprotective properties attributable to suppression of IL-1â mediated promatrix metalloproteinase production and proteoglycan release.

In patients with osteoarthritis of the knee, aceclofenac decreases pain, reduces disease severity and improves the functional capacity of the knee. It reduce joint reduces inflammation, pain intensity and the duration of morning stiffness in patients with rheumatoid arthritis. The duration of morning stiffness and pain intensity are reduced and spinal mobility improved, by aceclofenac in patients with ankylosing spondylitis.

1.2.2 Pharmacokinetics

Aceclofeanc is rapidly and completely absorbed after oral adiminstration. Peak plasma concetrations are reached to 1 to 3 hours after an oral dose. The drug is highly protient bound (99%). The presence of food does not alter the extent of absorption of aceclofenac but the absorption rate is reduced. The plama concentration of aceclofenac was approximately twice that in synovial fluid after multiple doeses of the durg in patients with knee pain and slynovial fludie effusiton.

Aceclofeance is metabolised to a major metabolite, 4'-hydroxyaceclofenac and to a number of other metabolites including 5-hydroxyaceclofenac, 4'-hydroxydiclofenac, diclofenac and 5-hydroxydiclofenac. These other metabolites account for the fate of approximatlely 20 % of each does of aceclofenac. Renal excretion is the main route of elemination of aceclofenac with 70 to 80% of an adminstred does found in the urine, mainly as the glucuronides of aceclofenac and its metabolites. Of each dose of aceclofenac, 20% is extreted in the faeces. The plasma elimination half life of the drug is approximately 4 hours.

1.2.3 Indication

Aceclofenac is indicated for the relied of pain and inflammation associated with rheumatoid arthrits, osteoarthritis or ankylosing spondylitis.

1.2.4 Contraindcations

Aceclofenac shoud not be administreted to patients hypersensititve to aceclofenac of other NSAIDs or patients with a history of aspirin of NSAID related allergic or anaphylatic reactions of with peptic ulcers of GI bleeding, moderate of sever renal impairment.

1.2.5 Drug Interactions

- Drug interactions associated with aceclofenac are similar to those observed with other NSAIDs.
- Aceclofenac may increase plasma concentrations of lithium, digoxin and methotrexate, increase the activity of anticoagulatns, inhibite the activity of diuretics, enhance cyclosporin nephrotoxicity and precipitate convulsions when coadministered with quinolone antibiotics.
- When concomitant administration with potassium sparing diuretics is empployed, serum potassium shoud be monitored.
- Furthermore, hypo or hyperglycamia may result for the concomitant administration of acelofenac and antidiabetic drugs, although this is rare. The condministration of acelofenac with other NSAIDs of corticosteroids may result in increased frequency of adverse events.
- Cautions shuold be exercised if NSAIDs and methoterxate are administred wthinin 2-4 hours of each other, since NSAIDs may increase methotrexate plasma levels, resulting in increased toxicity.

1.2.6 Dosage and Administration

The usual dose of acelofenac is 100 mg given tiwice daily by mouth. One tablet in the morning and one in the evening.

There is no evidence that the dosage of aceclofenac needs to be modified in patients with mild renal impairment but as with other NSAIDs cautins should be excercised. There is some evidence that the dose of aceclofenac should be reduced in patients with hepatic impairment and it is suggested that an intial daily dose of 100 mg be used.Aceclofenac tablets should be swallowed whole with a sufficient quantity of liquid. When aceclofenac was administred to fasting and fed healthy volunteers only the rate and not the extent of aceclofenac absorption was affected and as such aceclofenac can be taken with food.

2. Literature Review

There are many reported method for the determination of either tramadol hydrochloride and aceclofenac alone or in combination with other drug in pharmaceutical dosage forms or individually in biological fluids are as under:

2.1 Literature Review for Tramadol Hydrochloride

2.1.1 **Y. H. Ardakani, M. R. Rouini** have developed liquid chromatographic method for the simultaneous determination of tramadol and its three main metabolites in human plasma, urine and saliva. Chromatographic separation was achieved with a Chromolith Performance RP-18e (100 mm x 4.6 mm) column, using a mixture of methanol-water (19:81, v/v) adjusted to pH 2.5 by phosphoric acid, in an isocratic mode at flow rate of 2 ml/min. Fluorescence detection (ë$_{ex}$ 200 nm/ ë$_{em}$ 301 nm) was used. The developed procedure was applied to assess the pharmacokinetics of tramadol and its main metabolites [10].

2.1.2 **R. Mehvar, K. Elliott, R. Parasrampuria, O. Eradiri** have reported a stereospecific method for simultaneous quantitation of the enantiomers of tramadol and its active metabolites O-demethyl tramadol and O-demethyl-N-demethyl tramadol in human plasma The separation was achieved using a Chiralpak AD column with a mobile phase of hexanes-ethanol-diethylamine (94:6:0.2, v/v/v) and a flow rate of 1 ml/min. The fluorescence of analytes was then detected at excitation and emission wavelengths of 275 and 300 nm, respectively. The application of the assay was demonstrated by simultaneous measurement of plasma concentrations of tramadol, O-demethyl tramadol and O-demethyl-N-demethyl tramadol enantiomers in a healthy volunteer [11].

2.1.3 **M. Zeceviæ, Z. Stankoviæ, Lj. Zivanoviæ, B. Jociæ** have developed and validated a high performance liquid chromatographic method for the determination of tramadol hydrochloride and its three impurities. The method can simultaneously assay potassium sorbate, used as preservative and saccharin sodium, used as sweetener in tramadol pharmaceutical formulation. The separation was carried out on a C18 XTerra (150 mm x 4.6 mm i.d., 5 µm particle size) column using acetonitrile-0.015 M Na_2HPO_4 buffer (2:8, v/v) as mobile phase (pH value 3.0

was adjusted with orthophosphoric acid) at a flow rate 1.0 ml/ min, temperature of the column 20 ^{0}C and UV detection at 218 nm mother method can be use in routine quality control analysis [12].

2.1.4 **A. Küçük, Y. Kadioðlu** have reported a determination method of tramadol hydrochloride in ampoule dosage forms by using UV spectrophotometric and HPLC-DAD methods in methanol and water media. Measurements for spectrophotometric method were performed using UV-Vis Spectrophotometer in ranges of 200-400 nm. The solutions of standard and the samples were prepared in methanol and water media and the UV absorption spectrums of tramadol were monitored with maximum absorptions at 275 and 271 nm for both mediums, respectively. Reversed phase chromatography for HPLC method was conducted using a Phenomenex Bondclone C18 column with an isocratic mobile phase consisting of acetonitrile- 0.01 M phosphate buffer, pH 3 (25:75,v/v). The effluent was monitored on a DAD detector at 218 nm. The methods were applied to pharmaceutical ampoule forms [13].

2.1.5 **A. Küçük, Y. Kadýoðlu, F. Çelebi** have reported a determination method of tramadol concentration in rabbit plasma using simple liquid-liquid extraction and high-performance liquid chromatography. The method was applied to a pharmacokinetic study of intravenous tramadol injections in rabbits [14].

2.1.6 **Y. Gu, J. P. Fawcett** have developed an HPLC method for the determination of tramadol and its major active metabolite, O-desmethyltramadol, in human plasma. Tramadol, O-desmethyltramadol and the internal standard, sotalol, were separated by reversed phase HPLC using 35 % acetonitrile and an aqueous solution containing 20 mM sodium phosphate buffer, 30 mM sodium dodecyl sulphate and 15 mM tetraethylammonium bromide pH 3.9. Detection was by fluorescence with excitation and emission wavelengths of 275 and 300 nm, respectively. The method can apply to a pharmacokinetic study of tramadol in human volunteers. [15].

2.1.7 **L. M. Zhao, X. Y. Chen, J. J. Cui, M. Sunita, D. F. Zhong** have documented a determination of tramadol and its active metabolite O-desmethyl tramadol in plasma and amniotic fluid using LC/MS/MS. Samples containing tramadol, O-desmethyltramadol and diphenhydramine (internal standard, IS) were extracted using liquid-liquid extraction, followed by liquid chromatographic separation and online MS/MS using atmospheric pressure chemical ionization as an interface detection. The analytes were detected in the selected reaction-monitoring mode. Method can be suitable for clinical pharmacokinetics studies of tramadol and O-desmethyltramadol [16].

2.1.8 **A. Medvedovici, F. Albu, A. Farca, V. David** have validated HPLC determination of 2-[(dimethylamino) methyl] cyclohexanone, an impurity in tramadol, using a precolumn derivatisation reaction with 2,4-dinitrophenylhydrazine. The method is based on the derivatisation of 2-[(dimethylamino) methyl] cyclohexanone with 2,4-dinitrophenylhydrazine (2,4-DNPH) in acidic conditions followed by a reversed-phase liquid chromatographic separation with UV detection [17].

2.1.9 **M. A. Campanero, E. Garcia-Quetglas, B. Sadaba, J. R. Azanza** have reported a bioanalytical method involving a simple liquid–liquid extraction for the simultaneous HPLC determination of the enantiomers of tramadol, the active metabolite O-desmethyltramadol and the other main metabolite *N*-desmethyltramadol in biological samples. Chromatography was performed at 5 °C on a Chiracel OD-R column containing cellulose tris (3,5-dimethylphenylcarbamate) as chiral selector, preceded by an achiral end-capped C 8 column (250 mm × 4 mm i.d., 5 μm particle size). The mobile phase was a mixture of phosphate buffer containing sodium perchlorate (1 M) adjusted to pH 2.5-acetonitrile-*N, N*-dimethyloctylamine (74.8:25:0.2, v/v/v). The flow rate was 0.5 ml/min. Fluorescence detection ($ë_{ex}$ 200 nm/ $ë_{em}$ 301 nm) was used [18].

2.1.10 **V. Kmetec, R. Roskar** hav reported HPLC determination of tramadol in human breast milk. They chose the liquid-liquid extraction procedure using n-hexane as an organic phase with back extraction into aqueous phase since it was considered the most suitable and the most compatible with the subsequent HPLC analysis. The developed method is suitable for monitoring of tramadol in human breast milk [19].

2.1.11 **S. H. Gan, R. Ismail, W. A. Wan Adnan, Z. Wan** have been developed and validated a method for the determination of tramadol concentration in human plasma by using a simple liquid-liquid extraction and HPLC with UV detection.

The method has been applied to determine tramadol concentrations in human plasma samples for a pharmacokinetic study [20].

2.1.12 **M. Nobilis, J. Kopecký, J. Kvetina, J. Chládek, Z. Svoboda, V. Vorísek, F. Perlík, M. Pour, J. Kunes** have developed and validated simultaneous HPLC determination of the tramadol, its major pharmacodynamically active metabolite O-desmethyltramadol in human plasma. HPLC analysis was performed on a chromatographic column with LiChrospher 60 RP-selectB (250 mm x 4 mm i.d., 5 μm particle size) and consists of an analytical period where the mobile phase acetonitrile-0.01 M phosphate buffer, pH 2.8 (3:7, v/v) was used. Detection was carried out by fluorescence detectore. The validated analytical method was applied to pharmacokinetic studies of tramadol in human volunteers [21].

2.1.13 **H. E. Abdellatef** has reported kinetic spectrophotometric determination of tramadol hydrochloride in pharmaceutical formulation. The results obtained are compared statistically with those given by the reference spectrophotometric method. [22]

2.1.14 **S. H. Gan, R. Ismail** has been developed and validated a solid-phase extraction and HPLC with UV detection method in order to determine tramadol and O-desmethyltramadol concentrations in human plasma. The system has been applied to determine tramadol concentrations in human plasma samples for a pharmacokinetic study [23].

2.1.15 **F. Vanderbist Ceccato, J. Y. Pabst, B. Streel** has reported enantiomeric determination of tramadol and its main metabolite O-desmethyltramadol in human plasma by liquid chromatography tandem mass spectrometry. The method developed can used to investigate plasma concentration of enantiomers of tramadol and O-desmethyltramadol in a pharmacokinetic study [24].

2.1.16 **M. A. Campanero, B. Calahorra, M. Valle, I.F. Troconiz, J. Honorato** have been reported a stereo selective HPLC assay for the quantitative determination of the analgesic tramadol and O-demethyltramadol, an active metabolite. The assay involved liquid chromatography analysis with fluorescence detection. Chromatography was performed at 20 ºC on a Chiracel OD-R column containing cellulose tris- (3,5-dimethylphenylcarbamate) as stationary phase, preceded by an achiral end-capped C18 column. The mobile phase was a mixture of phosphate buffer [containing sodium perchlorate (0.2 M) and triethylamine (0.09 M) adjusted to pH 6] - acetonitrile (80:20, v/v). Applicability of the method was demonstrated by a pharmacokinetic study in normal volunteers [25].

2.1.17 **G. C. Yeh, M. T. Sheu, C. L. Yen, Y. W. Wang, C. H. Liu, H. O. Ho** have developed HPLC method using UV detection for determination of tramadol concentration in human plasma. Separation was performed on a reversed phase LiChrospher 60 RP-select B column with a particle size of 5 μm. The mobile phase consisted of 0.05 M KH_2PO_4 aqueous solution (pH 3.5) - acetonitrile (90:10, v/v). Metoprolol was used as the internal standard and UV detection at 225 nm was employed. This validated method was applied to the determination of tramadol concentrations in healthy volunteers [26].

2.1.18 **M. Valle, J. M. Pavon, R. Calvo, M. A. Campanero, I. F. Troconiz** have developed method for simultaneous determination of tramadol and its main active metabolite O-demethyltramadol in rat plasma. The method involves a single step extraction procedure and a specific determination by HPLC with electrochemical detection, using an ethoxy analogue of tramadol (L-233) as internal standard. The dual-electrode detector was operated in the oxidation-screening mode [27].

2.1.19 **P. Overbeck, G. Blaschke** have developed a reversed phase HPLC method for the direct determination of three glucuronides of the centrally acting analgesic tramadol. Separation of these glucuronides into their diastereomers was achieved by HPLC using ion pair chromatography with nonanesulfonic acid sodium salt and LiChrospher 100 RP 18 as stationary phase. Fluorescence detector performed detection [28].

2.1.20 **A. Ceccato, P. Chiap, P. Hubert, J. Crommen** have reported automated method for the separation and individual determination of tramadol enantiomers in plasma using solid-phase extraction on disposable extraction cartridges in combination with chiral liquid chromatography. The enantiomeric separation of tramadol was achieved using a Chiralcel OD-R column containing cellulose tris-(3,5-dimethylphenylcarbamate) as chiral stationary phase. The mobile phase was a mixture of phosphate buffer, pH 6.0, containing sodium perchlorate (0.2 M) - acetonitrile (75:25, v/v) [29].

2.1.21 **I. Y. Zaghloul, M. A. Radwan, J. Liq** have developed a HPLC method for determination of tramadol in pharmaceutical dosage forms. Reversed phase chromatography was conducted using ì-Bondapak C18 column (3.9 mm x 150 nm) with an isocratic mobile phase consisting of 0.005 M triethylamine in 0.01 M sodium phosphate buffer (pH 5.5) containing 17% acetonitrile. The effluent was monitored on a UV detector at 230 nm [30].

2.1.22 **M. Nobilis, J. Pastera, P. Anzenbacher, D. Svoboda, J. Kopecký, F. Perlík** have determined tramadol in human plasma samples using a sensitive HPLC method. Separation performed on reversed phase silica gel using ion-pair chromatography (verapamil as an internal standard) and fluorescence detection. The method was applied to the determination of tramadol levels in healthy volunteers [31].

2.1.23 **B. Elsing, G. Blaschke** have been developed a reversed phase HPLC method for the simultaneous determination of tramadol and its major metabolites O-demethyltramadol and N-demethyltramadol in urine. The determination of the enantiomeric ratios of the three compounds was achieved using a Chiralpak AD column and a Chiralcel OD column, respectively [32].

2.2 Literature Review for Aceclofenac

2.2.1 **P. Musmade, G. Subramanian, K. K.Srinivasan** have developed a simple HPLC method for quantification of aceclofenac in rat plasma. Ibuprofen was used as an internal standard. Separation was carried out on reversed-phase C18 column (250 mm x 4.6 mm, 5 µm particle size) and the column effluent was monitored by UV detector at 282 nm. The mobile phase used was methanol-0.3%(v/v) triethylamine, pH 7.0, (60:40, v/v) at a flow rate of 1.0 ml/min. The method was applied for pharmacokinetic study of aceclofenac in rats [33].

2.2.2 **A. Zinellu, C. Carru, S. Sotgia, E. Porqueddu, P. Enrico, L. Deiana** have reported a fast-free zone capillary electrophoresis method for the simultaneous determination of aceclofenac and diclofenac in human plasma. A separation was achieved using a 40 cm × 75 µm uncoated silica capillary, 300 mM/L sodium borate buffer, 200 mM/l *N*-methyl-d-glucamine, pH 8.9, in about 3 min. method is efficient mean for the comprehensive determination of aceclofenac and diclofenac in human plasma when pharmacokinetics studies are required [34].

2.2.3 **Y. Jin, H. Chen, S. Gu, F. Zeng** has established a reversed phase HPLC method for the determination of aceclofenac in human plasma. Chromatography was performed on a C18 column with methanol-0.1 M/L ammonium acetate, pH 6.0, (7:3, v/v) as the mobile phase. The flow rate was 1.0 ml/min. The UV- detector was set at 275 nm. This method can be used for clinical pharmacokinetic study of aceclofenac [35].

2.2.4 **N. Y. Hasan, M. Abdel-Elkawy, B. E. Elzeany, N. E. Wagieh** have established five new methods for the determination of aceclofenac in the presence of its degradation product diclofenac. Method-A utilizes third derivative spectrophotometry at 242 nm. Method-B was RSD_1 spectrophotometric method based on the simultaneous use of the first derivative of ratio spectra and measurement at 245 nm. Method-C was a Ph induced difference (Ä*A*) spectrophotometry using UV measurement at 273 nm. Method-D was a spectrodensitometric one, which depends on the quantitative densitometric evaluation of thin layer chromatogram of aceclofenac at 275 nm. Method-E was RP-HPLC that depends on using methanol- water (60:40, v/v) as mobile phase at a flow rate of 1 ml/min and UV detection at 275 nm. The methods could be applied for the analysis of the drug in its pharmaceutical formulation [36].

2.2.5 **B. Hinz, D. Auge, T. Rau, S. Rietbrock, K. Brune, U. Werner** have been reported a method for the simultaneous determination of aceclofenac and three of its metabolites in human plasma by HPLC. The analytes were separated using an acetonitrile-phosphate buffer gradient at a flow rate of 1 ml/min and UV- detection at 282 nm. The developed procedure was applied to assess the pharmacokinetics of aceclofenac and its metabolites [37].

2.2.6 **N. H. Zawilla, M. A. A. Mohammad, N. M. El Kousy, S. M. El-Moghazy Aly** have reported three sensitive and reproducible methods for quantitative determination of aceclofenac in pure form and in pharmaceutical formulation. The first method is based on Beer's law. Absorption measurements were carried out at 665.5 nm. The other two methods are high performance liquid chromatography and densitometric methods by which the drug was determined in the presence of its degradation products [38].

2.2.7 **H. S. Lee, C. K. Jeong, S. J. Choi, S. B. Kim, M. H. Lee, G. Il Ko and D. H. Sohn** have developed a narrow bore HPLC with column-switching for the simultaneous determination of aceclofenac and diclofenac from human plasma samples. Plasma sample (100 μl) was directly introduced onto a Capcell Pak MF Ph-1 column (20 mm × 4 mm i.d.) primary separation was occurred to remove proteins and concentrate target substances using acetonitrile- 0.1 M potassium phosphate, pH 7, (14:86, v/v). The drug molecules eluted from MF Ph-1 column were focused in an intermediate column (35 mm × 2 mm i.d.) by the valve-switching step. The substances enriched in intermediate column were eluted and separated on the narrow bore phenyl–hexyl column (100 mm × 2 mm i.d.) using acetonitrile-0.02M potassium phosphate, pH 7, (33:67, v/v)[39].

2.2.8 **X. Q. Liu, X. J. Chen, L. H. Zhao, J. H. Peng** have reported HPLC assay method for the determination of aceclofenac in plasma and its pharmacokinetics in dogs [40].

3. Aim of Present Work

The aim of the present work is to developed analytical method for combine fixed dose formulation, which is novel to the market. The work of interest is tramadol hydrochloride and aceclofenac in the combine dosage form. There is no analytical method reported for the simultaneous determination of tramadol hydrochloride and aceclofenac in novel combination. Various publication are available regarding determination method of tramadol hydrochloride and aceclofenac but most of the methods are applicable to alone tramadol hydrochloride or aceclofenac in pharmaceutical dosage form or in bilogical fluids.

Our work deals with the development and validation of stability-indicating high performance liquid chromatographic assay method for the determination of tramadol hydrochloride and aceclofenac in commercial tablet formulation. The combination formulation was subjected to ICH recommended stress condition like acid hydrolysis, base hydrolysis, oxidation, thermal and photolytic stress. Thus validated method can recommend for the routine quality control analysis and also stability sample analysis.

The aim and scope of the proposed work are as under

1. To developed suitable HPLC method for simultaneous determination tramadol hydrochloride and aceclofenac in tablet formulation.
2. Forced degradation study of tramadol hydrochloride and aceclofenac under stress condition.
3. To resolve all major impurities generated during the force degradation studies of tramadol hydrochloride and aceclofenac.
4. Perform the validation for the developed method.

4. Experimental

4.1 Materials

Pharmacopoeial grade standards of tramadol hydrochloride and aceclofenac were provided by reputed pharma company. A tablet containing 37.5 mg tramadol hydrochloride and 100 mg aceclofenac was commercially available (HIFENAC-TL, Intas Pharmaceuticals LTD.) HPLC grade acetonitrile, methanol and water were obtained from Spectrochem Pvt. Ltd., Mumbai (India). Analytical grade hydrochloric acid, sodium hydroxide pellets, glacial acetic acid and hydrogen peroxide solution (30% v/v) were obtained from Ranbaxy Fine Chemical, New Delhi (India).

4.2 Instrumentation

LC-10AT*vp* HPLC system was used as describe as general chapter (5A).

4.3 Mobile Phase Preparation

The mobile phase was consisted of 0.01M-ammonium acetate buffer pH 6.5 - acetonitrile (65:35, v/v). To prepare the buffer solution, 0.77 g ammonium acetate was weighed and dissolves in 1000 ml HPLC grade water. 3 ml triethylamine was added and then adjusted to pH 6.5 with glacial acetic acid. Mobile phase was filtered through a 0.45 μm nylon membrane (Millipore Pvt. Ltd. Benglore, India) and degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai).

4.4 Diluent Preparation

Diluent use during whole study was prepared with acetonitrile-water (50:50,v/v)

4.5 Standard Preparation

Standard solution containing tramadol hydrochloride (0.0375 mg/ml) and aceclofenac (0.100 mg/ml) were prepared by dissolving 18.75 mg tramadol hydrochloride and 50 mg aceclofenac in 50 ml volumetric flask by diluent (stock standard solution). Pipette out 5 ml stock solution into 50 ml volumetric flask and dilute up to mark with diluent (standard solution).

4.6 Test Preparation

Twenty tablets were weighed and the average tablet weight was determined. Tablets were crushed by mortar and pastel. Tablet powder was weighed equivalent to five times of average weight and transfer in to 500 ml volumetric flask. About 50 ml methanol and 300 ml mobile phase was added and sonicated for of 20 min. time interval with intermittent shaking. Content was brought back to room temperature and dilute to volume with diluent (stock test solution). The stock solution was filtered through 0.45 µm nylon syringe filter. Pipette out 5ml filtered stock solution in to 50 ml volumetric flask and dilute with diluent (test solution). The concentration obtain was 0.0375 mg/ml of tramadol hydrochloride and 0.100 mg/ml of aceclofenac.

4.7 Chromatographic Conditions

Chromatographic analysis was performed on a Phenomenex Gemini C18 (250 mm × 4.6 mm i.d., 5 µm particle size) column. The mobile phase was consisted of 0.01 M-ammonium acetate buffer pH 6.5 -acetonitrile (65:35, v/v). The flow rate of the mobile phase was adjusted to 1.0 ml/min and the injection volume was 20 µl. Detection was performed at 270 nm.

5. Result and Discussion

5.1 Development and Optimization of the HPLC Method

In the presence work, an analytical method based on high performance liquid chromatography using photodiode array detection was developed and validated for assay determination of tramadol hydrochloride and aceclofenac in tablet formulation. The analytical conditions were selected, keeping in mind the different chemical nature, molecular weight and solubility of tramadol hydrochloride and aceclofenac. The development trials were taken by using the degraded sample of each component was done, by keeping them in various extreme conditions.

Tramadol hydrochloride and aceclofenac are dissolved in polar solvent hence RP-HPLC was selected to estimate them. The column selection has been done on the basis of backpressure, resolution, peak shape, theoretical plates and day-to-day reproducibility of the retention time and resolution between tramadol hydrochloride and aceclofenac peak. After evaluating all these factors, Phenomenex Gemini C18 (250 mm × 4.6 mm i.d., 5 μm particle size) column was found to be giving satisfactory results. The selection of buffer based on chemical structure of both the drugs. Our preliminary trails with di-sodium hydrogen orthophosphate and ammonium acetate buffer. Result obtain with 0.01 M-ammonium acetate buffer was quite satisfactory but after adding triethylamine and changing pH from 2.5 to 7, at 6.5 pH excellent result were obtain. Finally, by fixing 0.01 M ammonium acetate buffer with addition of 3 ml triethylamine per 1000 ml of buffer, adjusted to pH 6.5 with glacial acetic acid and keeping mobile phase composition as of 0.01 M-ammonium acetate buffer-acetonitrile (65:35, v/v), best peak shape was obtained. Triethylamine was added to buffer to lower the peak asymmetry. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. Optimize mobile phase proportion was provide good resolution between tramadol hydrochloride and aceclofenac and also for degradation product which is generated during force degradation study. Figure 3 and Figure 4 represent the chromatograms of standard and test preparation respectively.

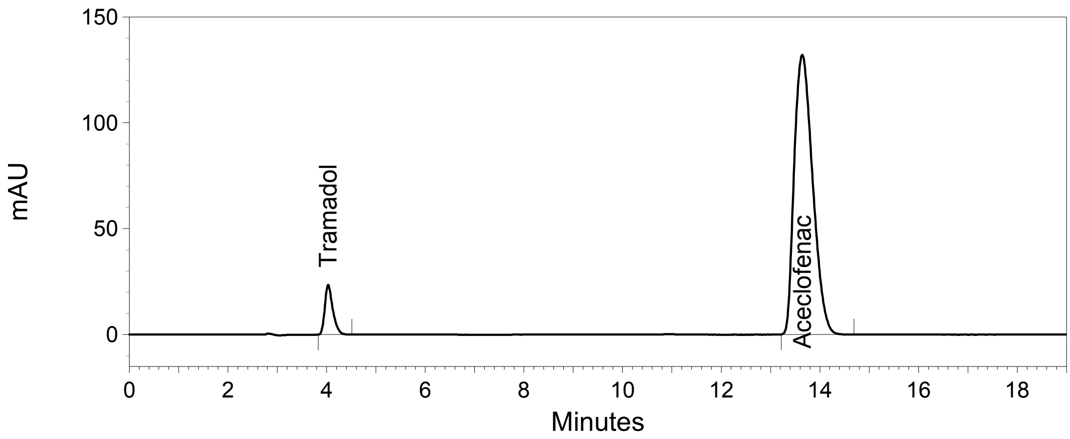


Figure 3: Chromatogram of standard preparation

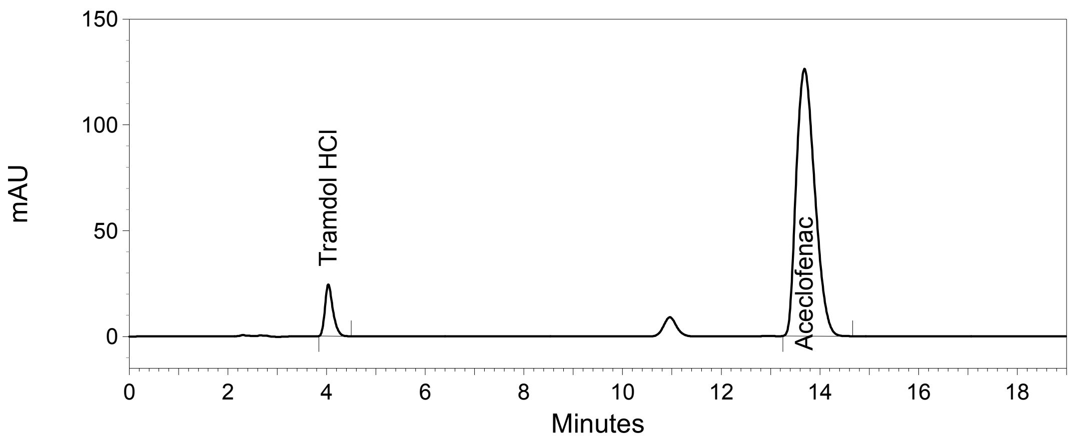


Figure 4: Chromatogram of test preparation

5.2 Degradation Study

In order to determine whether the analytical method or assay were stability-indicating, tramadol hydrochloride and aceclofenac combine tablets were stressed under various conditions to conduct forced degradation studies. Regulatory guidance in ICH Q2A, Q2B, Q3B and FDA 21 CFR section 211 all require the development and validation of stability-indicating potency assays. Unfortunately, the current guidance documents do not indicate detailed degradation conditions in stress testing. However, the used forced degradation conditions, stress agent concentration and times of stress, were found to effect a degradation and not complete degradation of active materials. The discovery of such conditions was based on development trial.

The degradation samples were prepared by transferring powdered tablets, equivalent to 37.5 mg tramadol hydrochloride and 100 mg aceclofenac into a 250 ml round bottom flask. Then prepared samples were employed for acidic, alkaline and oxidant media and also for thermal and photolytic stress conditions. After the degradation treatments were completed, the stress content solutions were allowed to equilibrate to room temperature and diluted with mobile phase to attain 0.0375 mg/ml of tramadol hydrochloride and 0.100 mg/ml of aceclofenac concentration. Specific degradation conditions were described as follows.

5.2.1 Acidic condition: Acidic degradation study was performed by refluxed the drug content in 1 N HCl (50 ml) for 30 min and mixture was neutralized with 1 N NaOH solutions. The drug content was found to be degrading up to 30.50% in acidic condition (Figure 5).

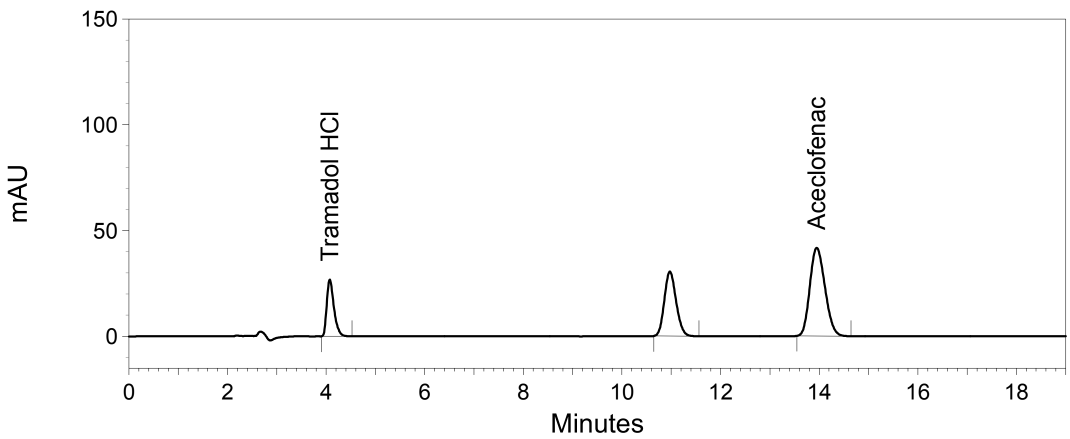


Figure 5: Chromatogram of acidic forced degradation study

5.2.2 Alkaline condition: Solutions of alkali degradation study were prepared in 0.05 N NaOH (50 ml) and the resultant solution analyzed 15 min after preparation, around 8.49% of the drug degraded (Figure 6). Our second trail with 0.10 N NaOH (50 ml) and the resultant solution analyzed 15 min after preparation, peak of aceclofenac was disappear and major degradent peak was found at 11.3 min. In alkali degradation, it was found that aceclofenac is highly unstable.

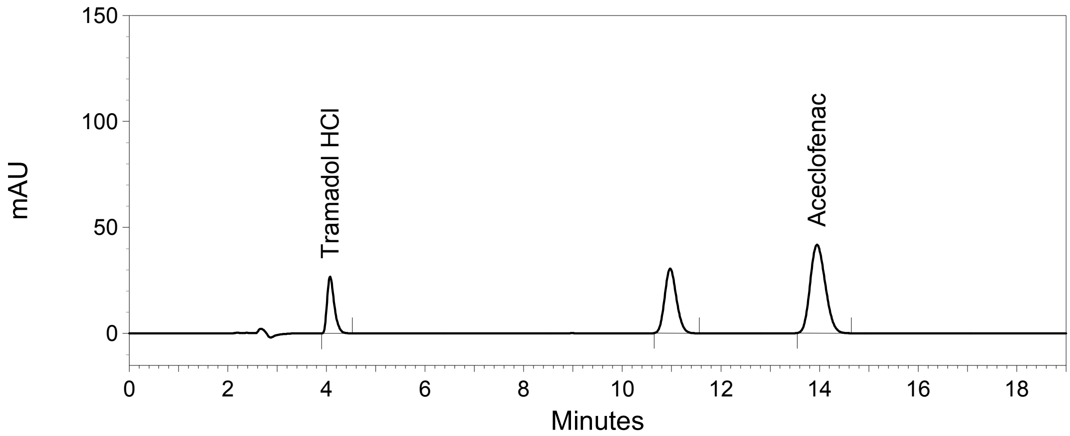


Figure 6: Chromatogram of alkali forced degradation study

5.2.3 Oxidative condition: Oxidation degradation study was performed by refluxed the drug content in 5% v/v H_2O_2 (50 ml) for 3 h. In oxidative degradation, it was found that around 7.62% of the drug degraded (Figure 7).

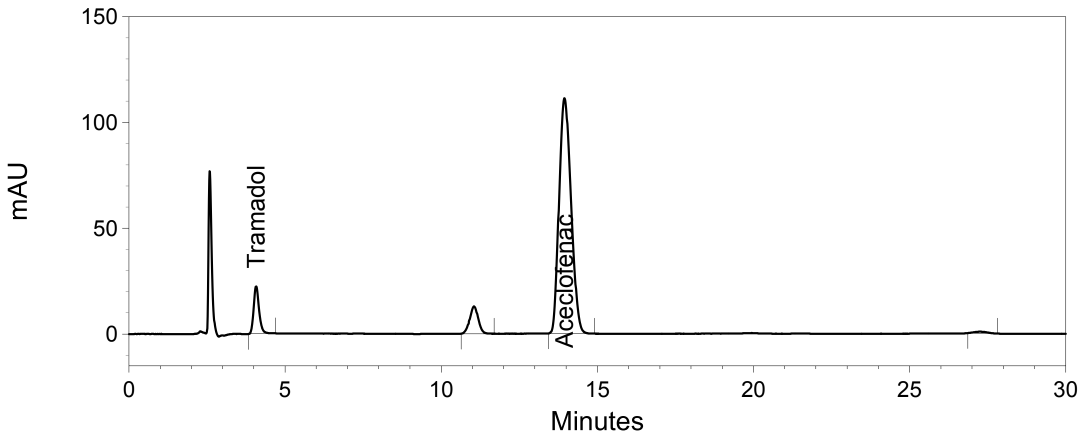


Figure 7: Chromatogram of oxidative forced degradation study

5.2.4 Thermal condition: Thermal degradation was performed by exposing solid drug to dry heat of 80 ^{0}C in a convection oven for 72 h. Drug content was found slightly stable under above specific condition. Only 3.0% drug content were degraded (Figure 8).

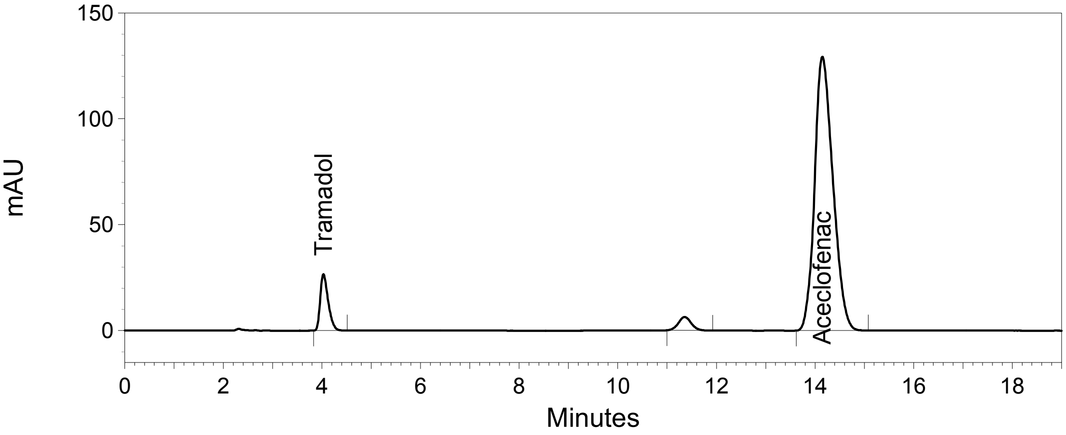


Figure 8: Chromatogram of thermal degradation study

5.2.5 Photolytic condition: Photolytic degradation study was performed by exposing the drug content in sunlight for 72 h. There was 8.9% degradation observed in above specific photolitic condition. Drug content was found to be more stable than other stress condition (Figure 9).

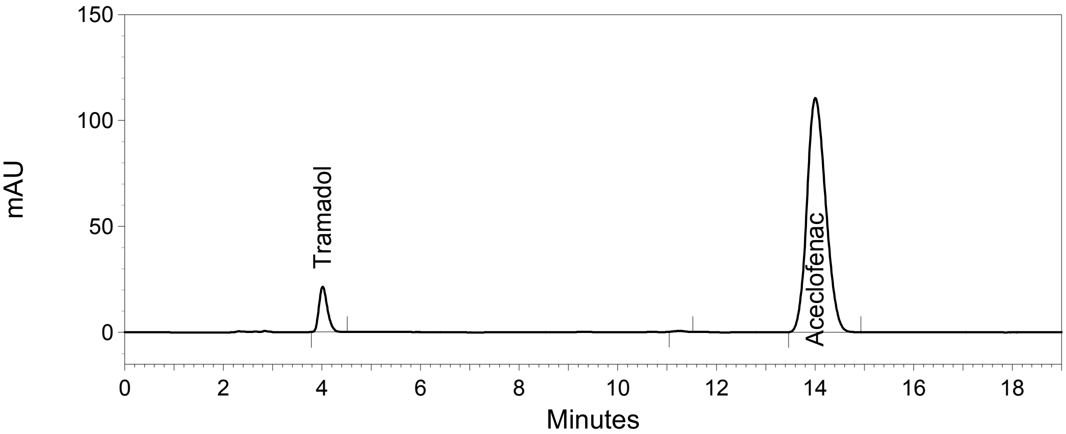


Figure 9: Chromatogram of sunlight degradation study

5.3 Method Validation

5.3.1 Specificity: The specificity of the method was determined by checking the interference of placebo with analyte and the proposed method were eluted by checking the peak purity of tramadol hydrochloride and aceclofenac during the force degradation study. The peak purity of the tramadol hydrochloride and aceclofenac were found satisfactory under different stress condition. There was no interference of any peak of degradation product with drug peak.

5.3.2 Linearity: For linearity, seven points calibration curve were obtained in a concentration range from 0.015-0.060 mg/ml for tramadol hydrochloride and 0.040-0.160 mg/ml for aceclofenac. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation for tramadol hydrochloride was y = 7030988.47x + 5215.35 with correlation coefficient 0.9999 (Figure 10) and for aceclofenac was y = 33843850.67x + 1174.16 with correlation coefficient 0.9999 (Figure 11). Where x is the concentration in mg/ml and y is the peak area in absorbance unit. Chromatogram obtain during linearity study were shown in Figure 12-18.

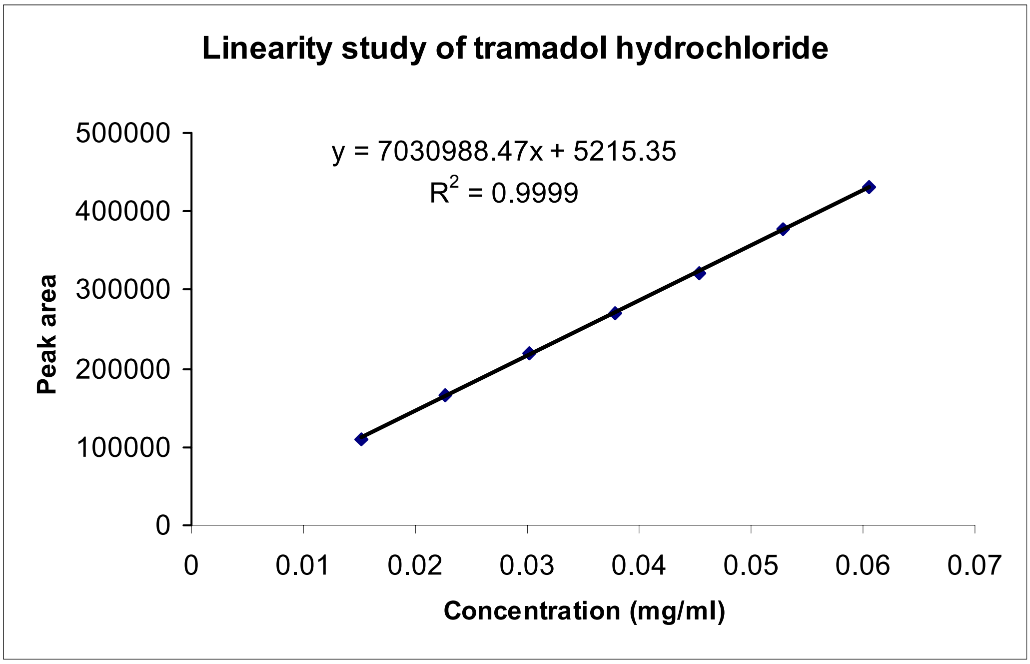


Figure 10: Linearity curve for tramadol hydrochloride

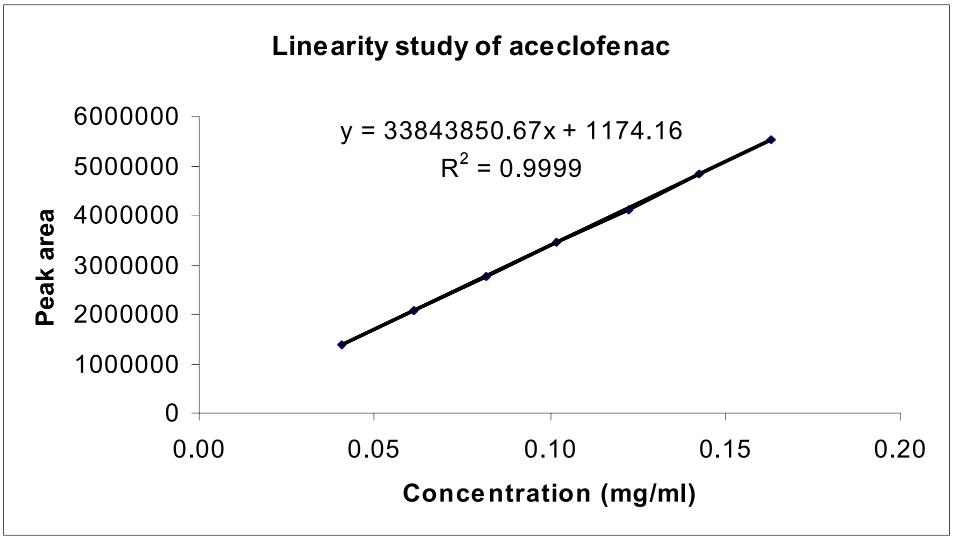


Figure 11: Linearity curve for aceclofenac

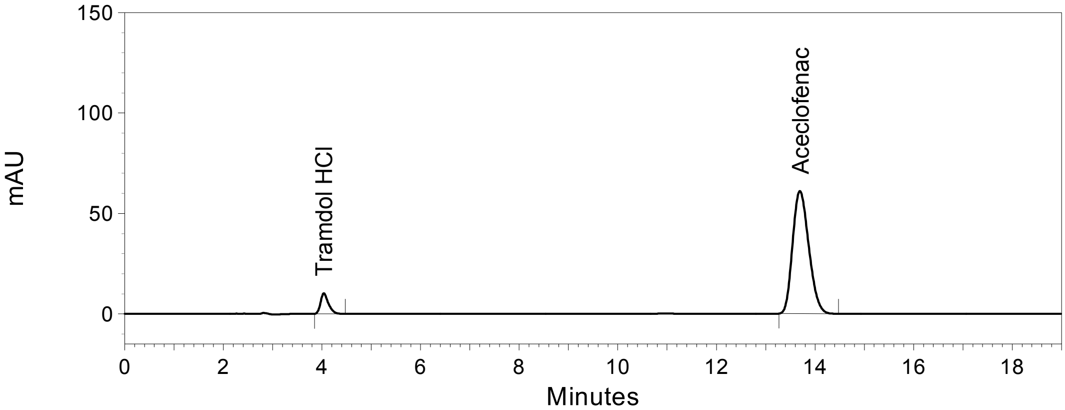


Figure 12: Linearity study chromatogram of level-1 (40%)

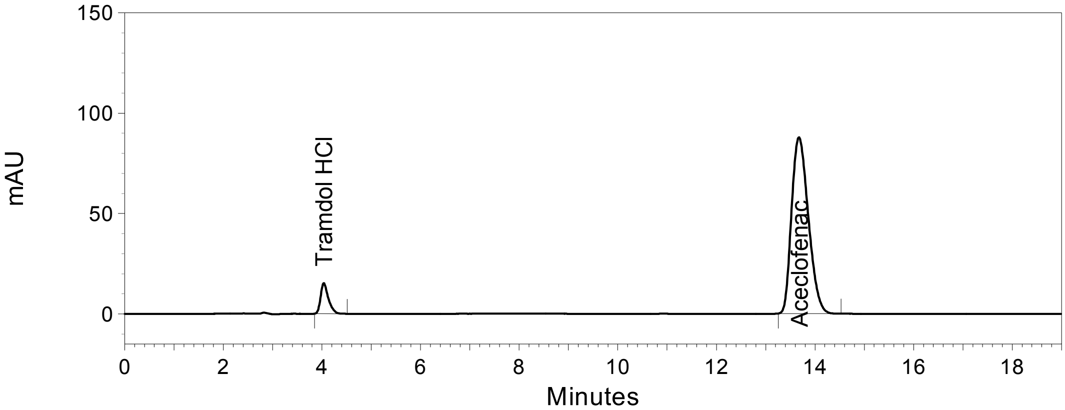


Figure 13: Linearity study chromatogram of level-2 (60%)

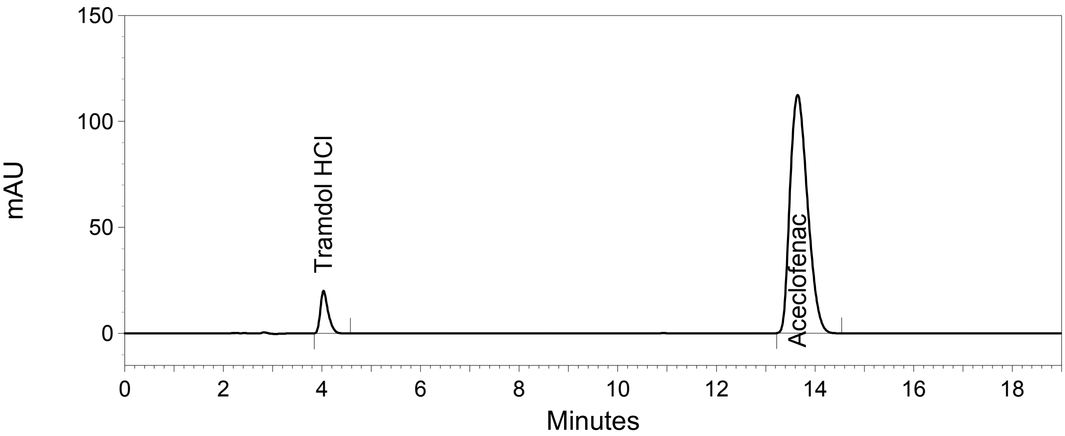


Figure 14: Linearity study chromatogram of level-3 (80%)

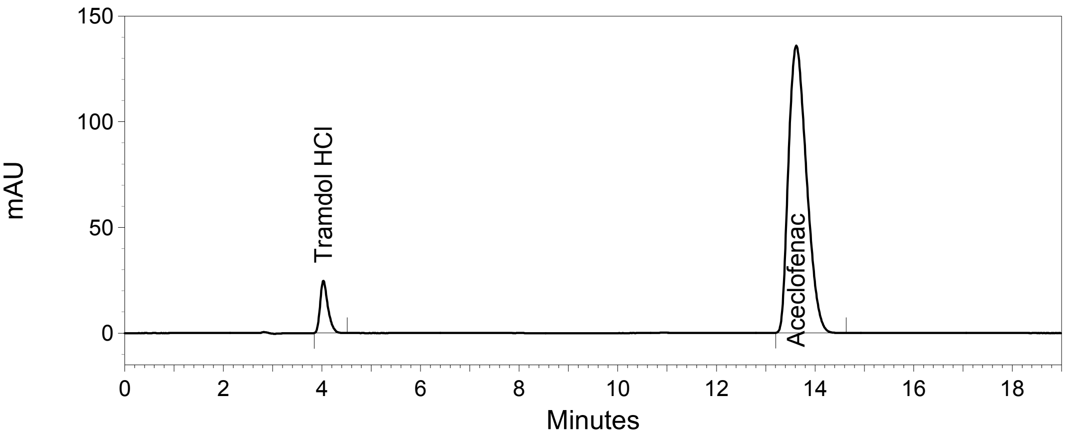


Figure 15: Linearity study chromatogram of level-4 (100%)

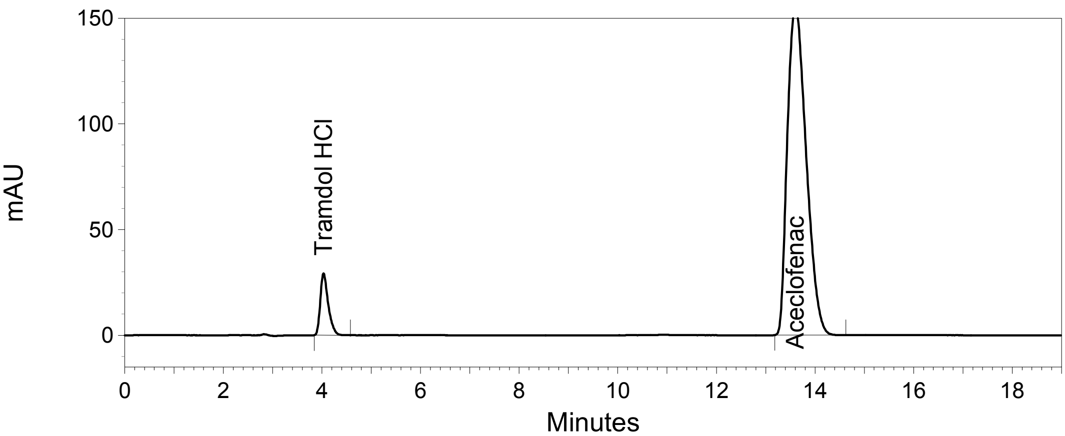


Figure 16: Linearity study chromatogram of level-5 (120%)

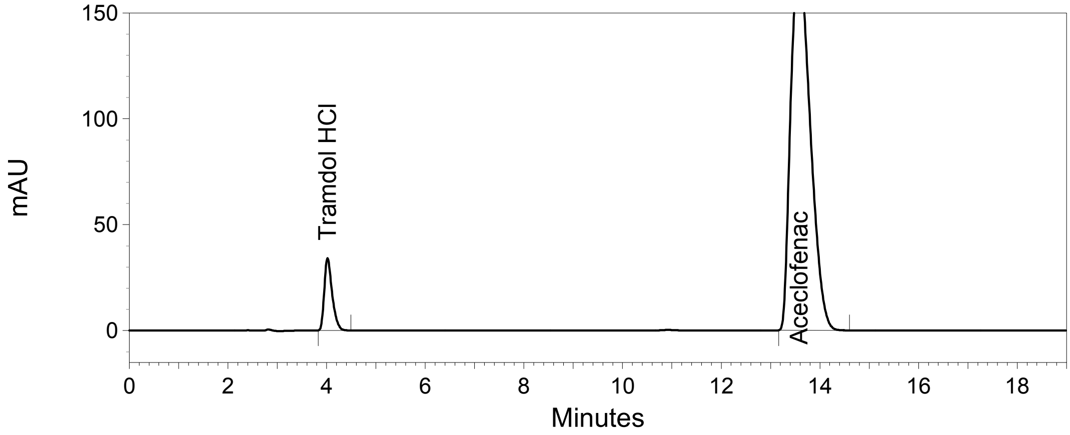


Figure 17: Linearity study chromatogram of level-6 (140%)

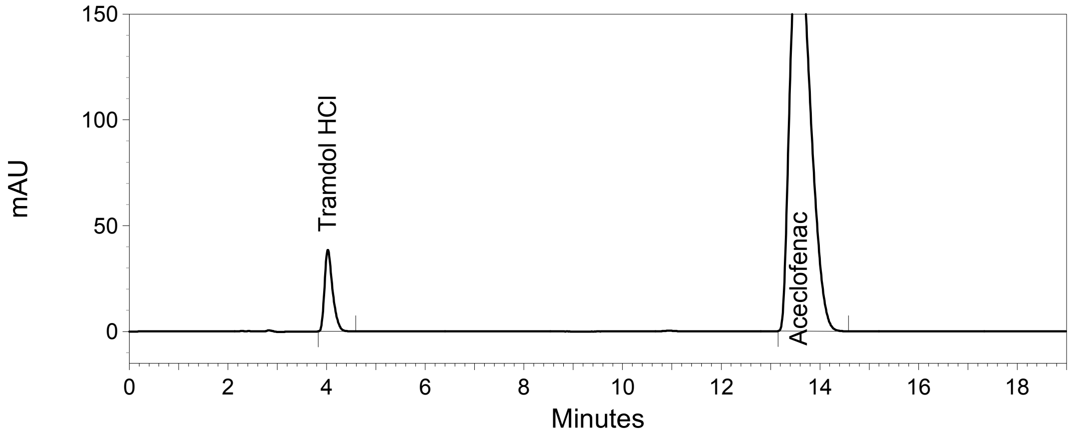


Figure 18: Linearity study chromatogram of level-7 (160%)

5.3.3 LOD and LOQ: The limit of detection and limit of quantification were evaluated by serial dilutions of tramadol hydrochloride and aceclofenac stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD value for tramadol hydrochloride and aceclofenac were found to be 0.1 ppm and 0.1 ppm, respectively and the LOQ value 0.5 ppm and 0.4 ppm, respectively. Chromatogram of LOD and LOQ study were shown in Figure 19-22.

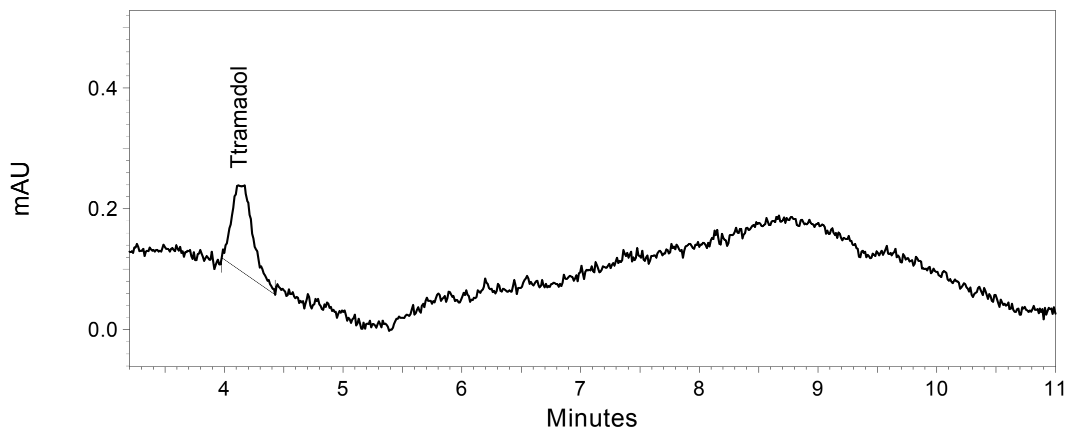


Figure 19: Chromatogram of LOD study of tramadol hydrochloride

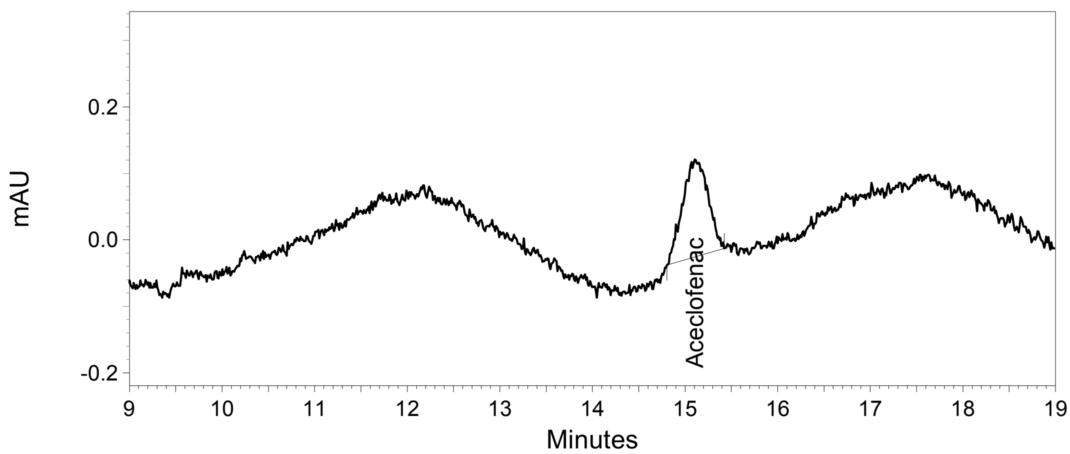


Figure 20: Chromatogram of LOD study of aceclofenac

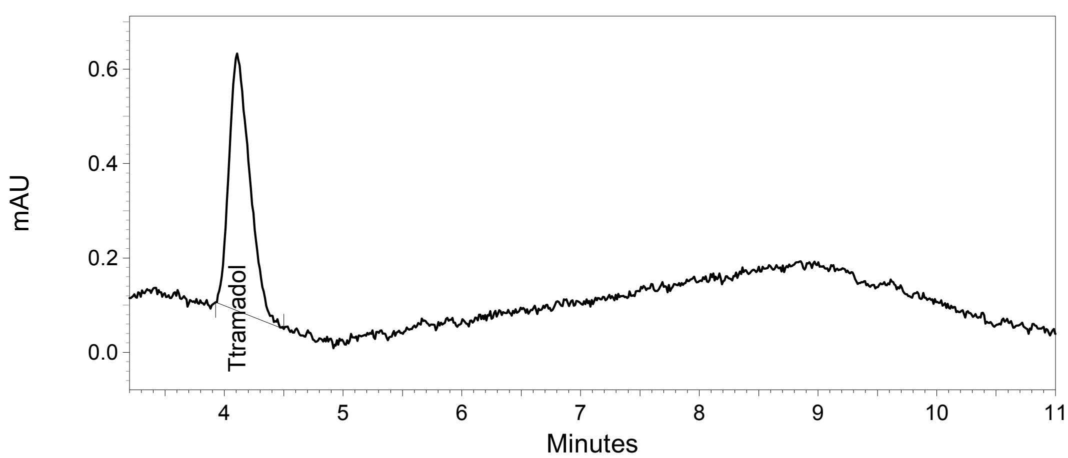


Figure 21: Chromatogram of LOQ study of tramadol hydrochloride

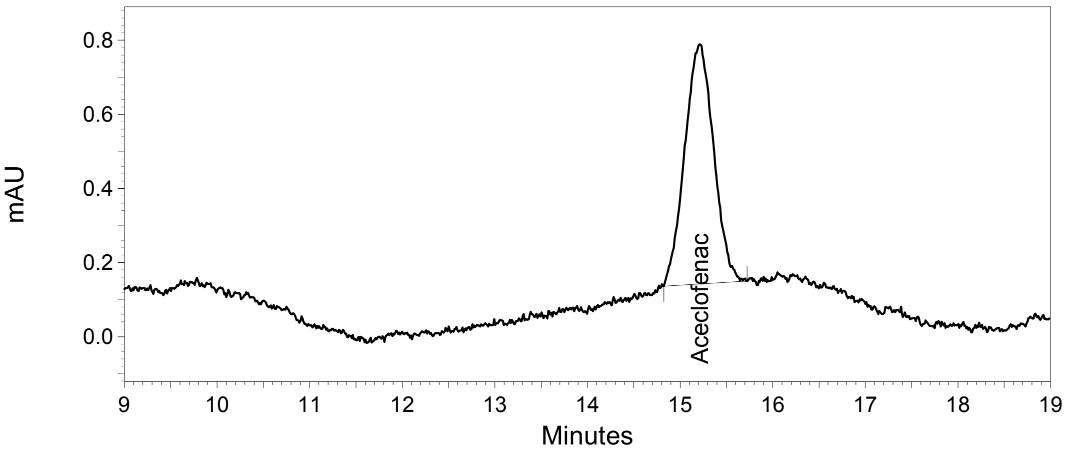


Figure 22: Chromatogram of LOQ study of aceclofenac

5.3.4 Precision: Data obtain from precision experiments are given in Table 1 for intraday and interday precision study for both tramadol hydrochloride and aceclofenac. The RSD values for intra day precision study and interday precision study was < 2.0% for tramadol hydrochloride and aceclofenac. Which confirm that the method was precise.

Table 1: Results of precision study

Set	Tramadol Hydrochloride (%Assay)		Aceclofenac (%Assay)	
	Intraday (n = 6)	**Interday (n = 6)**	**Intraday (n = 6)**	**Intraday (n = 6)**
1	102.0	102.0	98.1	98.1
2	102.7	101.6	98.1	98.1
3	101.6	102.0	98.3	98.3
4	103.1	101.8	99.2	98.5
5	101.7	102.4	98.6	98.9
6	102.4	101.8	99.1	98.6
Mean	102.3	101.9	98.6	98.4
Standard deviation	0.59	0.27	0.49	0.31
% RSD	0.58	0.27	0.50	0.32

5.3.5 Accuracy: Recovery of tramadol hydrochloride and aceclofenac were determined at three different concentration levels. The mean recovery for tramadol hydrochloride was 98.87-99.32% and 98.81-99.49% for aceclofenac (Table 2). The result indicating that the method was accurate. Chromatogram obtain during accuracy study were shown in Figure 23-25.

Table 2: Results of accuracy study

	Level (%)	Amount Added Concentration[a] (mg/ml)	Amount Found Concentration[a] (mg/ml)	% Recovery[a]	% RSD[a]
Tramadol Hydrochloride	50	0.01985	0.01962	98.87	1.12
	100	0.03844	0.03811	99.14	0.16
	150	0.05746	0.05707	99.32	0.58
Aceclofenac	50	0.05033	0.05008	99.49	0.42
	100	0.10040	0.09921	98.81	0.19
	150	0.15038	0.14955	99.45	0.06

[a] Each value corresponds to the mean of three determinations.

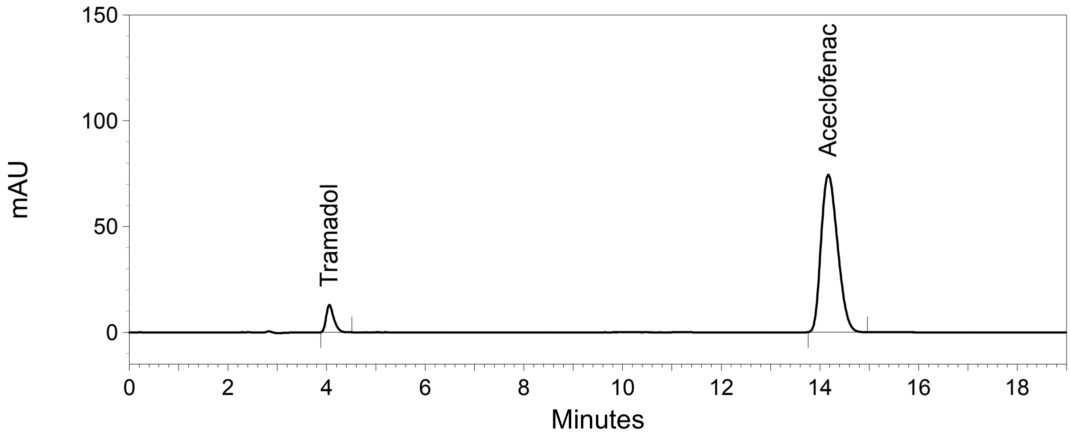


Figure 23: Accuracy study chromatogram of level-1 (50%)

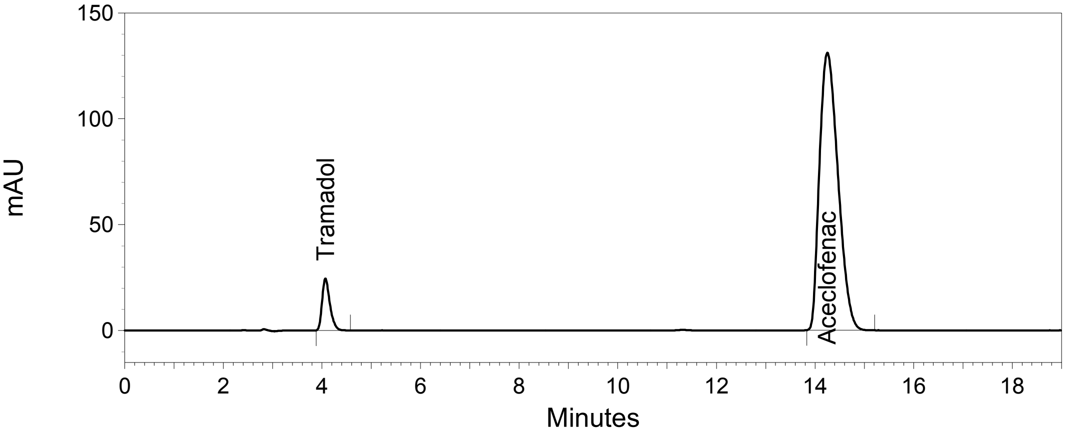


Figure 24: Accuracy study chromatogram of level-2 (100%)

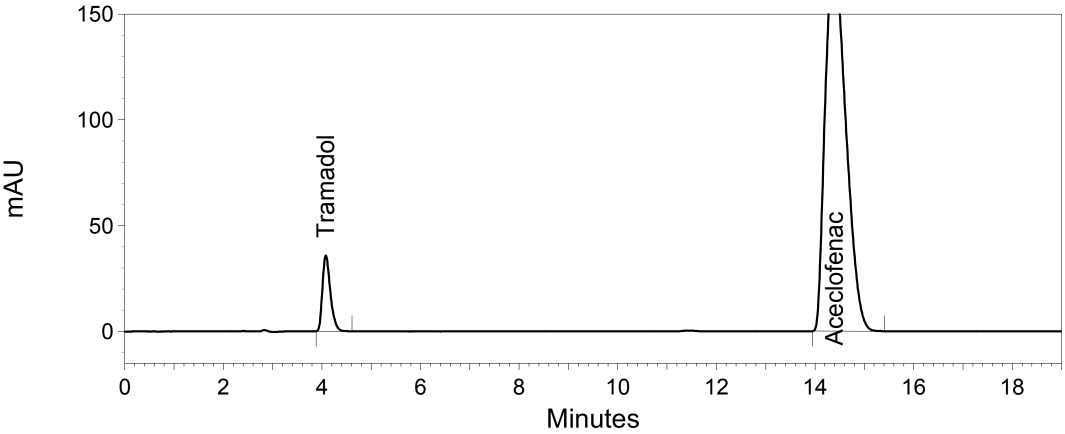


Figure 25: Accuracy study chromatogram of level-3 (150%)

5.3.6 Solution stability study: Table 3 shows the results obtain in the solution stability study at different time intervals for test preparation. It was concluded that the test preparation solution was found stable up to 48 h at 2 - 5 ^{0}C and ambient temperature with the consideration of < 2.0% in % assay value difference of interval value against initial value.

Table 3: Evaluation data of solution stability study

Intervals	% Assay for test solution stored at 2 –5 ^{0}C		% Assay for test solution stored at ambient temperature	
	Tramadol Hydrochloride	Aceclofenac	Tramadol Hydrochloride	Aceclofenac
Initial	102.4	98.9	102.4	98.9
12 h	102.1	98.8	102.0	99.0
24 h	102.0	98.8	101.9	98.8
36 h	102.0	98.9	101.3	98.6
48 h	101.8	98.8	101.0	98.4

5.3.7 Robustness: The result of robustness study of the developed assay method was established in Table 4 and Table 5. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory, hence the analytical method would be concluded as robust. Chromatogram obtain during robustness study were shown in Figure 26-32.

Table 4: Evaluation data of robustness study of tramadol hydrochloride

Robust Conditions	% Assay	System Suitability Parameters		
		Theoretical Plates	Asymmetry	% RSD
Flow 0.9 ml/min	102.6	2827	1.22	0.98
Flow 1.1 ml/min	102.0	2847	1.23	0.79
Buffer- ACN (63:37,v/v)	102.3	3125	1.40	0.38
Buffer- ACN (67:33,v/v)	103.2	2893	1.21	0.42
Buffer pH 6.7	101.9	3184	1.31	1.81
Buffer pH 6.3	103.0	3230	1.39	1.49
Column change	102.4	2767	1.27	0.80

Table 5: Evaluation data of robustness study for aceclofenac

Robust Conditions	% Assay	System Suitability Parameters			
		Theoretical Plates	Asymmetry	% RSD	Resolution
Flow 0.9 ml/min	98.3	6918	1.35	0.48	20.06
Flow 1.1 ml/min	97.8	6158	1.28	0.77	20.55
Buffer-ACN (63:37,v/v)	97.7	6259	1.37	0.19	17.25
Buffer-ACN (67:33,v/v)	97.9	6969	1.36	0.20	23.64
Buffer pH 6.7	98.1	6131	1.26	1.85	19.12
Buffer pH 6.3	98.6	7101	1.39	1.33	22.22
Column change	98.2	6371	1.31	0.09	19.73

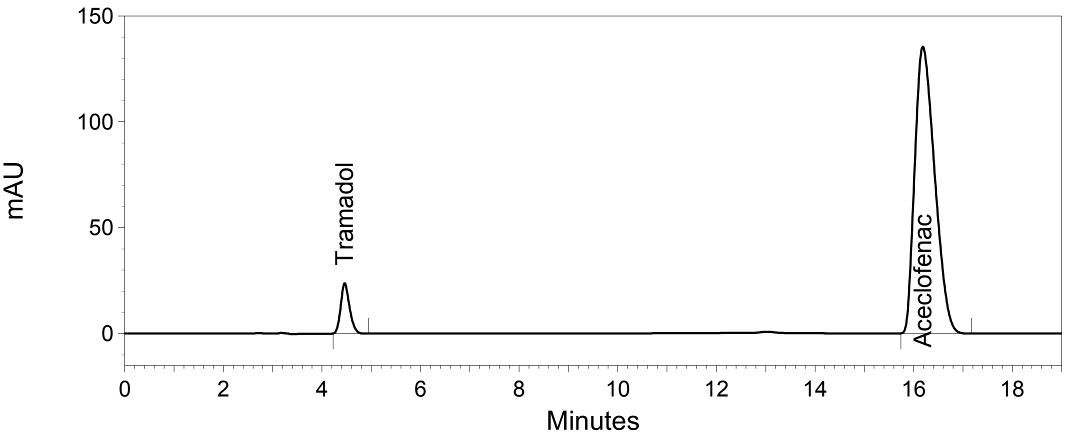


Figure 26: Standard chromatogram (0.9 ml/min flow rate)

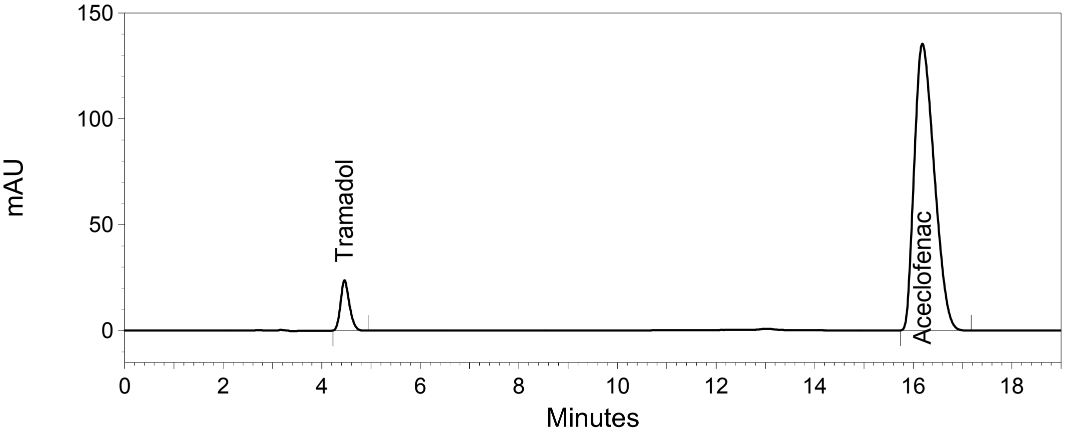


Figure 27: Standard chromatogram (1.1 ml/min flow rate)

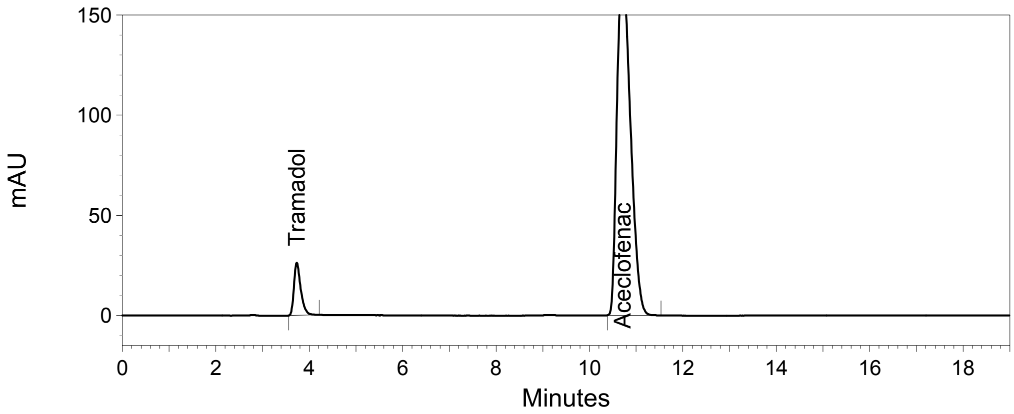


Figure 28: Standard chromatogram [Buffer-ACN (63: 37,v/v)]

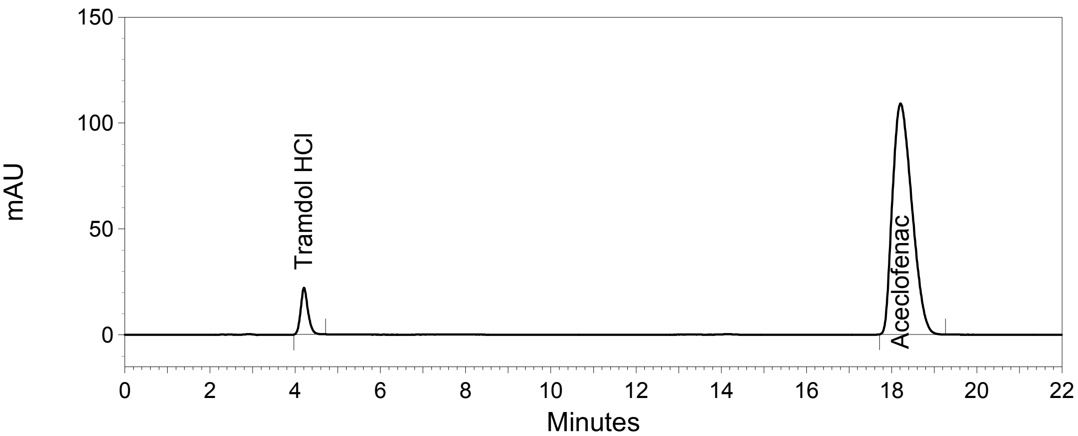


Figure 29: Standard chromatogram [Buffer-ACN (67: 33,v/v)]

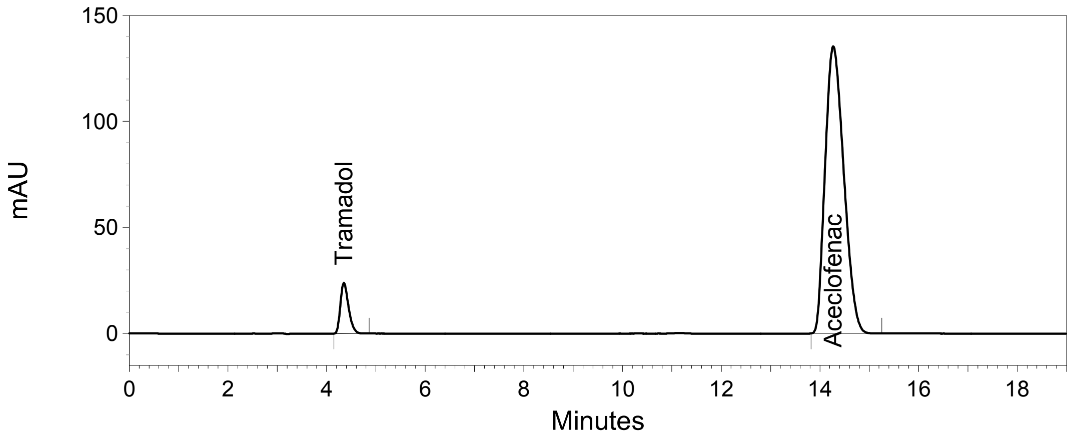


Figure 30: Standard chromatogram (pH 6.7)

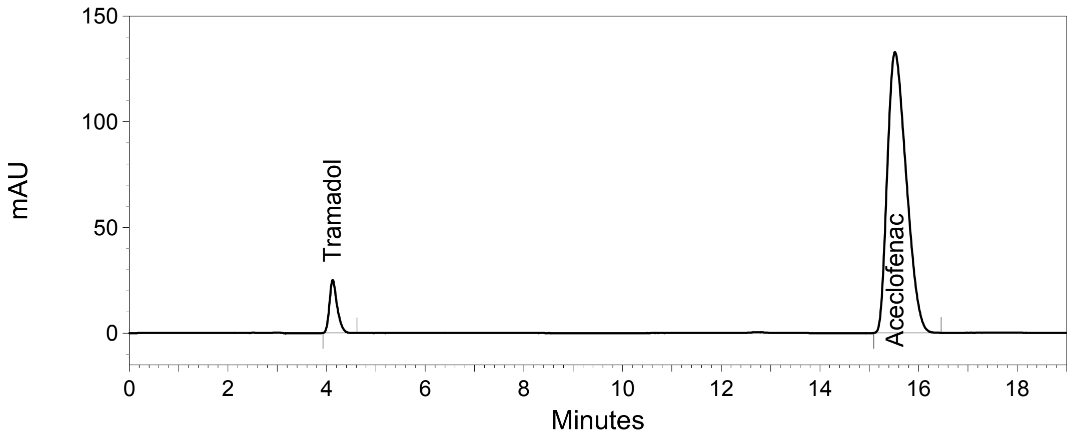


Figure 31: Standard chromatogram (pH 6.3)

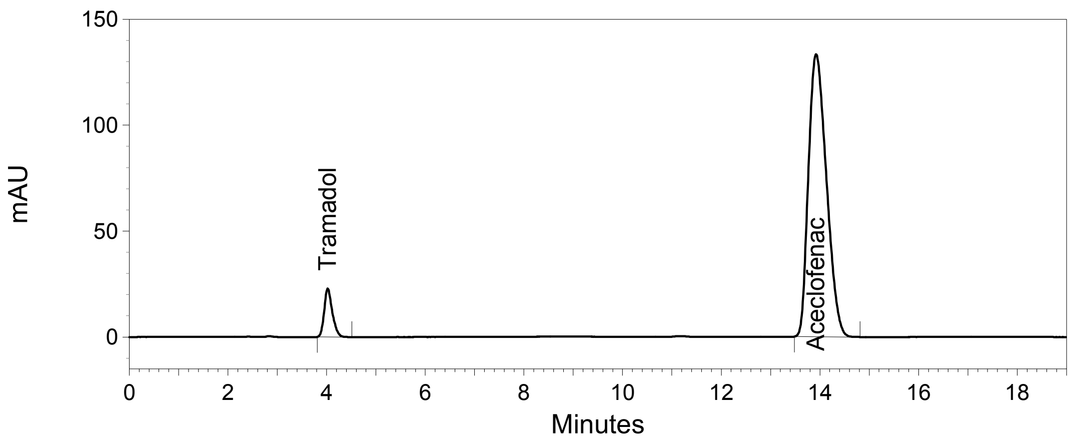


Figure 32: Standard chromatogram (Column change)

5.3.8 System suitability: A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate, resolution and % RSD of peak area were determined for same. Acceptance criteria for system suitability, asymmetry should not be more than 2.0, theoretical plate should not be less then 2000 and % RSD of peak area should not be more then 2.0, were full fill during all validation parameters.

6. Calculation and Data

Calculation formula used:

1. Calculation formula for % assay tramadol hydrochloride

$$\%\ \text{Assay} = \frac{\text{Mean Test Area}}{\text{Mean Standard Area}} \times \frac{\text{Standard Weight}}{50} \times \frac{5}{50} \times \frac{500}{\text{Test Weight}}$$

$$\times \frac{50}{5} \times \frac{\text{Average Weight}}{\text{Lable Claim}} \times \text{Potency of Standard}$$

2. Calculation formula for % assay aceclofenac

$$\%\ \text{Assay} = \frac{\text{Mean Test Area}}{\text{Mean Standard Area}} \times \frac{\text{Standard Weight}}{50} \times \frac{5}{50} \times \frac{500}{\text{Test Weight}}$$

$$\times \frac{50}{5} \times \frac{\text{Average Weight}}{\text{Lable Claim}} \times \text{Potency of Standard}$$

3. Relative standard deviation

$$\%\ \text{RSD} = \frac{\text{Standard Deviation of Measurments}}{\text{Mean value of Measurments}} \times 100$$

4. Recovery

$$\%\ \text{Recovery} = \frac{\text{Amount Found}}{\text{Amount Added}} \times 100$$

5. Amount found

$$\text{Amount found (mg/ml)} = \frac{\text{Mean Test Area}}{\text{Mean Standard Area}} \times \text{Standard Concentration}$$

6. Amount added

$$\text{Amount added (mg/ml)} = \frac{\text{Weight}}{\text{Volume}}$$

Specificity Study for Analytical Method Validation of Tramadol Hydrochloride Aceclofenac Tablets

➢ **For Tramadol Hydrochloride**

Standard Weight (mg)	18.2		
Standard Dilution	50	5	50
Standard Potency	100.0 %		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0364		

Replicate	1	2	3	4	5
Standard Area	252026	258953	253404	250062	253524
Mean Standard Area	253594				
Stdev.	3305.05				
% RSD	1.30				

Replicate	**Test Area**
1 2	272822 270132
Mean Area	271477
Test weight (mg)	1184.2
Label Claim (mg)	37.5
Mean Test weight (mg)	232.8
% Assay	102.1 %

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{271477}{253594} \times \frac{18.2}{50} \times \frac{5}{50} \times \frac{500}{1184.2} \times \frac{50}{5} \times \frac{232.8}{37.5} \times 100$$

$$= 102.1\ \%$$

- **For Aceclofenac**

Standard Weight (mg)	50.1		
Standard Dilution	50	5	50
Standard Potency	99.30 %		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.1002		

Replicate	1	2	3	4	5
Standard Area	3279160	3285344	3282303	3283417	3286086
Mean Standard Area	3283262				
Stdev	2742.25				
% RSD	0.08				

Replicate	**Test Area**
1 2	3290447 3291787
Mean Area	3291117
Test weight (mg)	1184.2
Label Claim (mg)	100.0
Mean Test weight (mg)	232.8
% Assay	98.0 %

Calculation:

Prototype calculation for one set:

$$\% \text{ Assay} = \frac{3291117}{3283262} \times \frac{50.1}{50} \times \frac{5}{50} \times \frac{500}{1184.2} \times \frac{50}{5} \times \frac{232.8}{100} \times 99.30$$

= 98.0 %

Linearity Study for Analytical Method Validation of Tramadol Hydrochloride Aceclofenac Tablets

- **For Tramadol Hydrochloride**

Standard Weight	18.9 mg		
Standard Dilution	50	5	50
Standard Potency	100.0 %		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0378		
Concentration of Linearity Stock Solution (mg/ml)	0.3780		

Replicate	1	2	3	4	5
Standard Area	261299	261449	266215	259101	258707
Mean Standard Area	261354				
Stdev	2988.48				
% RSD	1.14				

Concentration Level (%)	Volume of Linearity Stock Solution Taken (ml)	Diluted to (ml)	Con (
40	2.0	50	
60	3.0	50	
80	4.0	50	
100	5.0	50	
120	6.0	50	
140	7.0	50	
160	8.0	50	
		Correlation co-e	
		Slope	
		Intercept	

➢ **For Aceclofenac**

Standard Weight (mg)	51.0		
Standard Dilution	50	5	50
Standard Potency	99.30 %		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.1020		
Concentration of Linearity Stock Solution (mg/ml)	1.0200		

Replicate	1	2	3	4	5
Standard Area	3438103	3432879	3436263	3433501	3431984
Mean Standard Area	3434546				
Stdev	2551.23				
% RSD	0.07				

Concentration Level (%)	Volume of Linearity Stock Solution Taken (ml)	Diluted to (ml)	Final Concentration (mg/ml)	Mean Area
40	2.0	50	0.0408	1390024
60	3.0	50	0.0612	2070748
80	4.0	50	0.0816	2768981
100	5.0	50	0.1020	3447377
120	6.0	50	0.1224	4118809
140	7.0	50	0.1428	4837026
160	8.0	50	0.1632	5539765
		Correlation co-efficient		0.9999
		Slope		33843850.67
		Intercept		1174.16

Precision Study for Analytical Method Validation of Tramadol Hydrochloride Aceclofenac Tablets

- **For Tramadol Hydrochloride**

Standard Weight (mg)	18.3		
Standard Dilution	50	5	50
Standard Potency	100.0 %		
Label Claim (mg)	37.5		
Mean Test Weight (mg)	232.8		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0366		

Replicate	1	2	3	4	5
Standard Area	259646	261779	261378	262747	259980
Mean Standard Area	261106				
Stdev	1286.40				
% RSD	0.49				

Description	Mean Area	Test weight (mg)	% Assay
Set 1	273753	1168.1	102.0
Set 2	275894	1169.3	102.7
Set 3	272676	1167.9	101.6
Set 4	274325	1157.9	103.1
Set 5	271283	1161.0	101.7
Set 6	273052	1160.1	102.4
		Mean	102.3
		Stdev	0.59
		% RSD	0.58
		Confidence Level (95.0%)	0.62

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{273753}{261106} \times \frac{18.3}{50} \times \frac{5}{50} \times \frac{500}{1168.1} \times \frac{50}{5} \times \frac{232.8}{37.5} \times 100$$

$= 102.0\ \%$

- **For Aceclofenac**

Standard Weight (mg)	51.8		
Standard Dilution	50	5	50
Standard Potency	99.3 %		
Label Claim (mg)	100.0		
Mean Test Weight (mg)	232.8		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.1036		

Replicate	1	2	3	4	5
Standard Area	3425238	3427707	3431001	3434049	3437656
Mean Standard Area	3431130				
Stdev	4938.58				
% RSD	0.14				

Description	Mean Area	Test weight (mg)	% Assay
Set 1	3282936	1168.1	98.1
Set 2	3286875	1169.3	98.1
Set 3	3288107	1167.9	98.3
Set 4	3289935	1157.9	99.2
Set 5	3278731	1161.0	98.6
Set 6	3293697	1160.1	99.1
		Mean	98.6
		Stdev	0.49
		% RSD	0.50
		Confidence Level (95.0%)	0.51

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{3282936}{3431130} \times \frac{51.8}{50} \times \frac{5}{50} \times \frac{500}{1168.1} \times \frac{50}{5} \times \frac{232.8}{100} \times 99.3$$

$= 98.1\ \%$

Intermediate Precision Study for Analytical Method Validation of Tramadol Hydrochloride Aceclofenac Tablets

- **For Tramadol Hydrochloride**

Standard Weight (mg)	18.1		
Standard Dilution	50	5	50
Standard Potency	100.0 %		
Label Claim (mg)	37.5		
Mean Test Weight (mg)	232.8		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0362		

Replicate	1	2	3	4	5
Standard Area	251119	259443	261032	261607	260211
Mean Standard Area	258682				
Stdev	4306.80				
% RSD	1.66				

Description	Mean Area	Test weight (mg)	% Assay
Set 1	274319	1167.8	102.0
Set 2	273491	1169.2	101.6
Set 3	274349	1168.9	102.0
Set 4	273198	1165.4	101.8
Set 5	273605	1160.3	102.4
Set 6	272697	1163.2	101.8
		Mean	101.9
		Stdev	0.27
		% RSD	0.27
		Confidence Level (95.0%)	0.29

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{274319}{258682} \times \frac{18.1}{50} \times \frac{5}{50} \times \frac{500}{1167.8} \times \frac{50}{5} \times \frac{232.8}{37.5} \times 100$$

$$= 102.0\ \%$$

- **For Aceclofenac**

Standard Weight (mg)	51.5		
Standard Dilution	50	5	50
Standard Potency	99.3 %		
Label Claim (mg)	100.0		
Mean Test Weight (mg)	232.8		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.1030		

Replicate	1	2	3	4	5
Standard Area	3299469	3423933	3436241	3436863	3432344
Mean Standard Area	3405770				
Stdev	59647.36				
% RSD	1.75				

Description	Mean Area	Test weight (mg)	% Assay
Set 1	3278616	1167.8	98.1
Set 2	3279877	1169.2	98.1
Set 3	3287797	1168.9	98.3
Set 4	3283409	1165.4	98.5
Set 5	3282316	1160.3	98.9
Set 6	3280094	1163.2	98.6
		Mean	98.4
		Stdev	0.31
		% RSD	0.32
		Confidence Level (95.0%)	0.33

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{3278616}{3405770} \times \frac{51.5}{50} \times \frac{5}{50} \times \frac{500}{1167.8} \times \frac{50}{5} \times \frac{232.8}{100} \times 99.3$$

$= 98.1\ \%$

Comparison for Precision and Intermediate Precision Study for Analytical Method Validation for Tramadol Hydrochloride Aceclofenac Tablets

- **For Tramadol Hydrochloride**

	Set	%Assay
Precision study	1	102.0
	2	102.7
	3	101.6
	4	103.1
	5	101.7
	6	102.4
Intermediate precision study	1	102.0
	2	101.6
	3	102.0
	4	101.8
	5	102.4
	6	101.8
	Mean	102.1
	Stdev	0.47
	% RSD	0.46

- **For Aceclofenac**

	Set	%Assay
Precision study	1	98.1
	2	98.1
	3	98.3
	4	99.2
	5	98.6
	6	99.1
Intermediate precision study	1	98.1
	2	98.1
	3	98.3
	4	98.5
	5	98.9
	6	98.6
	Mean	98.5
	Stdev	0.40
	% RSD	0.40

Accuracy Study for Analytical Method Validation of Tramadol Hydrochloride Aceclofenac Tablets

- **For Tramadol Hydrochloride**

Standard Weight (mg)	18.2		
Standard Dilution	50	5	50
Standard Potency	100.0 %		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0364		

Replicate	1	2	3	4	5
Standard Area	271518	269386	275109	267286	268861
Mean Standard Area	270432				
Stdev	3021.31				
% RSD	1.12				

Recovery Level	Set No.	Mean Area	Weight (mg)	Volume-I (ml)	Volume-II (ml)	Volume-III (ml)	*Amount Added* Concentration (mg/ml)	*Amount Found* Concentration (mg/ml)	% Recovery	Mean % Recovery	Stdev	% RSD
50%	Set 1	139934	99.4	500	5	50	0.01988	0.01987	99.95	98.87	1.11	1.12
	Set 2	138269	99.2	500	5	50	0.01984	0.01963	98.94			
	Set 3	136423	99.1	500	5	50	0.01982	0.01937	97.73			
100%	Set 1	267994	192.2	500	5	50	0.03844	0.03805	98.99	99.14	0.16	0.16
	Set 2	268416	192.2	500	5	50	0.03844	0.03811	99.14			
	Set 3	268843	192.2	500	5	50	0.03844	0.03817	99.30			
150%	Set 1	399872	287.0	500	5	50	0.05740	0.05678	98.92	99.32	0.58	0.58
	Set 2	404056	286.9	500	5	50	0.05738	0.05737	99.98			
	Set 3	401787	288.0	500	5	50	0.05760	0.05705	99.05			

Calculation:

Prototype calculation for one set:

$$\%\ \text{Recovery} = \frac{0.01987}{0.01988} \times 100 \qquad = 99.95\ \%$$

$$\text{Amount Found (mg/ml)} = \frac{139934}{270432} \times 0.0364 \qquad = 0.01987\ \text{mg/ml}$$

$$\text{Amount Added (mg/ml)} = \frac{99.4}{500} \times \frac{5}{50} \qquad = 0.01988\ \text{mg/ml}$$

- **For Aceclofenac**

Standard Weight (mg)	49.6		
Standard Dilution	50	5	50
Standard Potency	99.3 %		
Factor 1	-		
Factor 2	-		
Standard Concentration (mg/ml)	0.0992		

Replicate	1	2	3	4	5
Standard Area	3420322	3421039	3421754	3424969	3425154
Mean Standard Area	3422648				
Stdev	2261.94				
% RSD	0.07				

Recovery Level	Set No.	Mean Area	Weight (mg)	Volume-I (ml)	Volume-II (ml)	Volume-III (ml)	*Amount Added* Concentration (mg/ml)	*Amount Found* Concentration (mg/ml)	% Recovery	Mean % Recovery	Stdev	% RSD
50%	Set 1	1727247	252.8	500	5	50	0.05056	0.05006	99.01	99.49	0.42	0.42
	Set 2	1729006	251.2	500	5	50	0.05024	0.05011	99.74			
	Set 3	1727330	251.0	500	5	50	0.05020	0.05006	99.72			
100%	Set 1	3419125	502.4	500	5	50	0.10048	0.09910	98.63	98.81	0.19	0.19
	Set 2	3423250	501.1	500	5	50	0.10022	0.09922	99.00			
	Set 3	3426074	502.5	500	5	50	0.10050	0.09930	98.81			
150%	Set 1	5157052	752.0	500	5	50	0.15040	0.14947	99.38	99.45	0.06	0.06
	Set 2	5162810	751.9	500	5	50	0.15038	0.14964	99.51			
	Set 3	5159992	751.8	500	5	50	0.15036	0.14955	99.46			

Calculation:

Prototype calculation for one set:

$$\%\ \text{Recovery} = \frac{0.05006}{0.05056} \times 100 \qquad = 99.01\ \%$$

$$\text{Amount Found (mg/ml)} = \frac{1727247}{3422648} \times 0.0992 \qquad = 0.05006\ \text{mg/ml}$$

$$\text{Amount Added (mg/ml)} = \frac{252.8}{500} \times \frac{5}{50} \qquad = 0.05056\ \text{mg/ml}$$

Solution Stability Study for Analytical Method Validation of Tramadol Hydrochloride Aceclofenac Tablets

➢ **For Tramadol Hydrochloride**

System suitability of standard preparation for solution stability					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	260392	261159	267935	264328	262170
2	254901	260317	264968	265192	261927
3	257240	256782	264130	263555	261336
4	255518	261812	265905	266558	261331
5	257058	258315	265036	266198	260358
Mean	257022	259677	265595	265166	261424
Stdev	2131.10	2085.68	1451.17	1255.08	700.41
%RSD	0.83	0.81	0.55	0.47	0.27

Solution stability for standard preparation at 2-8 °C				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	260392	260392	260392	260392
2	254901	254901	254901	254901
3	257240	257240	257240	257240
4	255518	255518	255518	255518
5	257058	257058	257058	257058
1	255324	258861	259349	260494
2	258516	257384	255916	258255
Mean	256993	257336	257196	257694
Stdev	1969.59	1870.29	2024.51	2182.52
%RSD	0.77	0.73	0.79	0.85

Solution stability for standard preparation at room temperature				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	260392	260392	260392	260392
2	254901	254901	254901	254901
3	257240	257240	257240	257240
4	255518	255518	255518	255518
5	257058	257058	257058	257058
1	261923	257993	259349	259334
2	262795	255794	255916	261031
Mean	258547	256985	257196	257925
Stdev	3142.20	1853.27	2024.51	2376.19
%RSD	1.22	0.72	0.79	0.92

Solution stability for test preparation at 2-8 °C					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	260392	261159	267935	264328	262170
2	254901	260317	264968	265192	261927
3	257240	256782	264130	263555	261336
4	255518	261812	265905	266558	261331
5	257058	258315	265036	266198	260358
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	273065	271328	271516	272799	272447
2	269238	274725	271750	275489	272828
Mean	271152	273027	271513	274144	272638
% Assay	102.4	102.1	102.0	102.0	101.8
Standard weight (mg)	18.2	18.2	18.7	18.5	18.3
Test weight (mg)	1164.0	1164.0	1164.0	1164.0	1164.0
% Absolute difference compare to that of initial		0.3	0.4	0.4	0.6

Solution stability for test preparation at room temperature					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	260392	261159	267935	264328	262170
2	254901	260317	264968	265192	261927
3	257240	256782	264130	263555	261336
4	255518	261812	265905	266558	261331
5	257058	258315	265036	266198	260358
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	273065	274081	271265	272175	271087
2	269238	271484	271580	272159	270236
Mean	271152	272783	271423	272167	270662
% Assay	102.4	102.0	101.9	101.3	101.0
Standard weight (mg)	18.2	18.2	18.7	18.5	18.3
Test weight (mg)	1164.0	1164.0	1164.0	1164.0	1164.0
% Absolute difference compare to that of initial		0.4	0.5	1.1	1.4

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{271152}{257022} \times \frac{18.2}{50} \times \frac{5}{50} \times \frac{500}{1164.0} \times \frac{50}{5} \times \frac{232.8}{37.5} \times 100$$

$$= 102.4\ \%$$

- **For Aceclofenac**

System suitability of standard preparation for solution stability					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	3347996	3382284	3385487	3391207	3316744
2	3344176	3387704	3384372	3376527	3326361
3	3358780	3388157	3383455	3385555	3331359
4	3356068	3391170	3392057	3382404	3329634
5	3357155	3390627	3384738	3385328	3331216
Mean	3352835	3387988	3386022	3384204	3327063
Stdev	6380.67	3526.13	3452.10	5345.72	6109.17
%RSD	0.19	0.10	0.10	0.16	0.18

Solution stability for standard preparation at 2-8 °C				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	3347996	3347996	3347996	3347996
2	3344176	3344176	3344176	3344176
3	3358780	3358780	3358780	3358780
4	3356068	3356068	3356068	3356068
5	3357155	3357155	3357155	3357155
1	3364445	3370161	3355610	3380903
2	3368077	3366295	3363435	3370000
Mean	3356671	3357233	3354746	3359297
Stdev	8435.62	9208.85	6549.26	12602.99
%RSD	0.25	0.27	0.20	0.38

Solution stability for standard preparation at room temperature				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	3347996	3347996	3347996	3347996
2	3344176	3344176	3344176	3344176
3	3358780	3358780	3358780	3358780
4	3356068	3356068	3356068	3356068
5	3357155	3357155	3357155	3357155
1	3360849	3359489	3355610	3362568
2	3350173	3358676	3363435	3365092
Mean	3353600	3354620	3354746	3355976
Stdev	6192.34	6040.71	6549.26	7513.73
%RSD	0.18	0.18	0.20	0.22

Solution stability for test preparation at 2-8 °C					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	3347996	3382284	3385487	3391207	3316744
2	3344176	3387704	3384372	3376527	3326361
3	3358780	3388157	3383455	3385555	3331359
4	3356068	3391170	3392057	3382404	3329634
5	3357155	3390627	3384738	3385328	3331216
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	3258806	3271689	3263250	3278056	3287915
2	3270233	3276823	3283054	3281618	3284992
Mean	3264520	3274256	3273152	3279837	3286454
% Assay	98.9	98.8	98.8	98.9	98.8
Standard weight (mg)	50.8	51.1	51.1	51.0	50.0
Test weight (mg)	1164.0	1164.0	1164.0	1164.0	1164.0
% Absolute difference compare to that of initial		0.1	0.1	0.0	0.1

Solution stability for test preparation at room temperature					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	3347996	3382284	3385487	3391207	3316744
2	3344176	3387704	3384372	3376527	3326361
3	3358780	3388157	3383455	3385555	3331359
4	3356068	3391170	3392057	3382404	3329634
5	3357155	3390627	3384738	3385328	3331216
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	3258806	3285429	3274892	3269432	3274922
2	3270233	3279243	3273532	3271832	3275400
Mean	3264520	3282336	3274212	3270632	3275161
% Assay	98.9	99.0	98.8	98.6	98.4
Standard weight (mg)	50.8	51.1	51.1	51.0	50.0
Test weight (mg)	1164.0	1164.0	1164.0	1164.0	1164.0
% Absolute difference compare to that of initial		-0.1	0.1	0.3	0.5

Calculation:

Prototype calculation for one set:

$$\%\ \text{Assay} = \frac{3264520}{3352835} \times \frac{50.8}{50} \times \frac{5}{50} \times \frac{500}{1164.0} \times \frac{50}{5} \times \frac{232.8}{100} \times 99.3$$

$$= 98.9\ \%$$

Robustness Study for Analytical Method Validation of Tramadol Hydrochloride Aceclofenac Tablets

➢ **For Tramadol Hydrochloride**

	Flow Rate at 0.9 ml/min	**Flow Rate at 1.1 ml/min**	**Buffer: ACN 63: 37**	**Buffer: ACN 67: 33**	**Buffer pH 6.7**	**Buffer pH 6.3**	**Column Change**
Replicate	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area
1	303478	241740	263306	259423	284336	276377	257684
2	295434	240573	265686	261282	274820	271564	259242
3	298696	243273	264818	259969	270696	270144	258121
4	297694	241172	263695	260836	265418	266552	262972
5	298648	245345	263608	262197	264298	261748	259138
Mean	298790	242421	264223	260741	271914	269277	259431
Stdev	2935.57	1917.98	999.04	1090.27	8130.74	5488.70	2087.36
% RSD	0.98	0.79	0.38	0.42	2.99	2.04	0.80
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area
1	306605	244492	273154	271529	272592	272471	273253
2	302247	246640	274216	273543	271692	272604	279094
Mean	304426	245566	273685	272536	272142	272538	276174
Standard weight (mg)	18.9	18.9	18.7	18.7	18.8	18.8	18.1
Test weight (mg)	1165.2	1165.2	1175.4	1175.4	1146.7	1146.7	1168.2
Label claim (mg)	37.5	37.5	37.5	37.5	37.5	37.5	37.5
Average test weight (mg)	232.8	232.8	232.8	232.8	232.8	232.8	232.8
% Assay	102.6	102.0	102.3	103.3	101.9	103.0	102.4

Calculation:

Prototype calculation for one set: $\%\ \text{Assay} = \frac{304426}{292790} \times \frac{18.9}{50} \times \frac{5}{50} \times \frac{500}{1165.2} \times \frac{50}{5} \times \frac{232.8}{37.5} \times 100 = 102.6\ \%$

- **For Aceclofenac**

	Flow Rate at 0.9ml/min	**Flow Rate at 1.1ml/min**	**Buffer: ACN 63: 37**	**Buffer: ACN 67: 33**	**Buffer pH 6.7**	**Buffer pH 6.3**	**Column Change**
Replicate	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area
1	3773862	3041954	3464662	3442705	3623564	3569151	3376959
2	3806151	3074647	3477913	3455713	3569215	3542703	3379883
3	3812891	3091682	3480193	3455743	3526308	3502585	3382690
4	3817707	3093673	3479795	3456071	3478339	3467828	3384750
5	3817072	3100742	3478568	3460925	3465130	3461979	3381953
Mean	3805537	3080540	3476226	3454231	3532511	3508849	3381247
Stdev	18295.69	23602.69	6529.39	6810.40	65481.13	46640.06	2962.23
% RSD	0.48	0.77	0.19	0.20	1.85	1.33	0.09
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area	Test Area
1	3665511	2954305	3306578	3293784	3293784	3282917	3283264
2	3664729	2949882	3301288	3284856	3284856	3282381	3277531
Mean	3665120	2952094	3303933	3289320	3289320	3282649	3280398
Standard weight (mg)	51.1	51.1	51.9	51.9	51.9	51.9	50.8
Test weight (mg)	1165.2	1165.2	1175.4	1175.4	1146.7	1146.7	1168.2
Label claim (mg)	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Average test weight (mg)	232.8	232.8	232.8	232.8	232.8	232.8	232.8
% Assay	98.3	97.8	97.7	97.9	98.1	98.6	98.2

Calculation:

Prototype calculation for one set: $\% \text{ Assay} = \frac{3665120}{3805537} \times \frac{51.1}{50} \times \frac{5}{50} \times \frac{500}{1165.2} \times \frac{50}{5} \times \frac{232.8}{100} \times 99.3 = 98.3\ \%$

REFERENCES

[1] S. Bodavari, Eds., In; The Merck Index, 14^{th} Edn., Merck & Co., Inc., Whitehouse Station, NJ. (2006) 23 and 9566.

[2] K. S. Lewis, N. H. Han, Tramadol: a new centrally acting analgesic, *Am. J. Health Syst. Pharm*. **54** (1997) 643.

[3] K. Budd, R. Langford, Tramadol revisited, *Br. J. Anaesth.* **82** (1999) 493.

[4] W. Lintz, H. Barth, G. Osterloh, E. Schmidt-Böthelt, Pharmacokinetics of tramadol and bioavailability of enteral tramadol formulations, Arzneim.-Forsch, *Drug Res*. **48** (1998) 889.

[5] R. N. Brogden, L. R. Wiseman, *Drugs*. **52** (1996) 113.

[6] M. Dooley, C. M. Spencer, C. J. Dunn, *Drugs*. **61** (2001) 1351-1378.

[7] J. Martel-Pelletier, J. M. Cloutier, J. P. Pelletier, *Clin. Drug Invest*. **14** (1997) 226.

[8] R. Yamazaki, S. Kawai, T. Matsuzaki, N. Kaneda, S. Hashimoto, T. Yokokura, R. Okamoto, T. Koshino, Y. Mizushima, *Eur. J. Pharmacol*. **28** (1999) 676.

[9] E. Legrand, Expert. Opin. *Pharmacother*. **5** (2004) 1347-1357.

[10] Y. H. Ardakani, M. R. Rouini, *J. Pharm. Biomed. Anal.* **44** (2007) 1168.

[11] R. Mehvar, K. Elliott, R. Parasrampuria, O. Eradiri, *J. Pharm. Biomed. Anal*. **852** (2007) 152.

[12] M. Zeceviæ, Z. Stankoviæ, Lj. Zivanoviæ, B. Jociæ, *J. Chromatogr. A* **1119** (2006) 251.

[13] A. Küçük, Y. Kadioðlu, *Farmaco*. **60** (2005) 163.

[14] A. Küçük, Y. Kadýoðlu, F. Çelebi, *J. Chromatogr. B* **816** (2005) 203.

[15] Y. Gu, J. P. Fawcett, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **821** (2005) 240.

[16] L. M. Zhao, X. Y. Chen, J. J. Cui, M. Sunita, D. F. Zhong, *Yao. Xue. Xue. Bao*. **39** (2004) 458.

[17] A. Medvedovici, F. Albu, A. Farca, V. David, *J. Pharm. Biomed. Anal*. **34** (2004) 67.

[18] M. A. Campanero, E. Garcia-Quetglas, B. Sadaba, J. R. Azanza, *J. Chromatogr. A* **1031** (2004) 219.

[19] V. Kmetec, R. Roskar. *J. Pharm. Biomed. Anal.* **32** (2003) 1061.

[20] S. H. Gan, R. Ismail, W. A. Wan Adnan, Z. Wan, *J. Chromatogr. B Analyt. Techno.l Biomed. Life. Sci.* **772** (2002) 123.

[21] M. Nobilis, J. Kopecký, J. Kvetina, J. Chládek, Z. Svoboda, V. Vorísek, F. Perlík, M. Pour, J. Kunes, *J. Chromatogr. A* **949** (2002) 11.

[22] H. E. Abdellatef, *J. Pharm. Biomed. Anal.* **29** (2002) 835.

[23] S. H. Gan, R. Ismail**,** *J. Chromatogr. B Biomed. Sci. Appl***.** **759** (2001) 325.

[24] F. Vanderbist Ceccato, J. Y. Pabst, B. Streel, *J. Chromatogr. B Biomed. Sci. Appl.* **748** (2000) 65.

[25] M. A. Campanero, B. Calahorra, M. Valle, I.F. Troconiz, J. Honorato, *Chirality*.**11** (1999) 272.

[26] G. C. Yeh, M. T. Sheu, C. L. Yen, Y. W. Wang, C. H. Liu, H. O. Ho, *J. Chromatogr. B Biomed. Sci. Appl.* **723** (1999) 247.

[27] M. Valle, J. M. Pavon, R. Calvo, M. A. Campanero, I. F. Troconiz, *J. Chromatogr. B* **724** (1999) 83.

[28] P. Overbeck, G. Blaschke, *J. Chromatogr. B* **732** (1999) 185.

[29] A. Ceccato, P. Chiap, P. Hubert, J. Crommen, *J. Chromatogr. B Biomed. Sci. Appl.* **698** (1997) 161.

[30] I. Y. Zaghloul, M. A. Radwan, *J. Liq. Chrom. Rel. Technol.* **20** (1997) 779.

[31] M. Nobilis, J. Pastera, P. Anzenbacher, D. Svoboda, J. Kopecký, F. Perlík., *J. Chromatogr. B Biomed. Appl.* **681** (1996) 177.

[32] B. Elsing, G. Blaschke, *J. Chromatogr.* ***612*** (1993) 223.

[33] P. Musmade, G. Subramanian, K. K.Srinivasan, *Anal. Chim. Acta*. **585** (2007) 103.

[34] A. Zinellu, C. Carru, S. Sotgia, E. Porqueddu, P. Enrico, L. Deiana, *Eur. J. Pharm. Sci.* **24** (2005) 375.

[35] Y. Jin, H. Chen, S. Gu, F. Zeng, *Se. Pu.* **22** (2004) 252.

[36] N. Y. Hasan, M. Abdel-Elkawy, B. E. Elzeany, N. E. Wagieh, *Il Farmaco.* **58** (2003) 91.

[37] B. Hinz, D. Auge, T. Rau, S. Rietbrock, K. Brune, U. Werner, *Biomed. Chromatogr.* **17** (2003) 268.

[38] N. H. Zawilla, M. A. A. Mohammad, N. M. El Kousy, S. M. El-Moghazy Aly, *J. Pharm. Biomed. Anal*. **27** (2002) 243.

[39] H. S. Lee, C. K. Jeong, S. J. Choi, S. B. Kim, M. H. Lee, G. Il Ko and D. H. Sohn, *J. Pharm. Biomed. Anal.* **23** (2000) 775.

[40] X. Q. Liu, X. J. Chen, L. H. Zhao, J. H. Peng, *Yao. Xue. Xue. Bao*. **32** (1997) 546.

Part-[B]

Studies on Synthesis of Bioactive Compounds

STUDIES ON SYNTHESIS OF BIOACTIVE COMPOUNDS

INTRODUCTION

The chemistry of the heterocyclic compounds is as logical as that of aliphatic or aromatic compounds. This study is of great interest both from the theoretical as well as practical stand point. A heterocyclic compound is one which possesses acyclic structure with at least two different kinds of atoms in the ring. The most common type, contain largely carbon atom, nitrogen, oxygen and sulphur are the most common heteroatoms, but many other elements, including even bromine, chlorine can also serve. The heterocyclic compounds containing the less common atoms have been subject to much investigation in recent years.

The variety of heterocyclic compounds is enormous, their chemistry is complex and synthesizing them requires great skill. Among large number of heterocycles found in nature nitrogen heterocycles are most abundant than those containing oxygen of sulphur owing to their wide distribution in nucleic acid instance and involvement in almost every physiological process of plants and animals.

Heterocyclic systems are encountered in many groups of organic compounds possessing great applicability in industry as well as in our life in various ways i. e. most of the sugars and their derivatives, including vitamin C, for instance, exist largely in the form of five membered (Furanosied str.) or six membered (Pyranoised str.) ring containing one oxygen atom. Most members of the vitamin B group possess heterocyclic rings containing nitrogen; one example is vitamin B_6 (Pyridoxine), which is a derivative of the pyridine essential in amino acid metabolism. Many other examples of the importance of heterocyclic compounds in biological systems can be given.

Natural products containing heterocyclic compounds such as alkaloids and glycosides have been used since old age, as remedial agents. Febrifagl alkaloid from ancient Chinese drug, Chang Shan, reserpine from Indian rouwopifia, Curen alkaloid from arrow poison, codenine, j-tropine and strychnine are all examples of heterocyclic compounds. Many antibiotics including penicillin, cephalosporin, norfloxacin, streptomycin etc. also contain heterocyclic ring systems. Majority of the large number of drugs being introduced in pharmacopeias in recent years are heterocyclic compounds.

Many veterinary products like pyrantel and morantel are the drug of choice as broad spectrum anthelmintics. The herbicides atrazine and simazine are well known example of heterocyclic agrochemicals. Plant pigments such as indigo, hemoglobin and anthiocyanins, chlorophyll has contributed much colour chemistry and many other heterocyclic colouring matters are in use since prehistoric times. The heterocyclic tetraselena fulvalene was the first ionic molecular crystal to demonstrate superconductivity.

Heterocyclic compounds are obtainable by the following methods.

a. Isolation from natural sources, i.e. alkaloids, amino acids, indigo dyes etc.

b. Degradation of natural products i.e. acridine, furfural, indol, pyridine, quinoline, thiophene etc.

c. Synthesis: Synthesis methods for obtaining heterocyclic compounds may be divided into ring closer reactions, addition reaction and replacement reaction. Cyclisation is usually accomplished by elimination of some small molecules such as water or ammonia from chain of suitable length.

Heterocyclic compounds have a great applicability as drugs because,

a. They have a specific chemical reactivity.

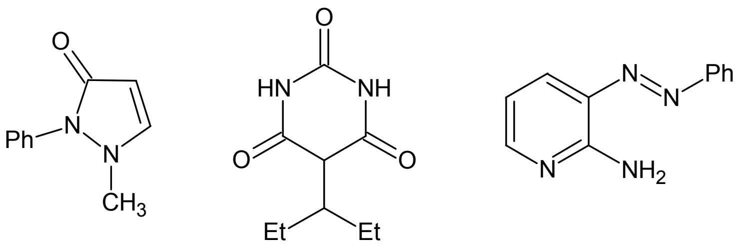
b. They resemble essential metabolism and can provide false synthons in biosynthetic process.

The current interest in the creation of large, searchable libraries of organic compounds has captured an imagination of organic chemists and the drug discovery community. Efforts in numerous laboratories focused on the introduction of chemical diversity have been recently reviewed and pharmacologically interesting compounds have been identified for libraries of widely different compositions.

Research in the field of pharmaceutical has its most important task in the development of new and better drugs and their successful introduction into clinical practice. Central to these efforts, accordingly stand the search for pharmaceutical substances and preparation which are new and original. In addition to these objectives the searching for drug which exhibit a clear advantage over a drug already known. Such advantages may be qualitative or quantitative improvement in activity, the absence of undesirable side effect, a lower toxicity, improved stability of decreased cost.

It is important at the outset to note that drug discovery is not an unambiguous term in the pharmaceutical R & D world. For example, it can be defined using either programmatic or organizational approaches (or both), with several options on each category. Hence, it is important first to understand this variability and to adopt a specific definition for the purpose of this discussion.

The contribution of organic chemistry to be development of scientific medicine in the 19th century mainly from acyclic and carbocyclic compounds, although the pyrazoline antipyrin **(1)** was introduced as an antipyretic and analgesic in 1984 and the first barbiturate baritone (veranol) **(2)** in 1903. Guttmann treated, malaria with methylene blue in 1891, with slight success, and in 1912 he introduced acriflavine as trypancide, it has proved to be more valuable as an antiseptic. Phenazopyridini (pyridium) **(3)** was introduced for the same purpose in 1926, and although it is relatively ineffective it has continued to be used since it has some analgesic action.



1 **2** **3**

Aims and objectives

Taking in view of the applicability of heterocyclic compounds, we have undertaken the preparation of heterocycles bearing triazole and pyrimidines nucleus. The placements of a wide variety of substituents of these nuclei have been designed in order to evaluate the synthesized products for their pharmacological profile against several strains of bacteria and fungi.

During the course of our research work, looking to the application of heterocyclic compounds, several entities have been designed, generated and characterized using spectral studies. The details are as under.

1. To generate several bioactive derivatives of 2-thioxotetrahydropyrimidines and their fused derivatives of thiazolo[3,2-*a*]pyrimidines.
2. To characterize these products for structure elucidation using various spectroscopic techniques like IR, PMR and mass spectral analysis.
3. To evaluate these new products for better drug potential against different strains of bacteria and fungi.
4. Purity of all compounds has been checked by thin layer chromatography.

Part-[B]

(Section-I)

Synthesis, Characterization and Antimicrobial screening of 2-Thioxotetrahydropyrimidines

SYNTHESIS CHARACTERIZATION AND ANTIMICROBIAL SCREENING OF 2-THIOXOTETRAHYDROPYRIMIDINES

1 INTRODUCTION

Pyrimidine is the most important member of all the diazines as this ring system occurs widely in living organisms. Purines, uric acid, barbituric acid, anti-malarial and anti-bacterial agents also contain the pyrimidine ring. The chemistry of pyrimidine has been widely studied. Pyrimidine was first isolated by Gabriel and Colman in 1899. Since pyrimidine is symmetrical about the line passing C-2 and C-5, the positions C-4 and C-6 are equivalent and so N-1 and N-3 are equivalent. When a hydroxyl or amino group is present at the 2-, 4- or 6- position than they are tautomeric with oxo and imino respectively (Figure-1).

Despite the importance of dihydroazines (particularly those containing the 1,4-dihydropyrimidine and dihydropyridine moiety[1]) for clarifying a wide range of theoretical, medicinal and biological problems, the chemistry of this group of compounds is still extremely spotty.[2-6] From the theoretical point of view, it is essential to predict the structure, binding properties, chemical reactivity, etc. of dihydro compounds from the number and positioning of nitrogen atoms in the ring, as well as from the disposition of double bonds. Such quantum mechanical calculations also enable an evaluation of the degree of aromatic character in potential homoaromatic and antiaromatic isomers. Availability of novel model compounds for verifying these predictions would open up new horizons in theoretical heterocyclic chemistry, particularly in clarifying the structures leading to spontaneous isomerization of a derivative or in verifying its redox properties.

From the biochemical point of view, dihydroazines are of intense interest because of presence of this group at the active site of the *hydrogen transferring coenzyme* (nicotinamide adenine dinucleotied hdrogenase-NADH or reduced nicotinamide adenine dinucleotide). This nucleotide, a central participant in metabolic processes in living organisms, participates in the reduction of various unsaturated functionalities.

1. A. L. Weis; *Adv. Heterocycl. Chem.,* **38,** 1 (1985).
2. S. Yasui, K. Nakamura, A. Ohno; *J. Org. Chem.*, **49,** 878 (1984).
3. N. Baba, M. Amano, J. Oda, Y. Inouye; *J. Am. Chem. Soc.*, **106,** 1481 (1984). *Annular Reports in Medicinal Chemistry*. **19,** 119 (1984).
4. U. Eisner, J. Kuthan; *J. Chem. Reu.,* **72,** 1 (1972).
5. J. Kuthan, A. Kurfurst; *Jnd. Eng. Prod. Res. Deu.,* **21,** 191 (1982).
6. D. M. Stout, A. I. Meyers, *J. Chem. Reu.,* **82,** 223 (1982).

In the area of drug development, dihydroazines show great promise, particularly since the 4-aryldihydropyridines exhibit powerful vasodilation activity via modifying the calcium ion membrane channel.[7-11] Additionally, dihydropyridines have been found to actively transport medication across biological membranes.[12]

Until recently, most of the information available on dihydroazines centered around dihydropyridines, with very little data extending to the related dihydropyrimidines.

This lacuna has motivated our deep involvement in developing dihydropyrimidine chemistry, particularly dihydropyrimidines containing no substituents on the ring nitrogen.[13] These molecules have long been considered unstable for oxidation, polymerization or disproportionation reactions.[14]

Figure **(1)** depicts the five possible isomeric structures of dihydropyrimidines, exhibiting different dispositions of the double bonds.

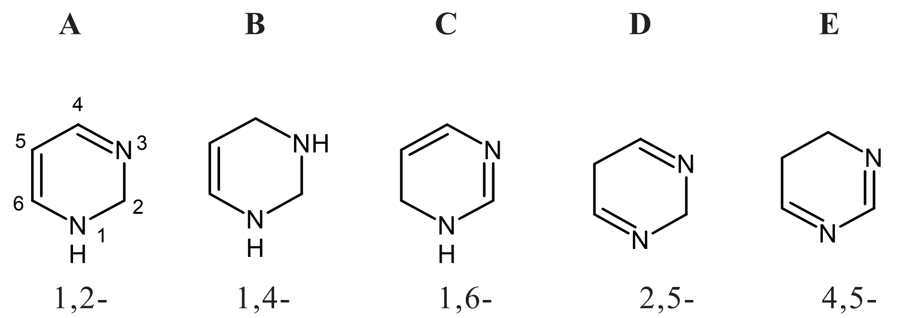


Figure-1

7. F. Bossert, W. Vater; *Naturwissenshaften,* **58,** 578 (1971).
8. W. Vater, G. Kronenberg; F. Hoffmeister, H. Keller, A. Meng, A. Oberdorf, W. Puls, K. Schlossmann, K. Stoepel; *Arzneim. Forsch.,* **22,** 1 (1972).
9. B. Loev, M. M. Goodman, K. M. Snader, R. Tedeschi, E. Macko, *J. Med. Chem.,* **17,** 956 (1974).
10. P. H. Stone; *J. Cardiouasc. Med.,* **7,** 181 (1982).
11. F. Bossert, H. Meyer, E. Wehinger; *Angew. Chem., Int. Ed. Engl.,* **20,** 762 (1981).
12. N. Bodor; In *Design of Biopharmaceutical Properties Through Prodrugs and Analogs;* E. B. Roche, Ed.; American Pharmaceutical Association: Washington, DC, 1977; p 98.
13. A. L. Weis, H. C. van der Plas, *Heterocycles,* **24,** 1433 (1986).
14. D. J. Brown, In *The Chemistry of Heterocyclic Compounds;* A. Weissberger, Ed.; Wiley (Interscience): New York, (1962). D. J. Brown, In *The Chemistry of Heterocyclic Compounds,* Suppl. 1; A. Weissberger; Ed.; Wiley: New York, (1970).

However, these structures are not easy to synthesize and, as a result, most of the known dihydropyrimidines have either 1,2- (A) or the tautomeric 1,4- (B) and 1,6- (C) geometry **(Figure 1)**. On the basis of data available in the literature,[15,16] the dihydropyrimidines can be conveniently divided into two groups, within each of which interconversion between isomers is possible under thermal conditions, namely, the 1,4- (B), 1,6- (C), and 4,5- (E) compounds, and the 1,2- (A) and 2,5- (D) isomers. It is worthwhile to note that, while thermal interconversion between the two groups is not observed, photochemical rearrangement of 1,4-(or 1,6-)dihydropyrmidines to 1,2-isomers has been reported.[17-18]

It should be stressed that dihydroazines take part in various isomerization processes, usually characterized by reversible or irreversible migrations within the ring, the study of which is still in its infancy. Hydrogen migration, for example, is classified either as rearrangement or tautomerism depending on its kinetic and thermodynamic parameters; the former term is reserved for irreversible processes, while the latter is used to describe fast reversible exchanges.[19] A study of isomerization in dihydropyrimidines provides an excellent opportunity for clarifying the factors regulating these processes.

After successfully developing versatile synthetic techniques for obtaining a variety of 1,4- and 1,6-dihydropyrimidines,[20-22] as well as the observation of amidinic tautomerism between the two,[23-24] A. L. Weis et al.[15] examining the possibility of preparative synthesis of similarly N-unsubstituted 1,2-dihydro derivatives and studying their properties. Particularly important goals of this study were the possible observation of the formally allowed hydrogen shift of homoaromaticity[25-26] or hydrogen shift of imine-enamine tautomerism[27] in these compounds, behaviors of which have been seen in other systems.

15. A. L. Weis, F. Frolow, R. Vishkautsan; *J. Org. Chem.*, **51,** 4623-4626 (1986).
16. A. L. Weis, R. Vishkautsan; *Heterocycles* **23,** 1077 (1985).
17. R. E. van der Stoel, H. C. van der Plas; *J. Chem. SOC., Perkin Trans.*-**1,** 1288 (1979).
18. R. E. van der Stoel, H. C. van der Plas; *J. Chem.* Soc., *Perkin Trans.*-**1,** 2393 (1979).
19. Y. I. Minkin, L. P. Olekhnovich, Y. A. Zhdanov; *Acc. Chem.* Res., **14,** 210 (1981).
20. A. L. Weis; *Synthesis.* 528 (1985).
21. A. L. Weis, F. *J.* Frolow; *Chem. Soc., Perkin Trans.*-**1,** 83 (1986).
22. A. L. Weis, R. Vishkautsan; *Isr. J. Chem.,* in press.
23. A. L. Weis; *Tetrahedron lett.,* **23,** 449 (1982).
24. A. L. Weis, Z. Luz Porat; *J. Am. Chem Soc.,* **106,** 8021 (1984).
25. L. A. Paquette, *Angew. Chem., Int. Ed. Engl.,* **7,** 565 (1968).

To date few reports on the formation of 1,2-dihydropyrimidines exist in the literature, and in those cases where a product could be isolated and characterized, the material was either an N-substituted derivative or else it contained geminal disubstitution at position 2, situations that prevent the molecule from oxidizing to the corresponding pyrimidine.

Pyrimidine ring carrying various substituents may be built up from two or three aliphatic fragments by the principle synthesis or by a variety of other synthesis, which are complimentary rather than alternative to it. A second type of synthesis is the isomerisation or break down of another heterocycles such as hydration of purine but such roots are frequently used.

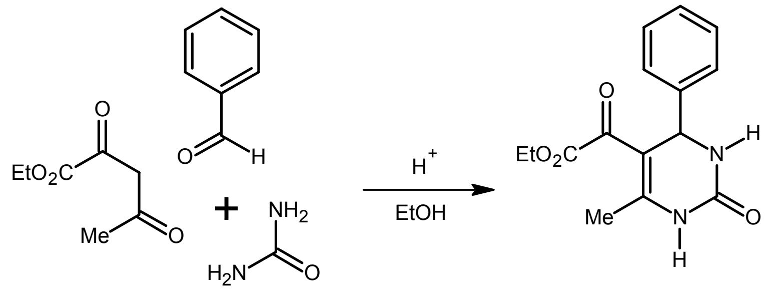
26. L. A. Paquette; *Acc. Chem. Res.,* **6,** 393 (1973).

27. J. Armond, K. Chekir, J. Pinson; *Can. J. Chem.,* **52,** 3971 (1974).

2 SYNTHETIC ASPECTS

2.1 Biginelli Reaction

In 1893, Italian chemist Pietro Biginelli reported an acid catalyzed cyclocondensation reaction of ethyl acetoacetate, benzaldehyde, and urea. The reaction was carried out by simply heating a mixture of the three components dissolved in ethanol with a catalytic amount of HCl at reflux temperature. The product of this novel one-pot, three-component synthesis that precipitated on cooling of the reaction mixture was identified correctly by Biginelli as 3,4-dihydropyrimidin-2(1*H*)-one.[28]



Biginelli Dihydropyrimidine Synthesis

2.2 Alternative synthetic routes for better yield, shorter reaction time to synthesize new analogs

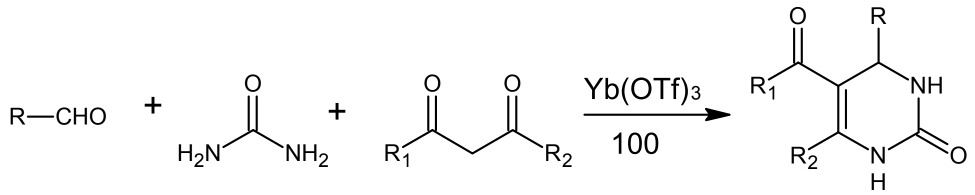
Various modifications have been applied to Biginelli reaction to get better yield and to synthesize biologically active analogs. Different catalysts have been reported to increase the yield of the reaction. Microwave synthesis strategies have also applied to shorten the reaction time. Solid phase synthesis and combinatorial chemistry has made possible to generate library of DHPM analogs.

2.2-A Catalysts

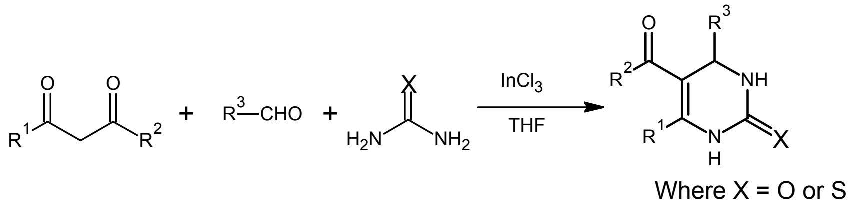
Min Yang and coworkers[29] have synthesized the different DHPMs by using different inorganic salts as a catalyst. They found that the yields of the one-pot Biginelli reaction can be increased from 20-50% to 81-99%, while the reaction time shorted for

28. P. Biginelli; *Gazz.Chem. Ital.,* **23,** 360-416 (1893).
29. Y. Ma, C. Qiana, L.Wang, M. Yang; *J. Org. Chem.,* **65,** 3864-3868 (2000).

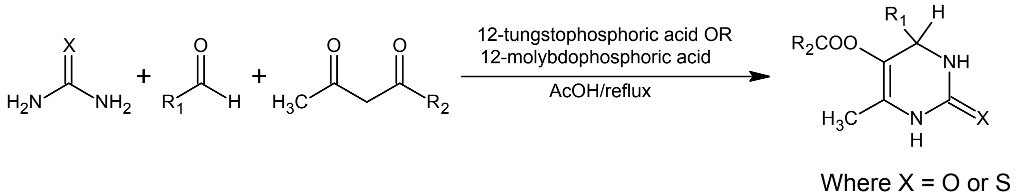
18-24 hr to 20 min. This report discloses a new and simple modification of the Biginelli-type reaction by using $Yb(OTf)_3$ and $YbCl_3$ as a catalyst under solvent free conditions. One additional important feature of the present protocol is the catalyst can be easily recovered and reused.



Indium(III) chloride was emerged as a powerful Lewis catalyst imparting high region and chemo selectivity in various chemical transformations. B. C. Ranu and coworkers[30] reported indium chloride($InCl_3$) as an efficient catalyst for synthesis of 3,4-dihydropyrimidn-2(1*H*)-ones. A variety of substituted aromatic, aliphatic, and heterocyclic aldehydes have been subjected to this condensation very efficiently. Thiourea has been used with similar success to provide the corresponding dihydropyrimidin-2(1*H*)-thiones.

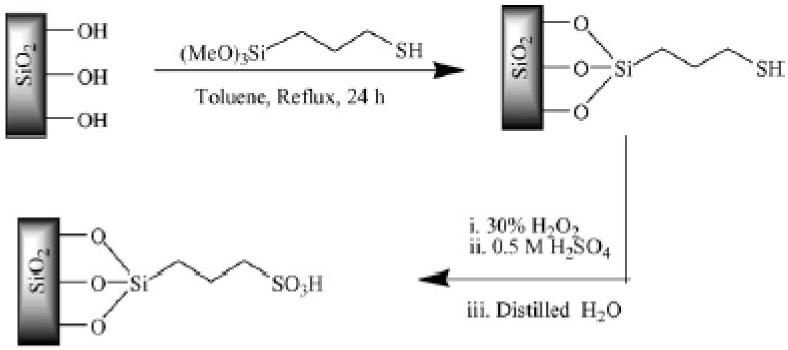


Majid M. Heravi et al.[32] have reported a simple, efficient and cost-effective method for the synthesis of 3,4-dihydropyrimidin-2(1*H*)-ones/thions by one pot three-component cyclocondensation reaction of a 1,3-dicarbonyl compound, an aldehyde and urea or thiourea using 12-tungstophosphoric acid[31] and 12-molybdophosphoric acid as a recyclable catalyst.

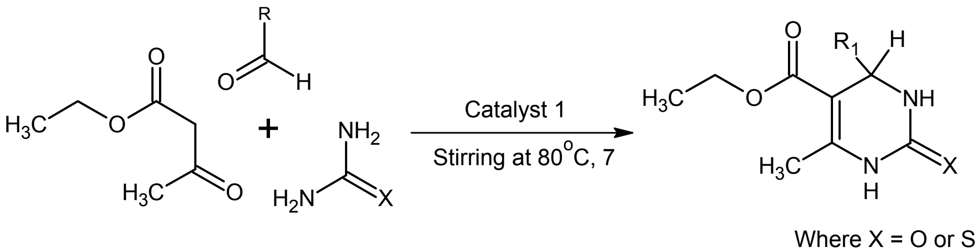


30. B. C. Ranu, A. Hajra, U. Jana; *J. Org. Chem.*, **65**, 6270-6272 (2000).
31. M. M. Heravi, F. Derikvand, F. F. Bamoharram; *J. Mol. Catal.,* **242,** 173-175 (2005).
32. M. M. Heravi, K. Bakhtiari, F. F. Bamoharram; *Catal. Commun.,* **7,** 373-377 (2006).

A novel covalently anchored sulfonic acid onto the surface of silica was prepared and investigated for the Biginelli reaction by Satya Paul and co-workers.[33] The catalyst is highly stable, completely heterogeneous and recyclable for several times. The work-up procedure is very simple and Biginelli compounds were obtained in good to excellent yields.



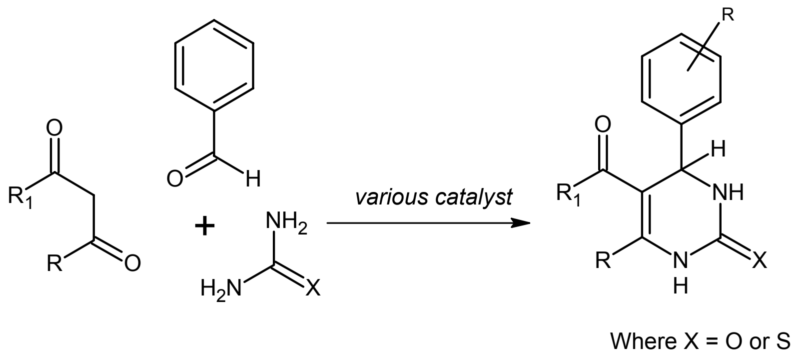
Catalyst 1



An efficient three-component synthesis of 3,4-dihydropyrimidinones using trichloroisocyanuric acid (TCCA) as mild, homogeneous and neutral catalyst for Biginelli reaction in ethanol or DMF under reflux condition.[34]

33. R. Gupta, S. Pal, R. Gupta; *J. Mol. Catal.,* **266,** 50-54 (2007) .
34. M. A. Bigdeli, S. Jafari, G. H. Mahdavinia, H. Hazarkhani; *Catal. Comm.,* **8** , 1641-1644 (2007).

Very recently, many researchers[35-41] have investigated an efficient Biginelli reaction under solvent-free conditions for one-pot synthesis of 3,4-dihydropyrimidi-2-(1*H*)ones/ thiones using various catalyst as described under.

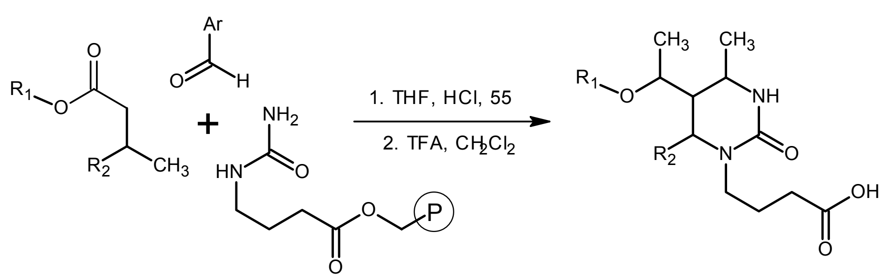


35. Yang Yu, Di Liu, Chunsheng Liu, Genxiang Luo, *Bioorg. Med. Chem. Lett.,* **17,** 3508–3510 (2007).
36. WeiYi Chen, SuDong Qin, JianRong Jin; *Catal.Commun.,* **8,** 123–126 (2007).
37. A. Kumar, R. A. Maurya; *J. Mol. Catal.,* **272,** 53–56 (2007).
38. X. Wang, Z. Quan, Z. Zhang; *Tetrahedron,* **xx,** 1–7 (2007).
39. N. Ahmed, J. E. van Lier; *Tetrahedron Lett.,* **48,** 5407-5409 (2007).
40. N. S. Nandurkar, M. J. Bhanushali, M. D. Bhor, B. M. Bhanage; *J. Mol. Catal.,* 271, 14-17 (2007).
41. F. Shirini, K. Marjani, H. T. Nahzomi, *Arkivoc,* **(i)** 51-57 (2007).

2.2-B Solid phase synthesis

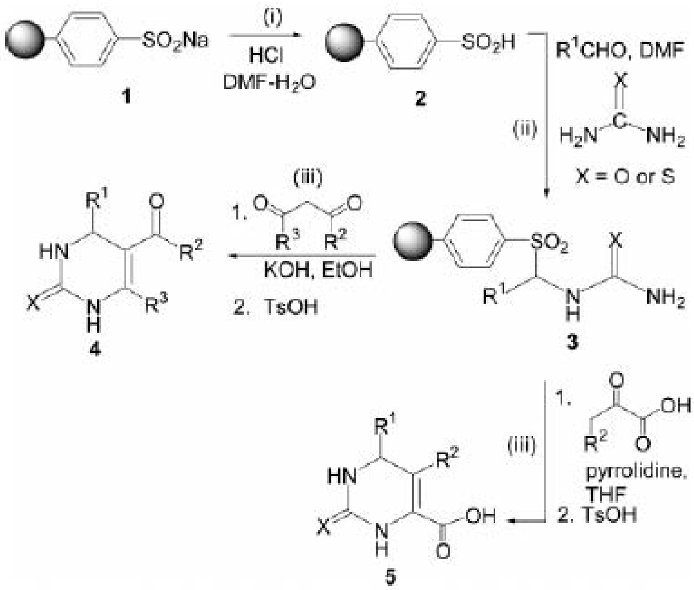
The generation of combinatorial libraries of heterocyclic compounds by solid phase synthesis is of great interest for accelerating lead discovery and lead optimization in pharmaceutical research.[42,43] Multicomponent reactions (MCRs) leading to heterocycles are particularly useful for the creation of diverse chemical libraries, since the combination of n≥3 small molecular weight building blocks in a single operation leads to high combinatorial efficiancy.[42-44] Therefore, solid phase modifications of MCRs are rapidly becoming the cornerstone of combinatorial synthesis of small-molecule libraries.[42-47] One such MCR that has attracted considerable attention in recent years is the Biginelli reaction, which involves the one-pot cyclocondensation of a b-ketoester with an aryl aldehyde and an urea/thiourea derivative.[48]

The first actual solid-phase modification of the Biginelli condensation was reported by Wipf and Cunningham[49] in 1995. In this sequence, g-aminobutyric acid-derived urea was attached to Wang resin using standard procedures. The resulting polymer-bound urea was condensed with excess β-ketoesters and aromatic aldehydes in THF at 55 °C in the presence of a catalytic amount of HCl to afford the corresponding immobilized DHPMs. Subsequent cleavage of product from the resin by 50% trifluoroacetic acid (TFA) provided DHPMs in high yields and excellent purity.

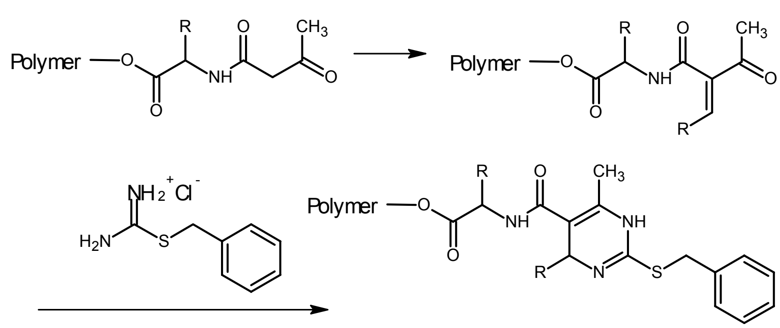


42. A. Nefzi, J. M. Ostresh, R. A. Houghton; *Chem. Rev.,* **97,** 449 (1997).
43. J. W. Corbett; *Org. Prep. Proced. Int.,* **30,** 489 (1998).
44. I. Ugi; *J. Prakt. Chem.,* **339,** 499 (1997).
45. A. Doemling; *Combinatorial Chemistry & High Throughput Screening,* **1,** 1 (1998).
46. L. F. Tietze, M. E. Lieb; *Curr. Opin. Chem. Biol.,* **2,** 363 (1998).
47. S. L. Dax, J. J. McNally, M. A. Youngman; *Curr. Med. Chem.,* **6,** 255 (1999).
48. For a review on DHPMs, see: C. O. Kappe; *Tetrahedron,* **49,** 6937 (1993).
49. P. Wipf, A. Cunningham; *Tetrahedron Lett.,* **36,** 7819-7822 (1995).

Weiwei Li and Yulin Lam[50] described the synthesis of 3,4-dihydropyrimidin-2-(1*H*)ones/thions using sodium benzenesulfinate as a traceless linker. The key steps involved in the solid-phase synthetic procedure include (i) sulfinate acidification, (ii) condensation of urea or thiourea with aldehydes and sulfinic acid, and (iii) traceless product release by a one-pot cyclization-dehydration process. Since a variety of reagents can be used in steps-ii and iii, the overall strategy appears to be applicable to library generation.



Recently, Gross et al[51] developed a protocol for based on immobilized α-ketoamides to increase the diversity of DHPM. The resulting synthetic protocol proved to be suitable for the preparation of a small library using different building blocks. They found that the expected DHPM derivatives were formed in high purity and yield if aromatic aldehyde and α-ketoamide building blocks were used. The usage of an aliphatic aldehyde leads to an isomeric DHPM mixture. Purities and yields were not affected if thiourea was used instead of urea.



50. Li Weiwei, Lam Yulin; *J. Comb. Chem.,* **7,** 721-725 (2005).
51. G. A. Gross, Hanns Wurziger, Andreas Schober; *J. Comb. Chem.,* **8,** 153-155 (2006).

2.1-C Liquid phase synthesis

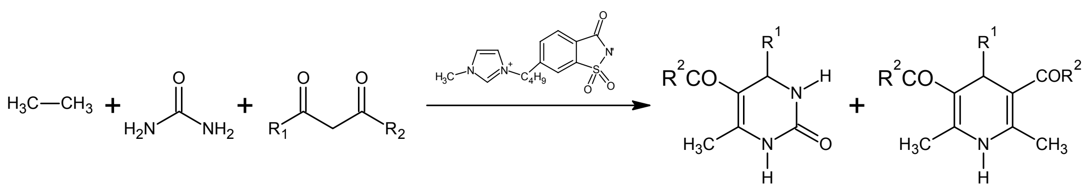
In the solid phase synthesis there are some disadvantages of this methodology compared to standard solution-phase synthesis, such as difficulties to monitor reaction progress, the large excess of reagents typically used in solid-phase supported synthesis, low loading capacity and limited solubility during the reaction progress and the heterogeneous reaction condition with solid phase.[52]

Recently, organic synthesis of small molecular compounds on soluble polymers, i.e. liquid phase chemistry[53] has increasingly become attractive field. It couples the advantages of homogeneous solution chemistry with those of solid phase chemistry. Moreover owing to the homogeneity of liquid-phase reactions, the reaction conditions can be readily shifted from solution-phase systems without large changes, and the amount of excessive reagents is less than that in solid-phase reactions.

In the recent years, Task Specific room temperature Ionic Liquids (TSILs) have emerged as a powerful alternative to conventional molecular organic solvents or catalysts. Liu Zuliang et al.[54] reported cheap and reusable TSILs for the synthesis of 3,4-dihydropyrimidin-2(1*H*)-ones via one-pot three component Biginelli reaction.

Ionic liquid-phase bound acetoacetate react with (thio)ureas and various aldehydes with a cheap catalyst to afford ionic liquid-phase supported 3,4-dihydropyrimidin-2(1*H*)-(thi)ones by Jean Pierre Bazureau and co-workers.[55]

3,4-Dihydropyrimidinones (Biginelli products) are synthesized in one-pot of aldehydes, â-dicarbonyl compounds and urea, catalyzing by non-toxic room temperature ionic liquid 1-*n*-butyl-3-methylimidazolium saccharinate (BMImSac).[56]

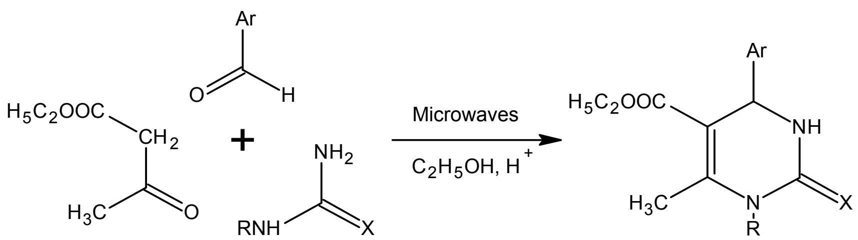


52. P. M. Toy, K. D. Janda; *Acc. Chem. Res.,* **33,** 546-554 (2000).
53. (a) D. J. Gravert, K. D. Janda; *Chem. Rev.,* **97,** 489 (1997). (b) P. Wentworth, K. D. Janda; *Chem. Rev.,* 1917 (1999). (c) P. H. Toy; K. D. Janda; *Acc. Chem. Res.,* **33,** 546 (2000).
54. Fang Dong, Luo Jun, Zhou Xinli, Ye Zhiwen, Liu Zuliang; *J. Mol. Catal.,* 274, 208-211 (2007).
55. Jean Christophe Legeay, Jean Jacques Vanden Eynde, Loic Toupet, Jean Christophe Legeay; *ARKIVOC,* **(iii)** 13-28 (2007).
56. Li Minga, Guo Wei-Si, Wen Li-Rong, Li Ya-Feng, Yang Hua-Zheng; *J. Mol. Catal.,* **258,** 133-138 (2006).

2.2-D Microwave assisted synthesis

In general, the standard procedure for the Biginelli condensation involves one-pot condensation of the three building blocks in a solvent such as ethanol using a strongly acidic catalyst, that is, hydrochloric acid.[57] One major drawback of this procedure, apart from the long reaction times involving reflux temperatures, are the moderate yields frequently observed when using more complex building blocks.

Microwave irradiation (MWI) has become an established tool in organic synthesis, because the rate enhancements, higher yields, and often, improved selectivity, with respect to conventional reaction conditions.[58] The publication by Anshu Dandia et al.[59] described 12 examples of microwave-enhanced solution-phase Biginelli reactions employing ethyl acetoacetate, (thio)ureas, and a wide variety of aromatic aldehydes as building blocks. Upon irradiation of the individual reaction mixtures (ethanol, catalytic HCl) in an open glass beaker inside the cavity of a domestic microwave oven the reaction times were reduced from 2–24 hours of conventional heating (80^{o}C, reflux) to 3–11 minutes under microwave activation (*ca.* 200–300 W). At the same time the yields of DHPMs obtained by us were markedly improved compared to those reported earlier using conventional conditions.



57. G. A. Gross, H. Wurziger, A. Schober; *J. Comb. Chem.,* **8,** 153-155 (2006).
58. (a) S. Caddick; *Tetrahedron,* **51,** 10403 (1995). (b) S. Deshayes, M. Liagre, A. Loupy, J. Luche, A.Petit; *Tetrahedron,* **55,** 10851(1999). (c) P. Lidstrom, J. Tierney, B. Wathey, J. Westman; *Tetrahedron*, **57,** 9225 (2001). (d) A. Kirschning, H. Monenschein, R. Wittenberg; *Angew. Chem. Int. Ed*., **73,** 193, (2001). (e) R.S. Varma; *Pure Appl. Chem*., **73,** 193 (2001). (f) A. Loupy; *Microwaves in Organic Synthesis*; Wiley-VCH: Weinheim, **2002**.
59. A. Dandia, M. Saha, H. Taneja, *J. Fluorine Chem.*, **90,** 17 (1998).

In recent years, solvent –free reactions using either organic or inorganic solid supports[60] have received increasing attention. There are several advantages to performing synthesis in dry media: (i) short reaction times, (ii) increased safety, (iii) economic advantages due to the absence of solvent. In addition, solvent free MWI processes are also clean and efficient.

Activated fly ash, an industrial waste (pollutant) is an efficient and novel catalyst for some selected organic reactions in solvent free conditions under microwave irradiation. M. Gopalakrishnan and co-workers[61] have reported Biginelli reaction under microwave irradiation in solvent-free conditions using activated fly ash as a catalyst.

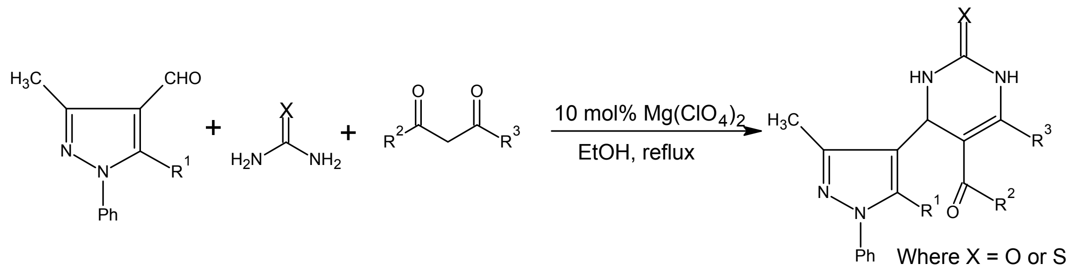
2.1-E Ultrasound assisted synthesis

Ultrasound as a green synthetic approach has gradually been used in organic synthesis over the last three decades. Compared with the traditional methods, it is more convenient, easier to be controlled, and consumes less power. With the use of ultrasound irradiation,[62] a large number of organic reactions can be carried out in milder conditions with shorter reaction time and higher product yields.

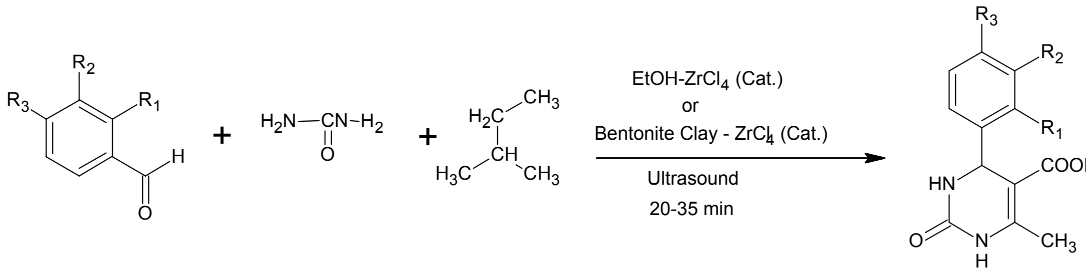
Ultrasound irradiated and amidosulfonic acid (NH_2SO_3H) catalyzed synthesis of 3,4-dihydropyrimidi-2-(1*H*)ones have reported by Ji-Taai Li and co-workers[63] using aldehydes, á-ketoester and urea.

60. P. Diddams, M. Butters, *In Solid Supports and Catalysts in Organic Synthesis;* K. Smith; Ed.; Ellis Harwood and PTR Prentice Hall: New York and London, 1992, Chapters 1, 3 and 5.
61. M. Gopalakrishnan, P. Sureshkumar, V. Kanagarajan, J. Thanusu, R. Govindaraju; *Arikvoc*, **(xiii)** 130-141 (2006).
62. (a) H. A. Stefani, C. M. P. Pereira, R. B. Almeida, R. C. Braga, K. P. Guzen, R. Cella; *Tetrahedron Lett.,* **46,** 6833 (2005). (b) Z. L. Shen, S. J. Ji, S.Y. Wang , X. F. Zeng; *Tetrahedron,* **61,** 10552 (2005) .
63. Ji-Tai Li, Jun-Fen Han, Jin-Hui Yang, Tong-Shuang Li; *Ultrasonics Sonochemistry* **10,** 119-122 (2003).

Chenjiang Liu et al.[64] have synthesized a novel series of 4-substituted pyrazolyl-3,4-dihydropyrimidin-2(1*H*)-(thio)ones under ultrasound irradiation using magnesium perchlorate [$Mg(ClO_4)_2$] as catalyst, by the condensation of 5-chloro/phenoxyl-3-methyl-1-phenyl-4-formylpyrazole, 1,3-dicarbonyl compound and urea or thiourea in moderate yields. The catalyst exhibited remarkable reactivity and can be recycled.



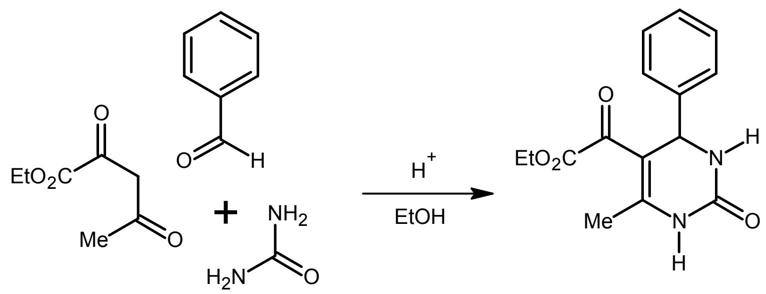
Sonication of aromatic aldehydes, urea and ethyl acetoacetate in presence of solvent (ethyl alcohol) or solvent-less dry media (bentonite clay) by supporting-zirconium chloride ($ZrCl_4$) as catalyst at 35 kHz gives 6-methyl-4-substitutedphenyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid ethyl esters proficiently in high yields reported by Harish Kumar.[65]



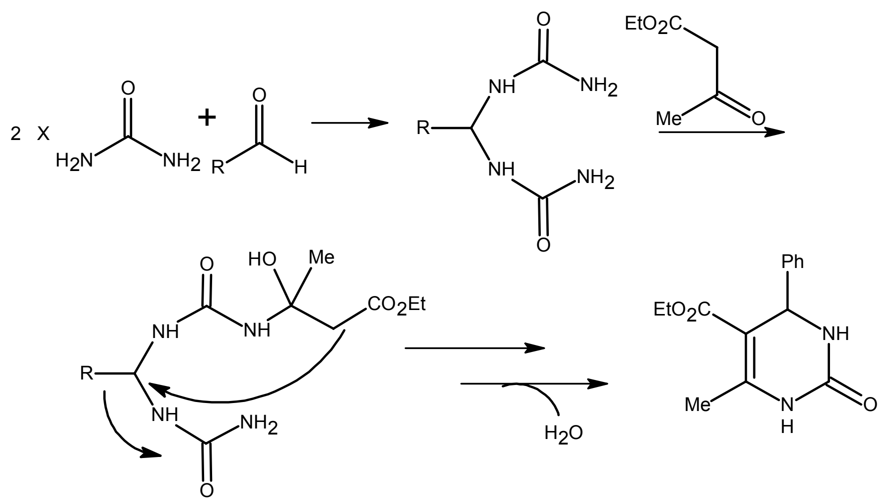
64. X. Zhang, Y. Li, C. Liu, J. Wang; *J. Mol. Catal.,* **253,** 207-211(2006).
65. H. Kumar, A. Parmar; *Ultrasonics Sonochemistry,* **XXX,** XXX (2003). Article in Press

3 MICHANISTIC STUDY

In 1893 Biginelli reported the first synthesis of dihydropyrimidines by a simple one-pot condensation reaction of ethyl acetoacetate, benzaldehyde and urea.[66]

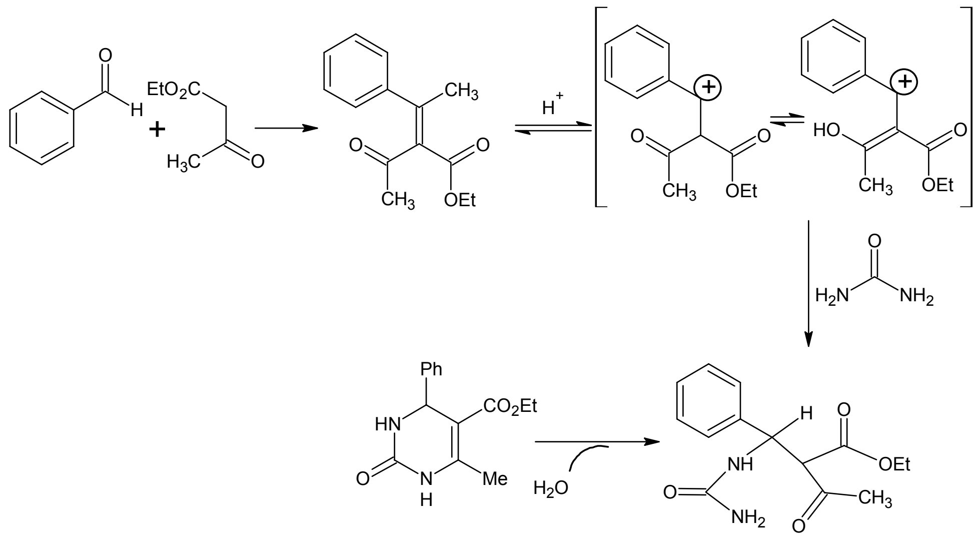


Despite the importance and current interest in dihydropyrimidines of the Biginelli type, the mechanism of the classical three-component Biginelli condensation has not been elucidated with certainty.[67] Early work by Folkers and Johnson[68] suggested that N,N"-benzylidienebisurea (i.e. the primary bimolecular condensation product of benzaldehyde and urea), is the first intermediate in this reaction.

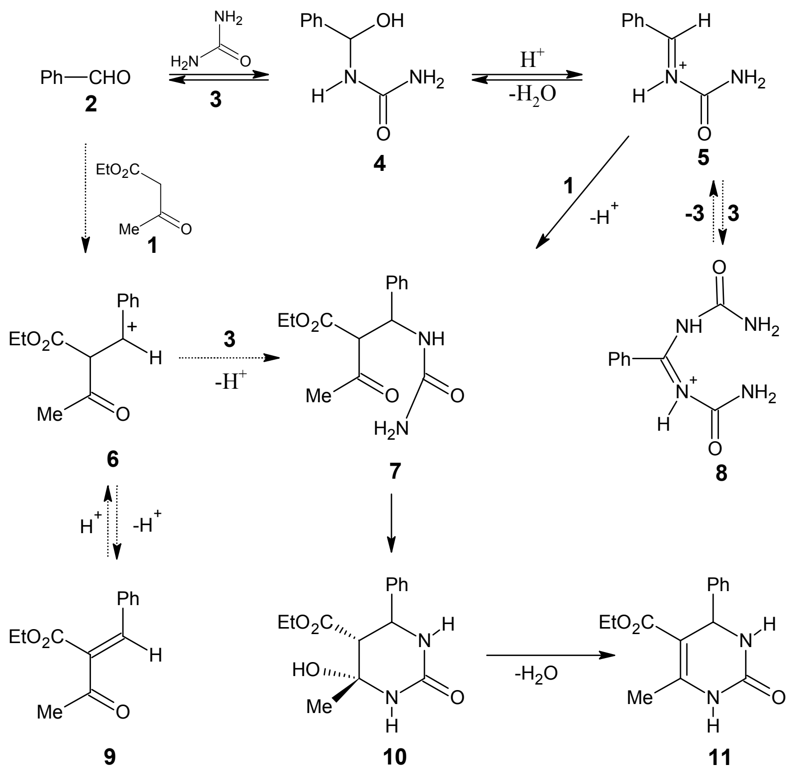


In 1973 Sweet and Fissekis[69] proposed that a "carbenium ion mechanism", produced by and acid-catalyzed aldol reaction of benzaldehyde with ethyl acetoacetate, is the key intermediate and is formed in the first and limiting step of the Biginelli reaction.

66. P. Biginelli; *Gazz.Chim. Ital.,* **23,** 360-416 (1893).
67. C. O. Kappe; *Tetrahedron,* **49,** 6937-6963 (1993).
68. K. Folkers, T. B. Johnson; *J. Am. Chem. Soc.,* **55,** 3784-3791 (1933).
69. F. Sweet, J. D. Fissekis; *J. Am. Chem. Soc.,* **95,** 8741-8749 (1973).



Kappe C. O.[70] carried out a detailed reinvestigation of the mechanism of the Biginelli condensation using ^{1}H-NMR and ^{13}C-NMR spectroscopy to identify possible intermediates.



70. C. O. Kappe; *J. Org. Chem.*, **62,** 7201-7204 (1997).

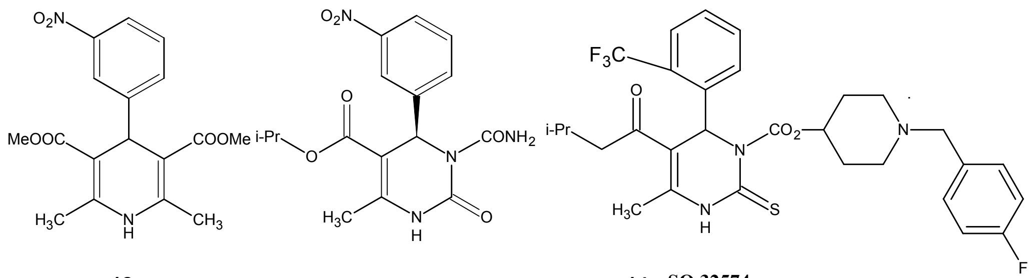
Kappe have established that the key step in this sequence involves the acid-catalyzed formation of an N-acyliminium ion intermediate of type **(5)** from the aldehyde **(2)** and urea **(3)** precursors. Interception of the iminium ion **(5)** by ethyl acetoacetate **(1)**, presumably through its enol tautoer, produces an opean chain ureide **(7)** which subsequently cyclizes to hexahydropyrimidine **(10)**. Acid-catalyzed elimination of water form **(10)** ultimately leads to the final DHPM product **(11)**. The reaction mechanism can therefore be classified as an á-amidoalkylation, or more specifically as an á-uridoalkylation.[71]

71. H. Petersen; *Synthesis*, 243-292 (1973).

4 BIOLOGICAL PROFILE

4-Aryl-1,4-dihydropyridines of the nifedipine type (DHPs, e.g nifedipine) are the most studied class of organic calcium channel modulators. More than 30 years after the introduction of nifedipine **(12)**, many DHP analogs have now been synthesized and numerous second-generation commercial products have appeared on the market (e.g. nitrendipine, nicardipine and amlodipine).[72]

The aza-analogs such as dihydropyrimidines of type **(13)** (DHPMs) which show a very similar pharmacological profile to classical dihydropyridine calcium channel modulators.[73-79] Over the past several lead-compounds were developed (e.g. **13,** SQ 32926 and **(14)** SQ 32574)[76-78] that are superior in potency and duration of antihypertensive activity to classical dihydropyridine drugs, and compare favorable with second-generation analogs such as amlodipine and nicardipine.[76,77]



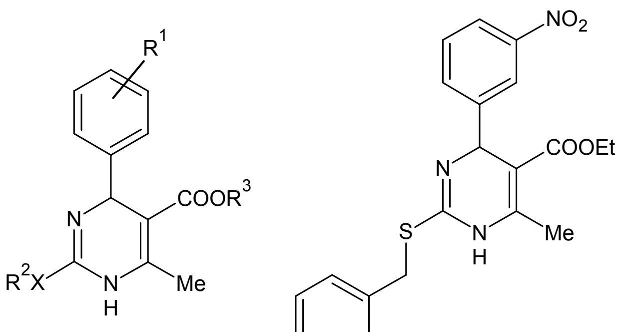
12 **13** **SQ 32926** **14** **SQ 32574**

---

72. F. Bossert, W. Vater, *Med. Res. Rev*., **9,** 291 (1989).
73. H. Cho, M. Ueda, K. Shima, A. Mizuno, M. Hayashimatsu, Y. Ohnaka, Y.Takeuchi, M. Hamaguchi, K. Aisaka, T. Hidaka, M. Kawai, M. Takeda, T. Ishihara, K. Funahashi, F. Sarah, M. Morita, T. J. Noguchi; *Meal. Chem.,* **32,** 2399-2406 (1989).
74. K. S. Atwal, G. C. Rovnyak, J. Schwartz, S. Moreland, A. Hedberg, J. Z. Gougoutas, ; M. F. Malley, D. M. Floyd, *J. Med Chem.,* **33,** 1510-1515 (1990).
75. K. S. Atwal, G. C. Rovnyak, S. D. Kimball, D. M. Floyd, S. Moreland, B. N. Swanson, J. Z. Gougoutas, J. Schwartz, K. M. Smillie, Malley, *M. F..1. Med. Chem.,* **33,** 2629-2635 (1990).
76. K. S. Atwal, B. N. Swanson, S. E. Unger, D. M. Floyd, S. Moreland, A. Hedberg, B. C. O'Reilly; *J. Med. Chem.,* , **34,** 806-811 (1991).
77. G. C. Rovnyak, K S. Atwal, A. Hedberg, S. D. Kimball, S. Moreland, J. Z. Gougoutas, B. C O'Reilly, J. Schwartz, M. F. Malley; *J. Med Chem.,* **35,** 3254-3263 (1992).
78. G. J. Grover, S. Dzwonczyk, D. M. McMullen, C. S. Normadinam, P. G. Sleph, S. J. Moreland; *J. Cardiovasc. Pharmacol.* , **26,** 289-294 (1995). M. Negwer, *Organic-Chemical Drugs and their Synonyms;* Akademie Verlag: Berlin, p. 2558 (1994).
79. G. C. Rovnyak, S. D. Kimball, B. Beyer, G. Cucinotta, J. D. DiMarco, J. Gougoutas, A. Hedberg, M. Malley, J. P. McCarthy, R. Zhang, S. Moreland, *J. Med. Chem.,* **38,** 119-129 (1995). For a discussion, see: D. J. Triggle, S. Padmanabhan; *Chemtracts: Org. Chem.,* **8,** 191-196 (1995).
80. C. O. Kappe, J. Birgit, P. Tetiana; *Molecules*, **5,** 227-239 (2000).

Calcium ion plays a vital role in a large number of cellular processes, including excitation-contraction and stimulus-secretion.[81,82] The regulation of the intracellular concentration of this ion makes possible the control of such Ca^{2+}-dependent processes. One means of accomplishing this is by the use of agents known as calcium channel antagonists, which inhibit the movement of calcium through certain membrane channel.[83-85]

K.S.Atwal[86] prepared the 2-heterosubstituted-4-aryl-l,4-dihydro-6-methyl-5 pyrimidinecarboxylic acid esters **(15)**, which lack the potential C_3 symmetry of dihydropyridine calcium channel blockers, were prepared and evaluated for biological activity. Biological assays using potassium-depolarized rabbit aorta and radioligand binding techniques showed that some of these compounds are potent mimics of dihydropyridine calcium channel blockers. The combination of a branched ester (e.g. isopropyl, sec-butyl) and an alkylthio group (e.g. SMe) was found to be optimal for

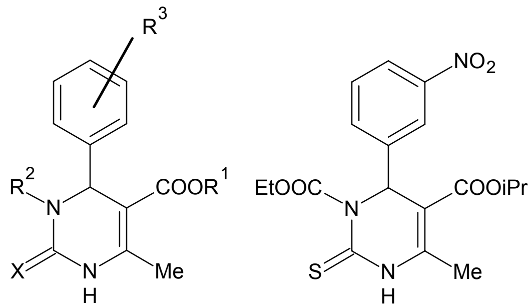


15 **16**

biological activity. Dihydropyrimidines **(15)** were found to be 30-fold less active than dihydropyridines. The solid-state structure of dihydropyrimidine analogue **(16)** shows that these compounds can adopt a molecular conformation which is similar to the reported conformation of dihydropyridine calcium channel blockers.

81. R. H. Kretsinger; *Adv. Cyclic Nucleotide Res.,* **11,** 1 (1979).
82. J. W. Putney Jr; *Pharmacol. Rev.,* **30,** 209 (1978).
83. L. B. Rosenberger D. J. Triggle; Calcium and Drug Action; G. B. Weiss, Ed.; Plenum Press: New York, 1978.
84. D. J. Triggle; New Perspectives on Calcium Antagonists; G. B. Weiss, Ed.; American Physiological Society: Bethesda, MD 1981.
85. A. Fleckenstein; *Annu. Rev. Pharmacol. Toxicol,* **17,** 149 (1977).
86. K. S. Atwal, C. Rovnyakg, J. Schwartz, S. Moreland, A. Hedberg, J. Z. Gougoutas, M. F. Malley, D. M. Floyd; *J.Med. Chem.,* **33,** 1510-1515 (1990).

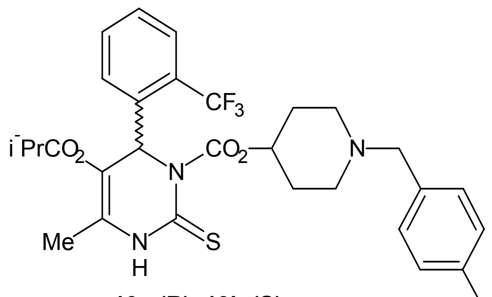
Atwal K. et al.[87] synthesized the 3-substituted 1,4-dihydropyrimidine **(17)** and documented that vasorelaxant activity was critically dependent on the size of the C5 ester group, isopropyl ester being the best, a variety of substituents (carbamate, acyl, sulfonyl, alkyl) were tolerated at N3. The dihydropyrimidines **(17)** are significantly more potent than corresponding 2-heteroalkyl-l,4-dihydropyrimidines. Dihydropyridine enantiomer usually show 10-15 fold difference in activity, while the enantiomers of dihydropyrimidine **(18)** show more than a 1000-fold difference in activity. These results strengthen the requirement of an enamino ester for binding to the dihydropyridine receptor and indicate a nonspecific role for the N3-substituent.



17 **18**

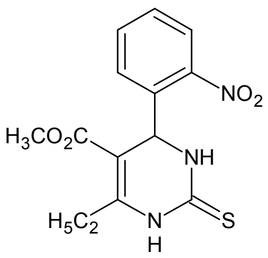
George C. Rovnyak et al.[88] examined a series of novel dihydropyrimidine calcium channel blockers that contain a basic group attached to either C5 or N3 of the heterocyclic ring. One of these compounds was identified as a lead, and the individual enantiomers **(19a)** (R) and **(19b)** (S) were synthesized. Dihydropyrimidine **(19a)** is equipotent to nifedipine and amlodipine *in vitro*. In the spontaneously hypertensive rat, dihydropyrimidine **(19a)** is more potent and longer acting than nifedipine and compares most favorably with the long-acting dihydropyridine derivative amlodipine. Dihydropyrimidine **(19a)** has the potential advantage of being a single enantiomer

87. K. S. Atwal, G. C. Rovnyak, D. S. Kimball, M. F. David, M. Suzanne, N. S. Brian, Z. G. Jack, S. Joseph, M. S. Kaye, F. M. Mary; *J. Med. Chem.,* **33,** 2629 (1990).
88. G. C. Rovnyak, K. S. Atwal, A. Hedberg, S. D. Kimball, S. Moreland: J. Z. Gougoutas, B. C. O'Reilly, J. Schwartz, M. F. Malleys; *J. Med. Chem.,* **35,** 3254-3263 (1992).

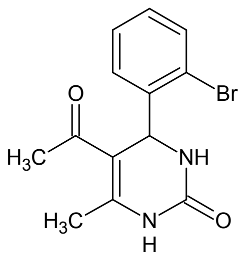


19a (R), 19b (S)

Selma Sarac and co-workers[89,90] have synthesized 4-arlyl-3,4-dihydropyrimidin-2(1H)-one/thione derivatives. The calcium channel blocker activities of all compounds performed on isolated rat ileum. Product **(20)**, 2-nitrophenyl-derivative and **(21)**, 2-bromophenyl-derivative have potent antispasmodic activity on $BaCl_2$-stimulated rat ileum.



20

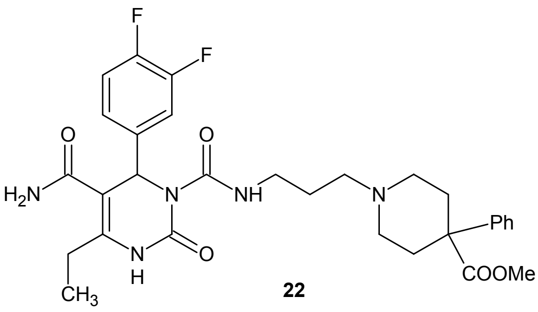


21

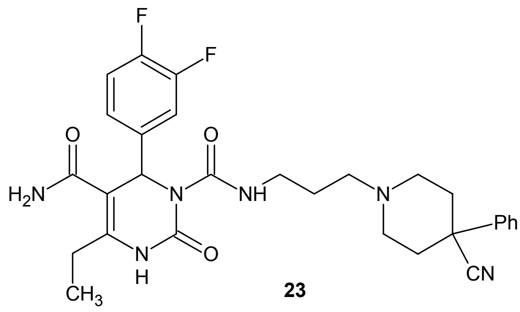
N. Dhanapalan and co-workers[91] have synthesized dihydropyrimidinones and describe compound **(22)** have a high binding affinity (*Ki* = 0.2nM) for á$_{1a}$ receptor and greater than 1500 fold selectivity over á$_{1b}$ and á$_{1d}$ adrenoceptors. Modification of the linker in **(22)** gave compounds **(23)** and **(24)**[92] viz ì-opioid receptor. Both these compounds showed good á$_{1a}$ binding affinity (*Ki* = 0.2nM) and selectivity (>800-fold over á$_{1b}$ and á$_{1d}$), also showed good selectivity over several other recombinant human G-protein

89. I. S. Zorkun, S. Sarac, S. Celebib, K. Erolb; *Bioorg. Med. Chem.,* **14,** 8582–8589 (2006).
90. M. Yarym, S. Sarac, M. Ertan, O. Sarnyc Batu, K. Erol; *Il Farmaco,* **54,** 359–36 (1999).
91. N. Dhanapalan, M. Shou Wu, L. Bharat, C. George, F. James, T. G. Murali, Z. Jack, T. Sriram, R. M. Mohammad, Z. Fengq, C. W. Wai, S. Wanying, T. Dake, M. W. John; *J. Med. Chem.,* **2,** 4764-4777 (1999).
92. T. G. Murali, N. Dhanapalan, R. M. Mohammad, L. Bharat, C. W. Wai, C. George, T. Sriram, M. Shou Wu, Z. Fengqi, S. Wanying, T. Dake, S. Quanrong, Z. Jack, M. W. John; *J. Med. Chem.,* **42,** 4778-4793 (1999).

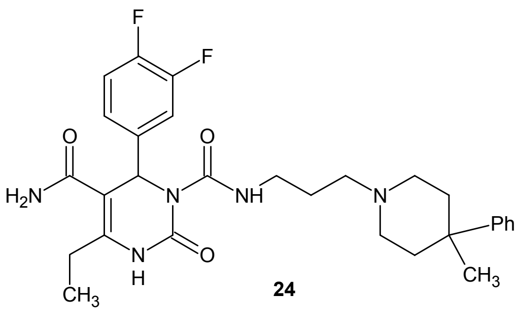
coupled receptors. They have also identify that compound **(25)**[93] was a lead compound with a binding and functional profile comparable to that of **(22)**. Compound **(25)** has negligible affinity for the ì-opioid receptor.



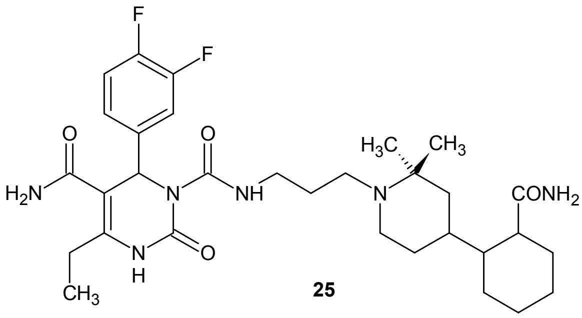
22



23



24

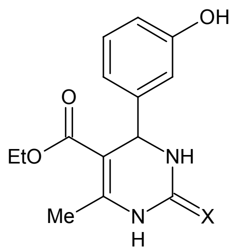


25

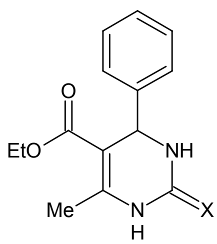
The synthesis and differential antiproliferative activity of monastrol **(26a)**, oxo-monastrol **(26b)** and eight oxygenated derivatives **(28a,b–31a,b)** on seven human cancer cell lines are described by Dennis Russowsky.[94] For all evaluated cell lines, monastrol **(26a)** was shown to be more active than its oxo-analogue, except for HT-29 cell line, suggesting the importance of the sulfur atom for the antiproliferative activity. Monastrol **(26a)** and the thio derivatives **(28a), (29a)** and **(31a)** displayed relevant antiproliferative properties with 3,4-methylenedioxy derivative **(31a)** being approximately more than 30 times more potent than monastrol **(26a)** against colon cancer (HT-29) cell line.

93. L. Bharat , T. Dake , N. Dhanapalan, R. M. Mohammad , C. W. Wai, W. M.Shou , Z. Fengqi, S. Wanying, C. George, F. James; *J. Med. Chem.,* **42,** 4794-4803 (1999).

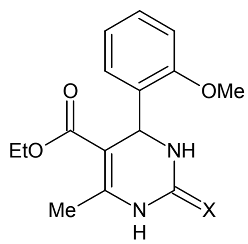
94. D. Russowsky, R. F. S. Canto, S. A. A. Sanches, M. G. M. DOca, A. de Fatima, R. A. Pilli, L. K. Kohn, M. A. Antonio, J. E. de Carvalho; *Bioorg. Chem.,* **34,** 173–182 (2006).



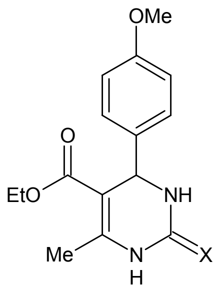
26a X = S Monastrol
26b X = O oxo-Monastrol



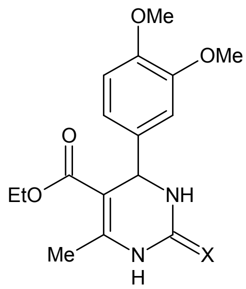
27a X = S
27b X = O



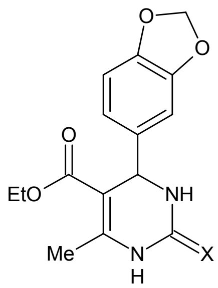
28a X = S
28b X = O



29a X = S
29b X = O



30a X = S
30b X = O



31a X = S
31b X = O

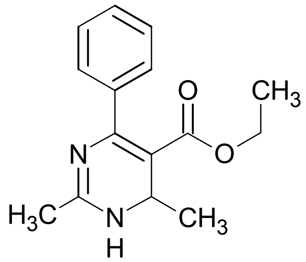
Y. Mizutani and co-workers identify that dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the pathway of uracil and thymine catabolism. DPD is also the principle enzyme involved in the degradation of 5-fluorouracil, and anticancer chemotherapeutic agent that is used clinically to treatment of bladder cancer[95] and renal cell carcinoma.[96]

95. Y. Mizutania, H. Wadab, M. Fukushimad, O. Yoshidac, O. Ukimura, A. Kawauchi, T. Mikia; *Euro. J. Cancer,* **37,** 569–575 (2001).

96. Y. Mizutania, H. Wadab, O. Yoshidac, M. Fukushimad, H. Nakanishia, M. Nakaoa, T. Mikia; *Euro. J. Cancer,* **39,** 541–547 (2003).

5 NEW DRUG MOLECULES UNDER CLINICAL STUDY

Recently many new molecules which are under study from phase-I to phase-IV clinical trials for different pharmacological action have shown that the basic characteristic of morpholine to behave as hidden amine has attracted many medicinal chemists to incorporate this feature in drug design. Some interesting compounds are as under.



32

Treatment of Hypertension

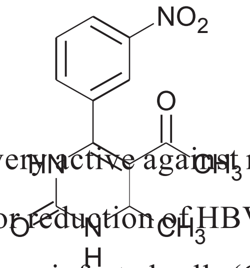
Calcium Channel Blokers

Drug Data Report, **8**(1), 35 (1986).

Calcium Channel Blokers[97]

Company Name: Merck & Co.

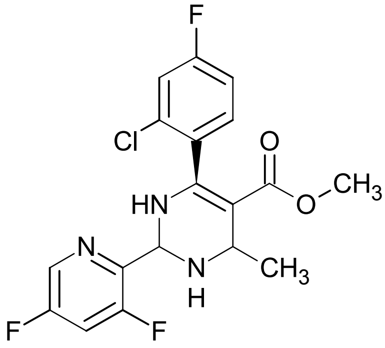
Drug Data Report, **10**(3), 200 (1988).



33

Moreover one compound **(33)** is very active against non-nucleoside inhibitor of human hepatitis *B virus* (IC50 = 53 nM for reduction of HBV DNA in human hepatoma HepG2.2.15 cells) with low cytotoxicity in uninfected cells (CC50 = 7 mcM). Compound inhibited both viral DNA and viral cores in HepG2.2.15 cells and HBV-transfected cell lines, whereas it did not affect the activity of endopolymerase and had no effect on other DNA or RNA viruses. *In vivo* in a transgenic mouse model, oral doses of 3-100 mg/kg b.i.d. or t.i.d. for up to 28 days dose-dependently.

Decreased viral DNA in the liver and plasma with efficacy comparable to lamivudine. However, unlike lamivudine, compound reduced cytoplasmic HBV core antigen (HBcAg) in the liver of mice. Pharmacokinetic studies in mice showed rapid absorption, 30% bioavailability and dose-proportional plasma levels.

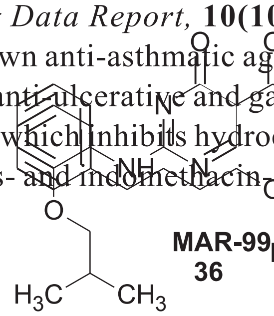


34

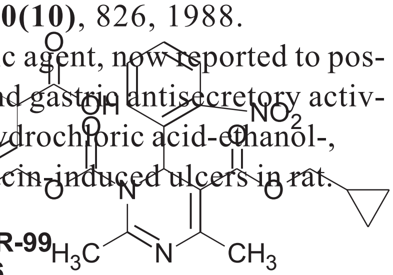
Compound Code: Bay-41-4109
Anti Hepatities B Virus Drugs
Bayer
Drug Data Report, **24(2)**, 165, 2002.

Calcium Channel Blocker
Drug Data Report, **8(5)**, 465, 1986.

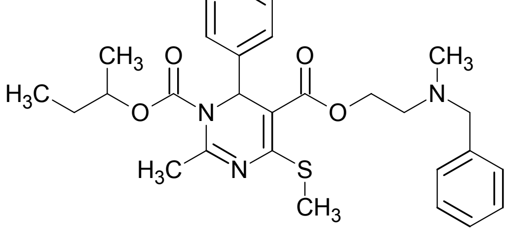
MAR-99
Leukotrine Antagonist[251]
Drug Data Report, **10(10)**, 826, 1988.
(Known anti-asthmatic agent, now reported to possess anti-ulcerative and gastric antisecretory activities, which inhibits hydrochloric acid-ethanol-, stress- and indomethacin-induced ulcers in rat.



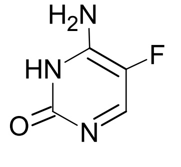
MAR-99
36



35



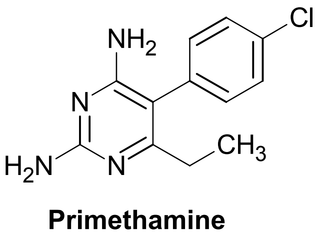
Calcium Channel Blocker
Drug Data Report, **10(11)**, 899, 1988.



Flucytosine
(flurocytosine)

Antifungal Agent.[253]

Clin Microbiol Infect 2003, 9, 1504.
In vitro susceptibility of Candida species isolated from cancer patients against some antifungal agents.



Primethamine

Antimalarial Agent.

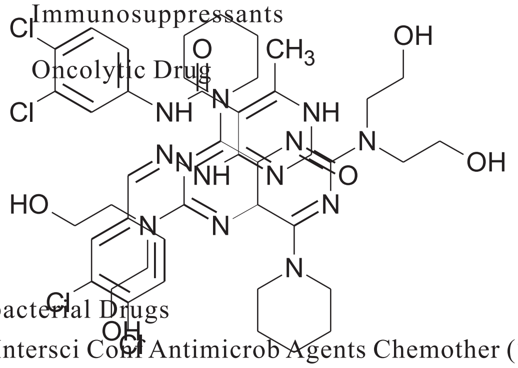
Iancet, **361** (9357), 577, 2003.

Acute Myocardial Infection

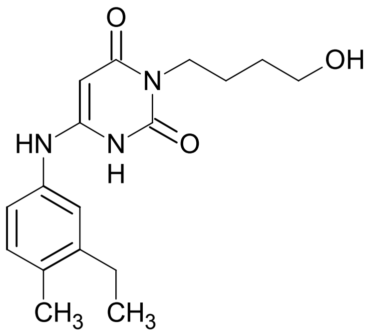
Treatment of Antiplatlet Therapy.

Immunosuppressants

Oncolytic Drug



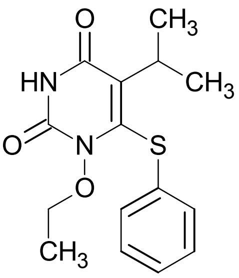
Dipyridamole



Antibacterial Drugs

39th Intersci Conf Antimicrob Agents Chemother (Sept 26-29, San Francisco) 1999, Abst F1808

In vitro activity of novel 6-anilinouracils targeted to DNA polymerase III of Gram-positive bacteria



TNK-6123

TNK-6123

Anti HIV Agent

Reverse Transcripase Inhibitors.

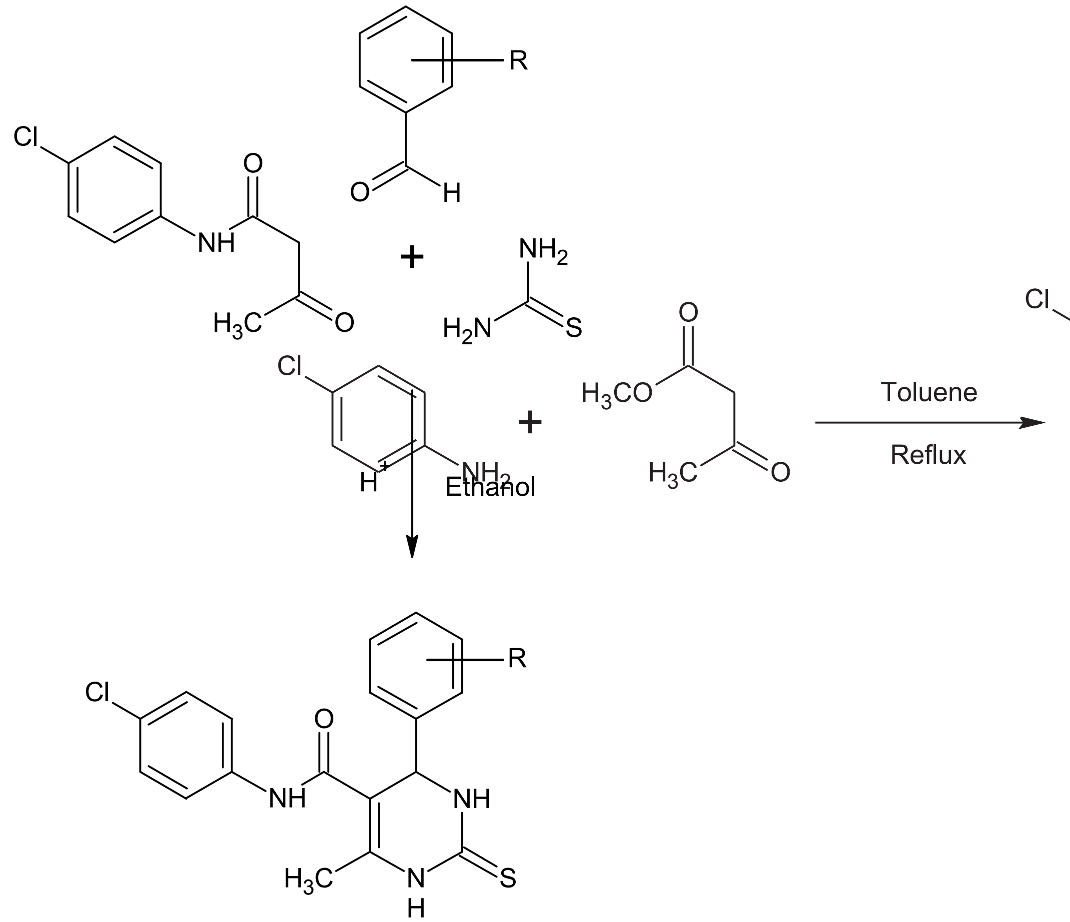
Non-nucleoside HIV-1 reverse transcriptase inhibitor

Compound was active not only against wild-type HIV-1 strains (IC50 = 3 nM against IIIB and NL4-3 HIV-1 strains) but also showed nanomolar

6 REACTION SCHEME

Step-1

Step-2



7 EXPERIMENTAL

Melting points of all the synthesized compounds were taken in open capillary bath on controlled temperature heating mental. The crystallization of all the compounds was carried out in appropriate solvents. TLC was carried out on silicagel-G as stationary phase. Ethyl acetate: Hexane (5:5) was used as a mobile phase. The other solvent system like acetone: benzene was also employed.

Step-1 ***Synthesis of N-(4-chlorophenyl)-3-oxobutanamide.***

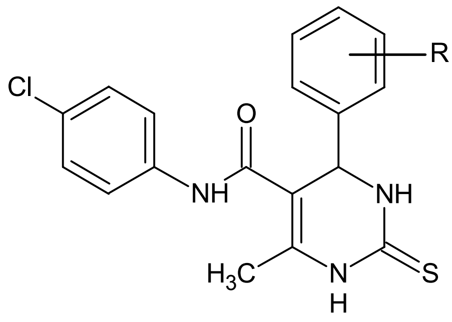
A suspension of ethyl acetoacetate (0.01 mol, 1.16 gm) and 4-choloroaniline (0.01 mol, 1.27 gm) in toluene (50 ml) containing catalytic amount of NaOH (0.05 ml, 40%) was refluxed on an oil bath for 8 hr. After completion of the reaction (TLC monitoring) the solvent was removed under reduced pressure, separated solid was filtered and washed with petroleum ether and crystallized from ethanol to give pure product. Yield 67% m.p. 87-89 °C.

Step-2 ***Synthesis of N-(4-chlorophenyl)-6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.***

The intimate mixture of *N*-(4-chlorophenyl)-3-oxobutanamide (0.01 mol, 2.11 gm), benzaldehyde (0.01 mol, 1.06 gm) and thiourea (0.01 mol, 0.76 gm) in ethanol (8 ml), containing 0.4 ml of concentrated HCl was heated under reflux for 6 hr. After completion of the reaction, the reaction mixture was allowed to stand at 0 °C for several hours and precipitation was obtained. The product was filtered, washed with chilled methanol and isolated product crystalized from ethanol.Yield 53%. m.p. 272-273 °C, Anal. Calcd. for $C_{18}H_{16}ClN_3OS$ Calcd: C, 60.14; H, 4.51; N, 11.74%, Found: C, 60.38; H, 4.41; N, 11.81%.

Similarly, other *N*-(4-chlorophenyl)-6-methyl-4-aryl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamides were prepared and crystallized from appropriate solvents, in some cases the products were purified by column chromatography. The reaction time and percentage yields of the respective reactions are depicted in the physical data **Table-1a**.

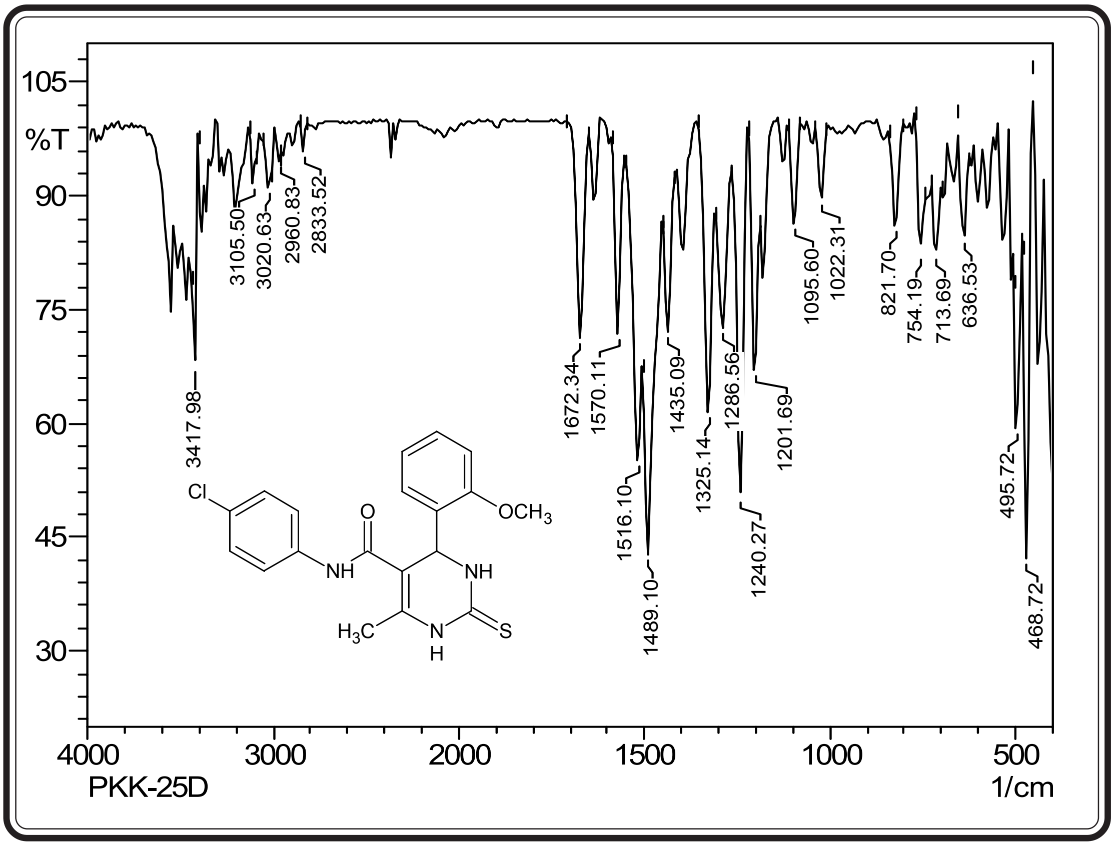
Table-1a: Physical constatnt of *N*-(4-chlorophenyl)-6-methyl-4-aryl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamides.



Sr. No.	Substitution R	Molecular Formula/ Molecular weight	M.P. °C	Yield %	% Composition Calcd./Found		
					C	H	N
1a	H	$C_{18}H_{16}ClN_3OS$ 357.85	272-273	53	60.41 60.38	4.51 4.41	11.74 11.81
1b	4-OCH_3	$C_{19}H_{18}ClN_3O_2S$ 387.88	240-241	52	58.83 58.76	4.68 4.66	10.83 10.79
1c	3-NO_2	$C_{18}H_{15}ClN_4O_3S$ 402.85	262-264	41	53.67 53.59	3.75 3.68	13.91 13.87
1d	4-Cl	$C_{18}H_{15}Cl_2N_3OS$ 392.30	275-276	42	55.11 55.06	3.85 3.79	10.71 10.73
1e	3,4-$(OCH_3)_2$	$C_{20}H_{20}Cl_2N_3OS$ 417.90	240-241	46	57.48 57.38	4.82 4.76	10.05 10.03
1f	2,5-$(OCH_3)_2$	$C_{20}H_{20}ClN_3O_3S$ 417.90	255-257	48	57.48 57.41	4.82 4.85	10.05 10.01
1g	2-OCH_3	$C_{19}H_{18}ClN_3O_2S$ 387.88	235-236	48	58.83 58.74	4.68 4.70	10.83 10.80
1h	2-OH	$C_{18}H_{16}ClN_3O_2S$ 373.85	281-282	35	57.83 57.71	4.31 4.29	11.24 11.20
1i	4-NO_2	$C_{18}H_{15}ClN_4O_3S$ 402.85	264-265	49	53.67 53.60	3.75 3.71	13.91 13.85
1j	3-OC6H_5	$C_{24}H_{20}ClN_3O_2S$ 449.95	267-269	57	64.06 64.01	4.48 4.52	9.34 9.29
1k	4-F	$C_{18}H_{15}ClFN_3OS$ 375.84	256-258	45	57.52 57.41	4.02 3.96	11.18 11.15

7 SPECTRAL STUDIES

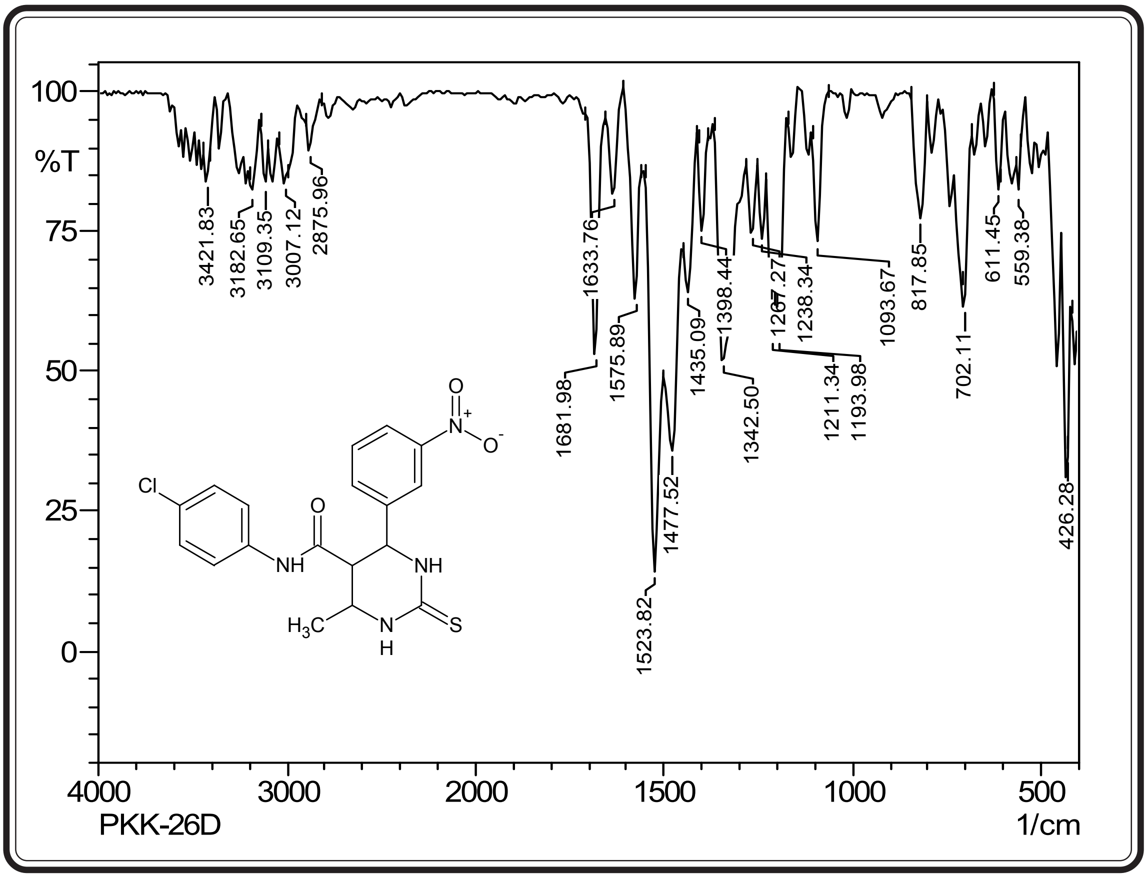
IR spectrum of *N*-(4-chlorophenyl)-6-methyl-4-(2-methoxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.



Instrument: Shimadzu FTIR-8400 using KBR DRS techniques. The percentage transmittance is given in cm^{-1} and frequence range is between 400-4000cm^{-1}.

Type	Vibration Mode	Frequency cm^{-1}
Alkane $-CH_3$	C-H str. (asym.)	2960
	C-H str. (sym.)	2832
	C-H i.p.d (asym)	1435
	C-H o.o.d (sym)	1325
Aromatic	C-H str.	3105
	C=C (skeleton)	1516, 1498
	C-H i.p. bending	1095
	C-H o.p bending	821
Carbonyl	-C=O	1672
Amine	-NH str.	3417
	-NH def.	1570
Halide	-C-Cl	754

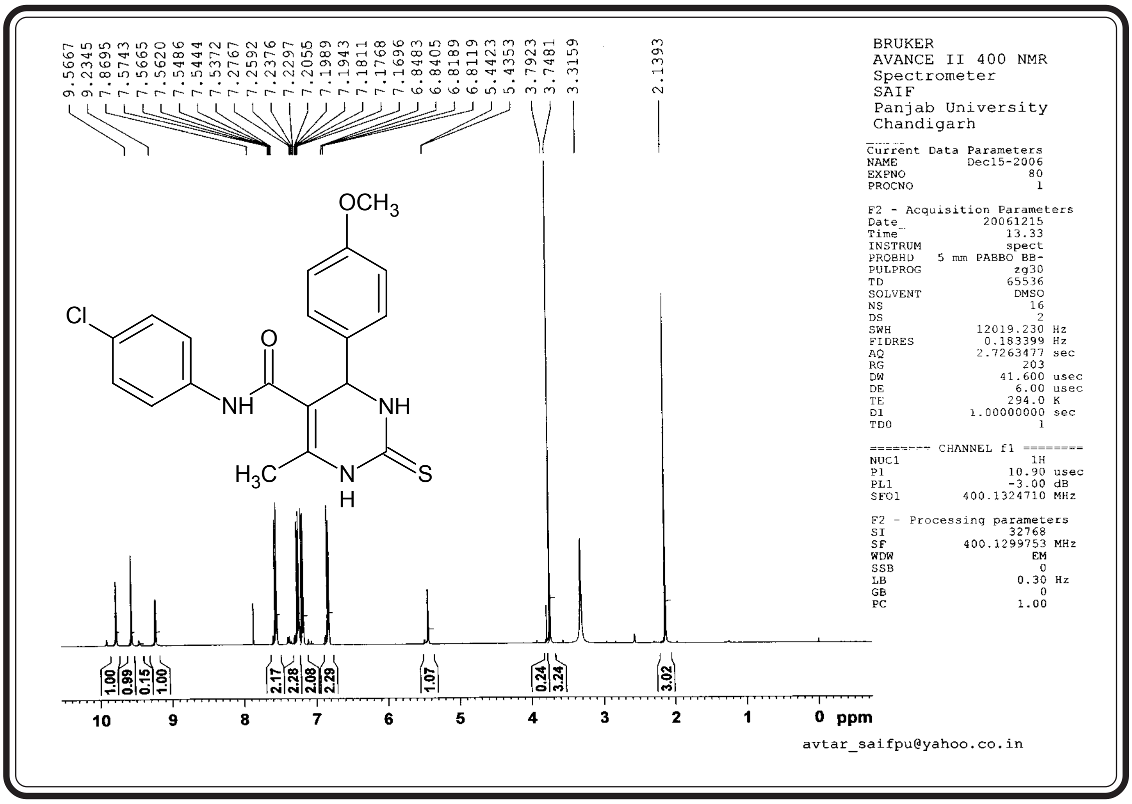
IR spectrum of *N*-(4-chlorophenyl)-6-methyl-4-(3-nitrophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.

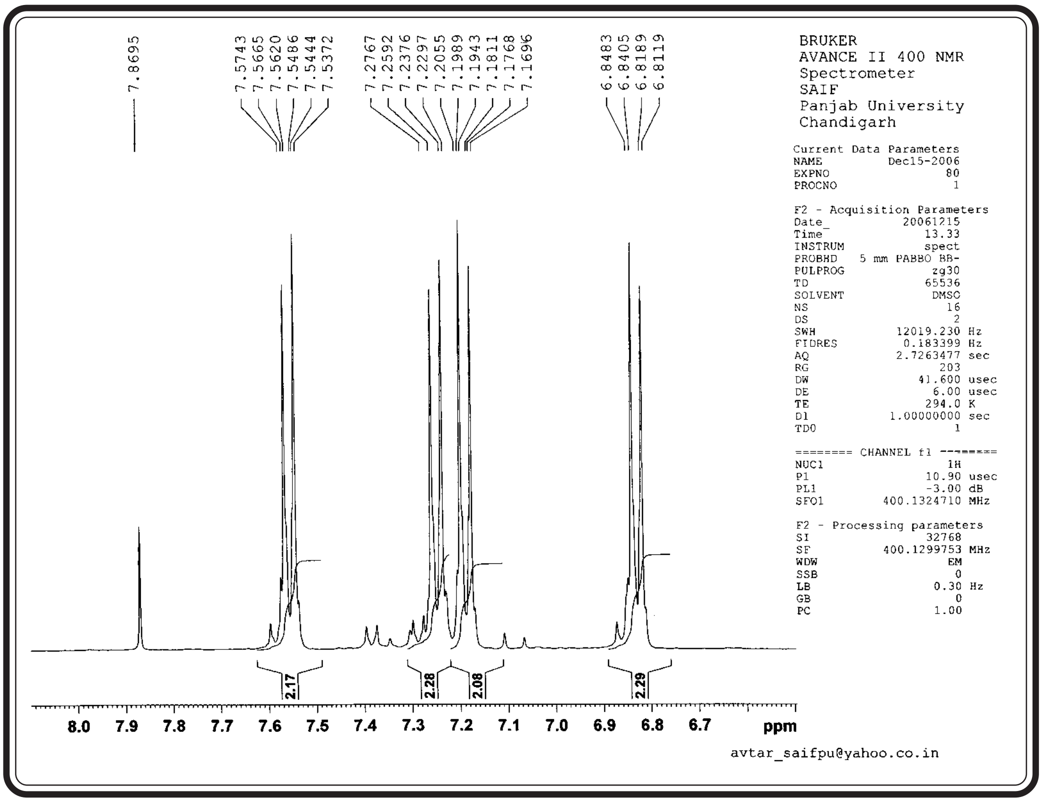


Instrument: Shimadzu FTIR-8400 using KBR DRS techniques. The percentage transmittance is given in cm^{-1} and frequence range is between 400-4000cm^{-1}.

Type	Vibration Mode	Frequency cm^{-1}
Alkane $-CH_3$	C-H str. (asym.)	3009
	C-H str. (sym.)	2875
	C-H i.p.d (asym)	1435
	C-H o.o.d (sym)	1342
Aromatic	C-H str.	3109
	C=C (skeleton)	1523, 1477
	C-H i.p. bending	1093
	C-H o.p bending	817
Carbonyl	-C=O	1681
Amine	-NH str.	3421
	-NH def.	1575
Halide	-C-Cl	702

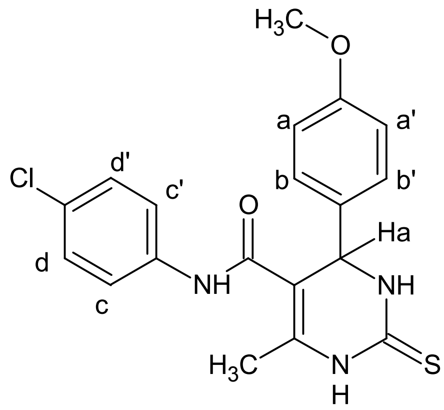
[1]H-NMR spectrum of *N*-(4-chlorophenyl)-6-methyl-4-(4-methoxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.





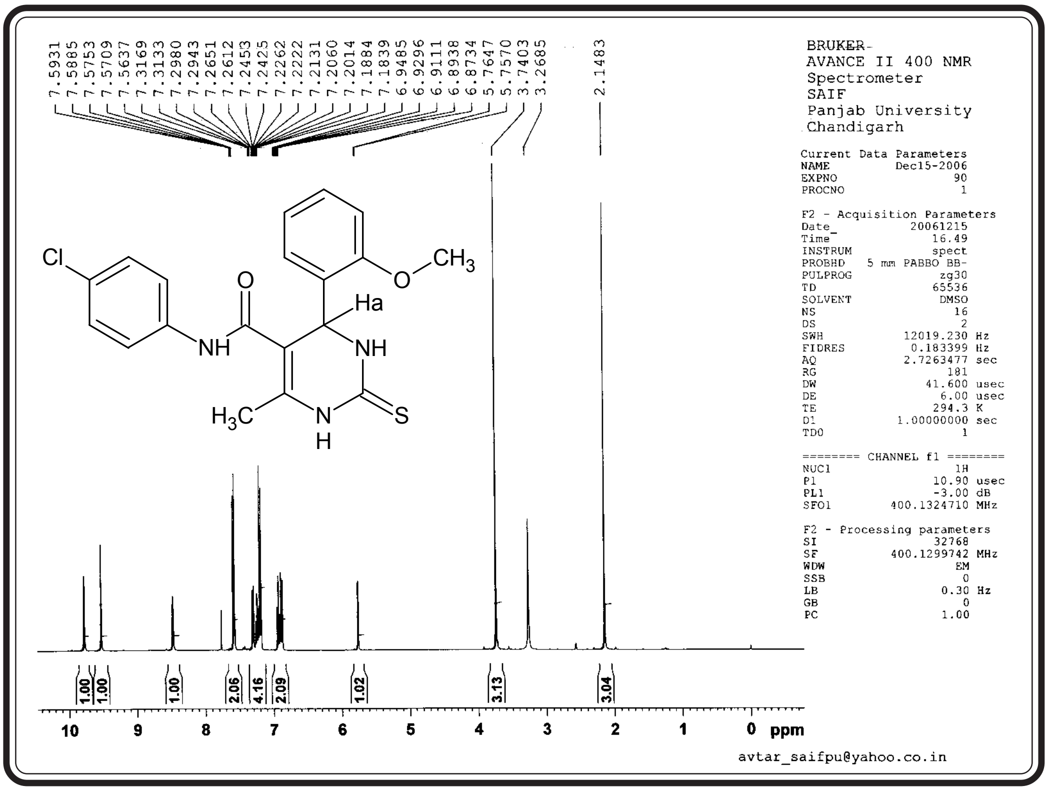
Instrument: BRUKER 400 MHz (Avance - II), **Internal reference:** TMS,
Solvent: DMSO [d_6].

Assignment of proton vlues of *N*-(4-chlorophenyl)-6-methyl-4-(4-methoxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.



Sr. No.	Chemical shift in ppm	Relative No. of Protons	Multiplicity	Inference	J value in Hz
1	2.14	3H	singlet	$-CH_3$	-
2	3.74	3H	singlet	$-OCH_3$	-
3	5.44	1H	singlet	Ha	-
4	6.81-6.84	2H	double doublet	Ar-Haa'	11.44
5	7.17-7.19	2H	double doublet	Ar-Hbb'	7.00
6	7.25-7.27	2H	doublet	Ar-Hdd'	
7	7.54-7.56	2H	double doublet	Ar-Hcc'	7.04
8	7.86	1H	singlet	-NH (pyrimidine ring)	-
9	9.23	1H	singlet	-NH (pyrimidine ring)	-
10	9.56	1H	singlet	-NH (Amide)	-

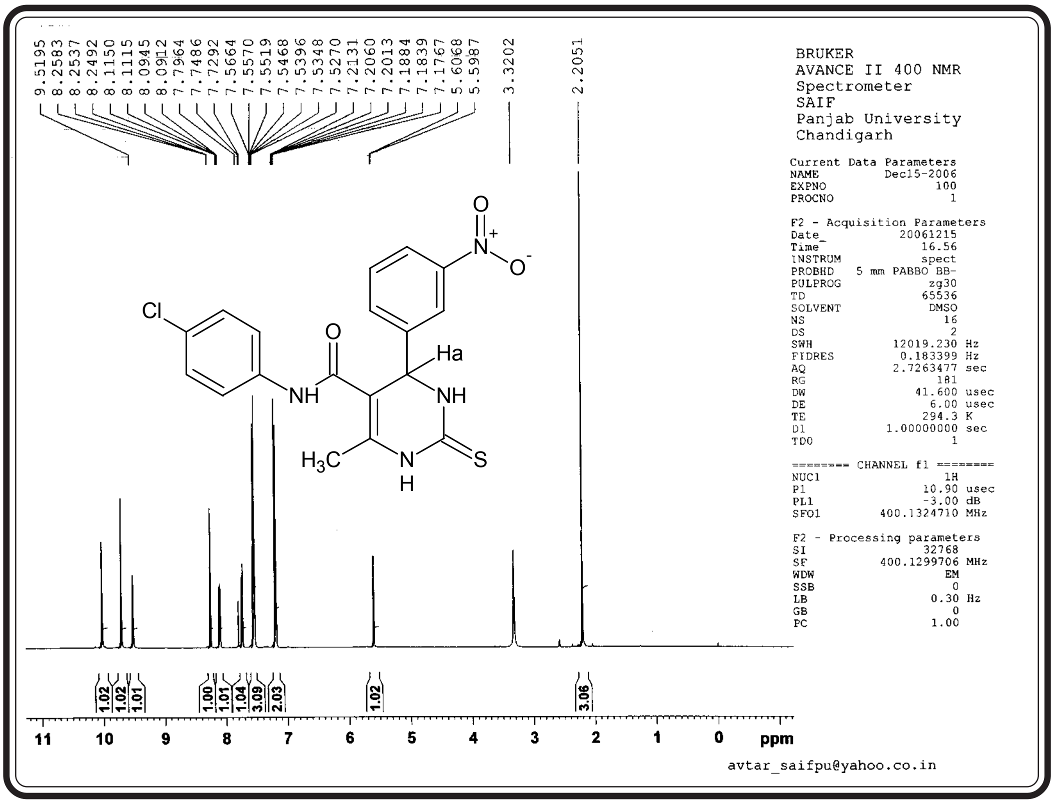
[1]H-NMR spectrum of *N*-(4-chlorophenyl)-6-methyl-4-(2-methoxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.



Instrument: BRUKER 400 MHz (Avance - II), **Internal reference:** TMS, **Solvent:**DMSO [d_6].

Sr. No.	Chemical shift in ppm	Relative No. of Protons	Multiplicity	Inference	J value in Hz
1	2.14	3H	singlet	$-CH_3$	-
2	3.74	3H	singlet	$-OCH_3$	-
3	5.76	1H	singlet	Ha	-
4	6.87-7.59	8H	multiplet	Ar-H	-
5	8.47	1H	singlet	-NH (pyrimidine ring)	-
6	9.56	1H	singlet	-NH (pyrimidine ring)	-
7	9.82	1H	singlet	-NH (Amide)	-

[1]H-NMR spectrum of *N*-(4-chlorophenyl)-6-methyl-4-(3-nitrophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.



Instrument: BRUKER 400 MHz (Avance - II), **Internal reference:** TMS,
Solvent: DMSO [d_6].

Sr. No.	Chemical shift in ppm	Relative No. of Protons	Multiplicity	Inference	J value in Hz
1	2.20	3H	singlet	$-CH_3$	-
2	3.32	3H	singlet	$-OCH_3$	-
3	5.60	1H	singlet	Ha	-
4	7.17-8.25	8H	multiplet	Ar-H	-
5	9.51	1H	singlet	-NH (pyrimidine ring)	-
6	9.72	1H	singlet	-NH (pyrimidine ring)	-
7	10.07	1H	singlet	-NH (Amide)	-

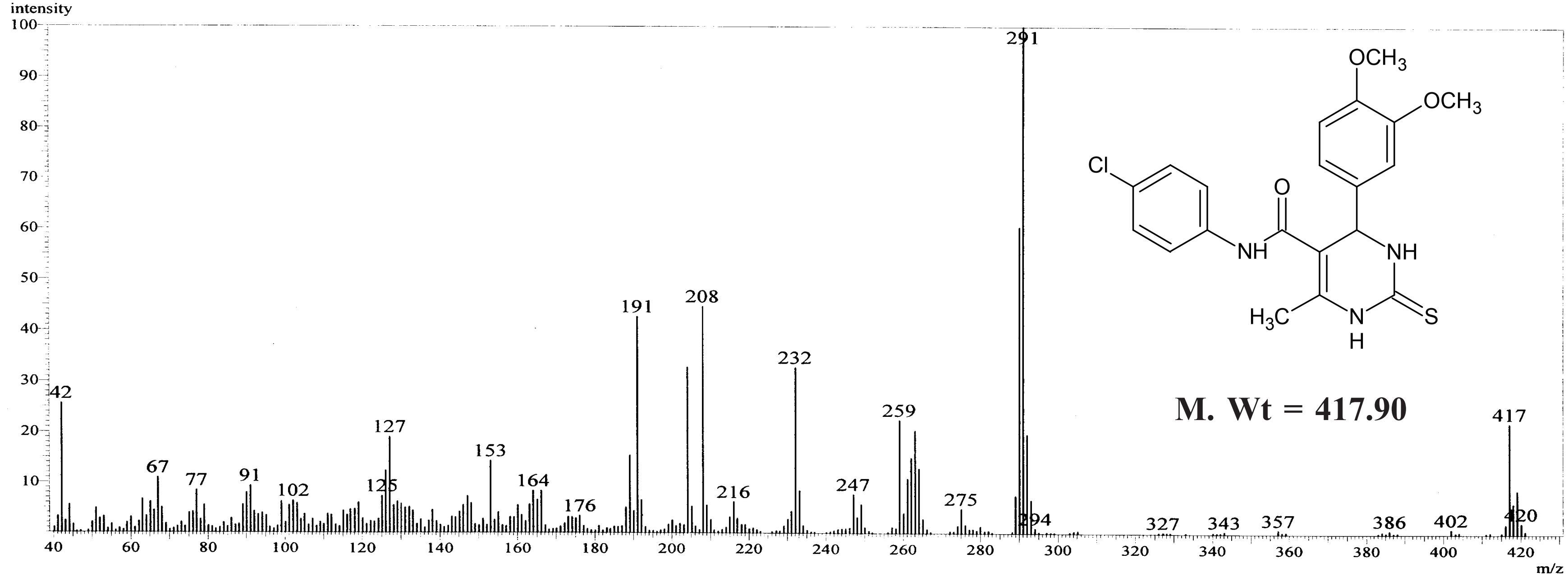
EI-Mass spectrum of *N*-(4-chlorophenyl)-6-methyl-4-(3,4-dimethoxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.

SAURASHTRA UNIVERSITY - RAJKOT
DEPT. OF CHEMISTRY

Sample Information

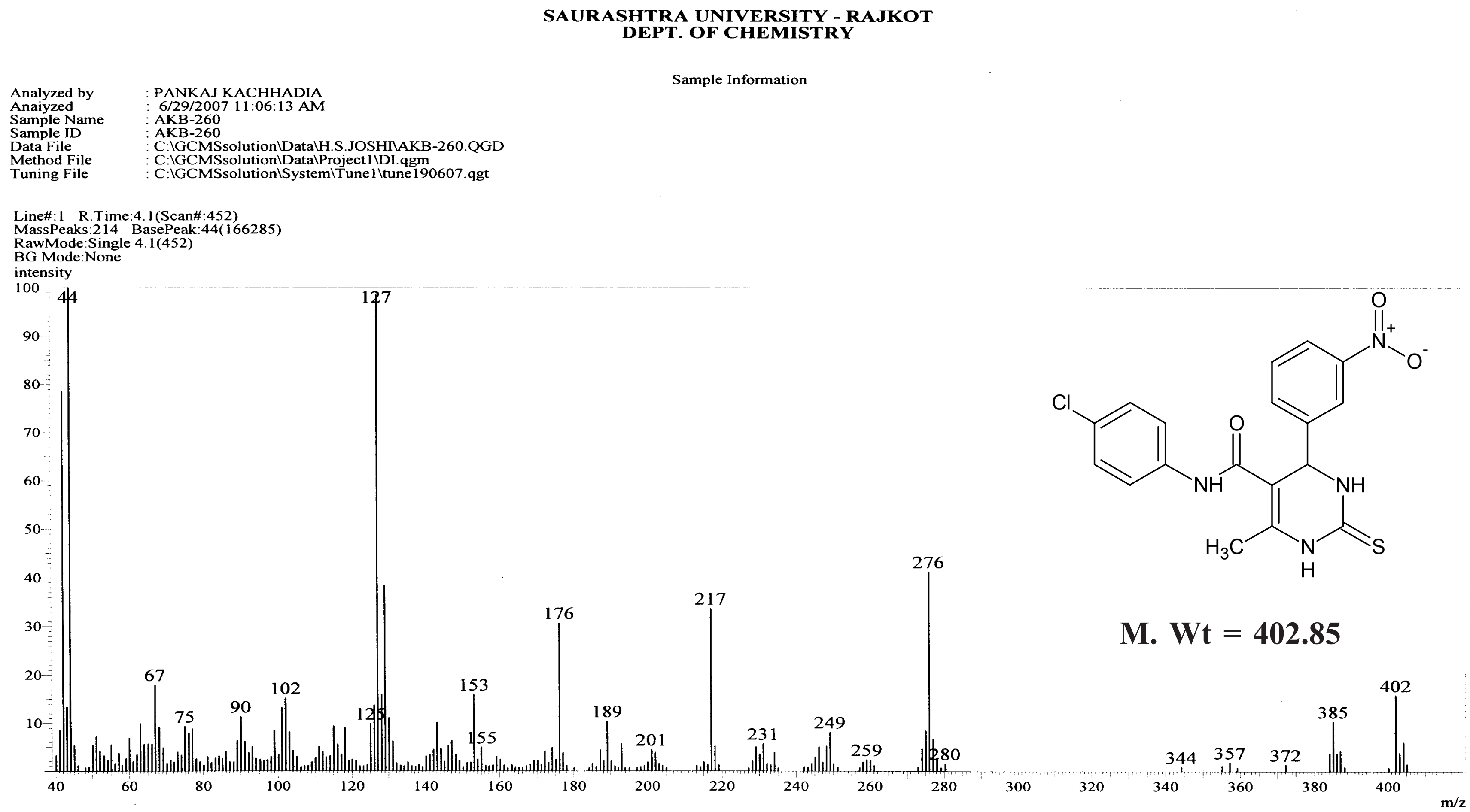
Analyzed by	: PANKAJ KACHHADIA
Anaiyzed	: 6/28/2007 2:53:24 PM
Sample Name	: AKB-270
Sample ID	: AKB-270
Data File	: C:\GCMSsolution\Data\H.S.JOSHI\AKB-270.QGD
Method File	: C:\GCMSsolution\Data\Project1\DI.qgm
Tuning File	: C:\GCMSsolution\System\Tune1\tune190607.qgt

Line#:1 R.Time:9.8(Scan#:1143)
MassPeaks:276 BasePeak:291(751899)
RawMode:Single 9.8(1143)
BG Mode:None



Instrument: Shimadzu GC-MS QP-2010, DI-probe, EI-method.

EI-Mass spectrum of *N*-(4-chlorophenyl)-6-methyl-4-(3-nitrophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.



Instrument: Shimadzu GC-MS QP-2010, DI-probe, EI-method.

9 ANTIMICROBIAL ACTIVITY

Method	:	Cup-Plate[97]
Gram positive bacteria	:	*Staphylococcus aureus* ATCC 6538
		Staphylococcus epidermidis ATCC 12228
Gram negative bacteria	:	*Escherichia coli* ATCC 8739
		Pseudomonas aeruginosa ATCC 1539
Fungi	:	*Aspergillus niger* ATCC 16888
Concentration	:	1000 µg/ml
Solvent	:	Dimethyl formamide
Standard drugs	:	Amoxicillin, Ciprofloxacin, Cephalexin, Erythromycin, Greseofulvin

The antimicrobial activity was compared with standard drug viz Amoxicillin, Ciprofloxacin, Cephalexin, Erythromycin and antifungal activity was compared with viz Greseofulvin. The inhibition zones measured in mm.

(a) Antibacterial activity

The purified products were screened for their antibacterial activity using cup-plate agar diffusion method. The nutrient agar broth prepared by the usual method was dispensed in 50 ml quantities of different conical flasks. Then, add the 0.5 ml culture of each bacteria (*Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 1539) in nutrient agar broth and inoculate at 37 °C for 24 hr.

The nutrient agar was melted at 100 °C and after cooling to 56 °C, was poured into petri plates of 13 cm diameter in quantities of 20 ml, and left on a flat surface to solidify and the surface of the medium was dried at 37 °C. Then, above subcultures of each bacteria pipetted in to the nutrient agar plate. The cups (10 mm diameter) were formed by the help of borer in agar medium and filled with 0.04 ml (40 ì g) solution of sample in DMF. The plates were incubated at 37 °C for 24 hr. and the control was also maintained with 0.04 ml of DMF in a similar manner. After the completion of incubation period, the zone of inhibition of growth in the form of diameter in mm was measure and recorded in **Table-1b**

97. A. L. Barry; The antimicrobial susceptibility test: Principle and practices, edited by *Illuslea & Febiger*, *(Philadelphia), USA*, 180; *Biol. Abstr.*, **64**, 25183 (1977).

(b) Antifungal activity

Aspergillus niger ATCC 16888 was employed for testing antifungal activity using cup-plate agar diffusion method. The culture was maintained on sabourauds agar slants, sterilized sabourauds agar medium was inoculated with 72 hr. old 0.5 ml suspension of fungal spores in a separate flask.

The sabourauds agar was melted at 100 ^{o}C and after cooling to 56 ^{o}C, was poured into petri plates of 13 cm diameter in quantities of 20 ml, and left on a flat surface to solidify and the surface of the medium was dried at 37 ^{o}C. Then, above subculture of fungi pipetted in to the sabourauds agar plate. The cups (10 mm diameter) were formed by the help of borer in agar medium and filled with 0.04 ml (40 ì g) solution of sample in DMF. The plates were incubated at 30 ^{o}C for 48 hr. and the control was also maintained with 0.04 ml of DMF in a similar manner. After the completion of incubation period, the zone of inhibition of growth in the form of diameter in mm was measure and recorded in **Table-1b**

Table-1b: Antimicrobial activities of *N*-(4-chlorophenyl)-6-methyl-4-aryl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamides.

Sr. No.	Antibacterial Activity				Antifungal Activity
	S. aureus	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*A. niger*
1a	**16** (0.80)C_1, (0.76)C_2 (0.88)C_3, (0.80)C_4	**16** (0.66)C_1, (0.66)C_2 (0.94)C_3, (0.88)C_4	**16** (0.72)C_1, (0.60)C_2 (0.66)C_3, (0.88)C_4	**18** (0.85)C_1, (0.72)C_2 (0.72)C_3, (1.20)C_4	**13** (0.54)C_5
1b	**18** (0.90)C_1, (0.85)C_2 (1.00)C_3, (0.90)C_4	**18** (0.75)C_1, (0.75)C_2 (1.05)C_3, (1.00)C_4	**15** (0.66)C_1, (0.60)C_2 (0.62)C_3, (0.83)C_4	**14** (0.66)C_1, (0.56)C_2 (0.56)C_3, (0.93)C_4	**20** (0.83)C_5
1c	**13** (0.65)C_1, (0.61)C_2 (0.72)C_3, (0.65)C_4	**14** (0.58)C_1, (0.58)C_2 (0.82)C_3, (0.77)C_4	**14** (0.63)C_1, (0.56)C_2 (0.58)C_3, (0.77)C_4	**12** (0.57)C_1, (0.48)C_2 (0.48)C_3, (0.80)C_4	**15** (0.62)C_5
1d	**12** (0.60)C_1, (057)C_2 (0.66)C_3, (0.60)C_4	**16** (0.66)C_1, (0.66)C_2 (0.94)C_3, (0.88)C_4	**13** (0.59)C_1, (0.52)C_2 (0.54)C_3, (0.72)C_4	**18** (0.85)C_1, (0.72)C_2 (0.72)C_3, (1.20)C_4	**14** (0.58)C_5
1e	**19** (0.95)C_1, (0.90)C_2 (1.05)C_3, (0.95)C_4	**14** (0.58)C_1, (0.58)C_2 (0.82)C_3, (0.77)C_4	**14** (0.63)C_1, (0.56)C_2 (0.58)C_3, (0.77)C_4	**21** (1.00)C_1, (0.84)C_2 (0.84)C_3, (1.40)C_4	**16** (0.66)C_{54}
1f	**10** (0.50)C_1, (0.47C_2 (0.55)C_3, (0.50)C_4	**12** (0.50)C_1, (0.50C_2 (0.70)C_3, (0.66)C_4	**12** (0.54)C_1, (0.48)C_2 (0.50)C_3, (0.66)C_4	**14** (0.66)C_1, (0.56)C_2 (0.56)C_3, (0.93)C_4	**16** (0.66)C_{54}

1g	**11** (0.55)C_1, (0.52)C_2 (0.61)C_3, (0.55)C_4	**18** (0.75)C_1, (0.75)C_2 (1.05)C_3, (1.00)C_4	**15** (0.68)C_1, (0.60)C_2 (0.62)C_3, (0.83)C_4	**13** (0.61)C_1, (0.52)C_2 (0.52)C_3, (0.86)C_4	**18** (0.75)C_5
1h	**10** (0.50)C_1, (0.47)C_2 (0.55)C_3, (0.50)C_4	**11** (0.45)C_1, (0.45)C_2 (0.67)C_3, (0.61)C_4	**14** (0.63)C_1, (0.56)C_2 (0.58)C_3, (0.77)C_4	**11** (0.52)C_1, (0.44)C_2 (0.44)C_3, (0.73)C_4	**13** (0.54)C_5
1i	**18** (0.90)C_1, (0.85)C_2 (1.00)C_3, (0.90)C_4	**12** (0.50)C_1, (0.50)C_2 (0.70)C_3, (0.66)C_4	**10** (0.45)C_1, (0.40)C_2 (0.41)C_3, (0.55)C_4	**17** (0.80)C_1, (0.68)C_2 (0.68)C_3, (0.88)C_4	**18** (0.75)C_5
1j	**23** (1.15)C_1, (1.09)C_2 (1.27)C_3, (1.15)C_4	**17** (0.70)C_1, (0.70)C_2 (1.00)C_3, (0.94)C_4	**11** (0.50)C_1, (0.44)C_2 (0.45)C_3, (0.61)C_4	**15** (0.71)C_1, (0.60)C_2 (0.60)C_3, (1.00)C_4	**14** (0.58)C_5
1k	**11** (0.55)C_1, (0.52)C_2 (0.61)C_3, (0.55)C_4	**15** (0.62)C_1, (0.62)C_2 (0.88)C_3, (0.83)C_4	**17** (0.77)C_1, (0.68)C_2 (0.70)C_3, (0.94)C_4	**11** (0.52)C_1, (0.44)C_2 (0.44)C_3, (0.73)C_4	**14** (0.58)C_5
C_1	**20**	**24**	**22**	**21**	**00**
C_2	**21**	**24**	**25**	**25**	**00**
C_3	**18**	**17**	**24**	**25**	**00**
C_4	**20**	**18**	**18**	**15**	**00**
C_5	**00**	**00**	**00**	**00**	**24**

Activity index = Inhibition zone of the sample / Inhibition zone of the standard

For antibacterial activity : C_1 = Amoxicillin, C_2 = Ciprofloxacin, C_3 = Cephalexin, C_4 = Erythromycin.

For antifungal activity : C_5 = Greseofulvin.

Part-[B]

(Section-II)

Synthesis, Characterization and Antimicrobial screening of Thiazolo[3,2-*a*]pyrimidines

SYNTHESIS CHARACTERIZATION AND ANTIMICROBIAL SCREENING OF THIAZOLO[3,2-*a*]PYRIMIDINES

1 INTRODUCTION

In section one, importance of DHPM moiety and various modifications applied to Biginelli reaction for better yield, shorter reaction time and to synthesize various analogs is surveyed in detail. The biological profile of this heterocyclic moiety is also briefly reported in section one.

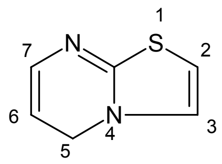
Biginelli reaction is not only important to synthesize analogs of DHPM ring using different building block as potent bioactive heterocycles, but also various scaffolds can be synthesized from this heterocyclic scaffold.

Tetrahydro/dihydropyrimidine-2-thiones are the key intermediate for the synthesis of fused pyrimidines. They have become of considerable interest during the last forty years[1-12]. Many of these compounds have proved to be active anticancer[5-10], antipyretic and antiinflammatory agents,[8-12] calcium antagonist,[13-15] analgesic,[16] antitumor,[17,18] antidepresant,[19] antibacterial and antifungal[20-22] agents.

---

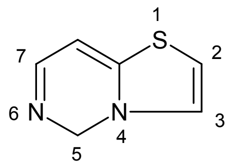
1. M. Reimlinger, M. A. Reiren, R. Merenyi; *Chem. Ber.,* **103,** 3252 (1970).
2. Q. Hayashi; Yakugaku Zasshi, 85, 442 (1965); *Chem Abstr.,* **63,** 5644 (1965).
3. M. H. Elangdi, S. A. Abdalla; *J. Pract. Chem.,* **315,** 1009 (1973).
4. M. H. Elangdi, M. Ohta, Bull. Chem. *Soc. Japan,* **46,** 1830 (1973).
5. Y. Masisumi; Japanese Patent **13,** 640 (63); *Chem. Abstr.,* **60,** 531 (1964).
6. Y. Masisumi; Japanese Patent **13,** 641 (63); *Chem. Abstr.,* **60,** 531 (1964).
7. Y. Masisumi; Japanese Patent **7,** 982 (62); *Chem. Abstr.,* **59,** 8764 (1963).
8. A. Takamizawa; Y. Hamashima; Japanese Patent **14,** 423 (66); *Chem. Abstr.,* **65,** 20144b (1966).
9. A. Takamizawa; Y. Hamashima; Japanese Patent **17,** 595 (65); *Chem. Abstr.,* **63,** 19112h (1965).
10. Ito, Japan, 7030, 101 (1970); *Chem. Abstr.,* **74,** 22827e(1971).
11. V. Sprio, S. Plescia, *J. Heterocyclic Chem.,* **9,** 951 (1972).
12. A. Takamizawa, H. Sato, Japanese Patent **72,** 45,595 (1972); *Chem. Abstr.,* **78,** 58454c (1973).
13. A. Balkan, M. Ertan, T. Burgemeister; *Arch. Pharm., (Weinheim)*, **325,** 499 (1992).
14. A. Balkan, S. Uma, M. Ertan W. Wiegrebe; *Pharmazie,* **47,** 687 (1992).
15. A. Balkan, B. Tozkoparan, M. Ertan, Y. Sara, N. Ertekin; *Boll. Chim. Farm.,* **135,** 648 (1996).
16. J. Baetz; *Brit.,* 1,334,628 (1973); *Chem. Abstr.,* **80,** 48028u (1974).
17. B. Dash, M. Patra, P.K. Mohapatra; *J. Inst. Chem.,* **52,** 92 (1980); *Chem. Abstr.,* **93,** 239353z (1980).
18. Teijin Ltd., Jpn. Kokai Tokkyo Koho JP, **58,** 24,590 [**83,** 24,590] (1983); *Chem. Abstr.,* **98,** 215611j (1983).
19. D. Bigg (Synthelabo S.A.); Fr. Demande FR, 2,479,831 (1981); *Chem. Abstr.,* **96,** 162725z (1982).
20. J. Mohan, V.K. Chadha, H.S. Chaudhary, B.D. Sharma, H.K. Pujari; *Indian J. Exp. Biol.,* **10,** 37 (1972).
21. S.N. Dehuri, A. Nayak; *J. Ind. Chem. Soc.,* **60,** 970 (1983).
22. H.R. Champaneri, S.R. Modi, H.B. Naik; *Asian J. Chem.,* **6,** 737 (1994); *Chem. Abstr.,* **121,** 300842y (1994).

The parent ring systems of known thiazolopyrimidine derivatives are shown in structures **1-4**.



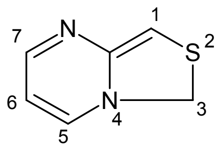
1

5H-Thiazolo[3,2-*a*]pyrimidine



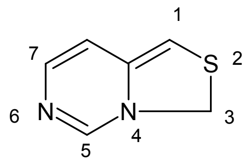
2

5H-Thiazolo[3,2-*c*]pyrimidine



3

6H-Thiazolo[3,4-*a*]pyrimidine



4

3H-Thiazolo[3,4-*c*]pyrimidine

2 SYNTHETIC ASPECTS

The following routs have been employed for the synthesis of thiazolopyrimidines.

2.1 Azole Approaches

Starting from the thiazole ring **(5)** and subsequent construction of the pyrimidine ring in the terminal step.

- Thiazolo[3,2-*a*]pyrimidine **(7)** was prepared in 30% yield by the reaction of 2-aminothiazole **(5)** with ethyl cyanoacetate **(6)** in a sodium ethoxide/ethanol mixture or using polyphosphoric acid[23] or acetic acid.[24,25]
- However, oxothiazolopyrimidine **(9)** was obtained upon treatment with phosphorous pentoxide and methanesulfonic acid **(8)**.[23]
- The reaction of **(5)** with ethyl acetoacetate **(10)** at 140–150 °C resulted in the formation of **(11)** that was then converted to the *Z*-isomer upon heating at 250 °C and cyclized to give **(12)**.[26]
- 2-Aminothiazole **(5)** cyclized with acetylacetone **(13)** at 100 °C, in the presence of methanesulfonic acid-phosphorus pentoxide or formic acid–phosphorus pentoxide, followed by treatment with 70% perchloric acid, to give the thiazolopyrimidin-4-ium salt **(14)**.[27]
- The ester **(16)** was obtained from 2-aminothiazole **(5)** with an excess of methyl methanetricarboxylate **(15)** in 61% yield.[28]
- Cyclocondensation of **(5)** with diethyl ethoxymethylene malonate **(17)** in acetic acid followed by hydrolysis of the ester **(18)** gave **(19)**.[29-30]
- Similarly, 2-aminothiazole **(5)** reacted with **(20)** in ethanol to give **(21)**.[31]
- Stanovink et al.[32-36] reported the synthesis of a series of thiazolopyrimidine derivatives upon reacting 2-aminothiazole with a variety of different reagents. Thus, dimethylaminobut-2-enoate (or pentenoate), **(22a–d),** reacted with **(5)** to give thiazolopyrimidines **(23a–d) (Chart 1).**

---

23. T. Tsuji; *J. Heterocycl. Chem.,* **28,** 489 (1991).
24. M. A. Jacobson, R. Norton; PCT Int. Appl. WO 97 33,879 (1997); *Chem. Abstr.,* **127,** 293241v (1997).
25. M.M. El-kerdawy, M.A. Moustafa, L.M. Gad, S.M.E. Badr; *Bull Fac. Pharm. (Cairo Univ.),* **31(1),** 67 (1993); *Chem. Abstr.,* **121,** 57472b (1994).
26. S. Sharma, M.S. Khanna, C.P. Garg, R.P. Kapoor, A. Kapil, S. Sharma; *Indian J. Chem. Sec. B,* **32B(6),** 693 (1993).

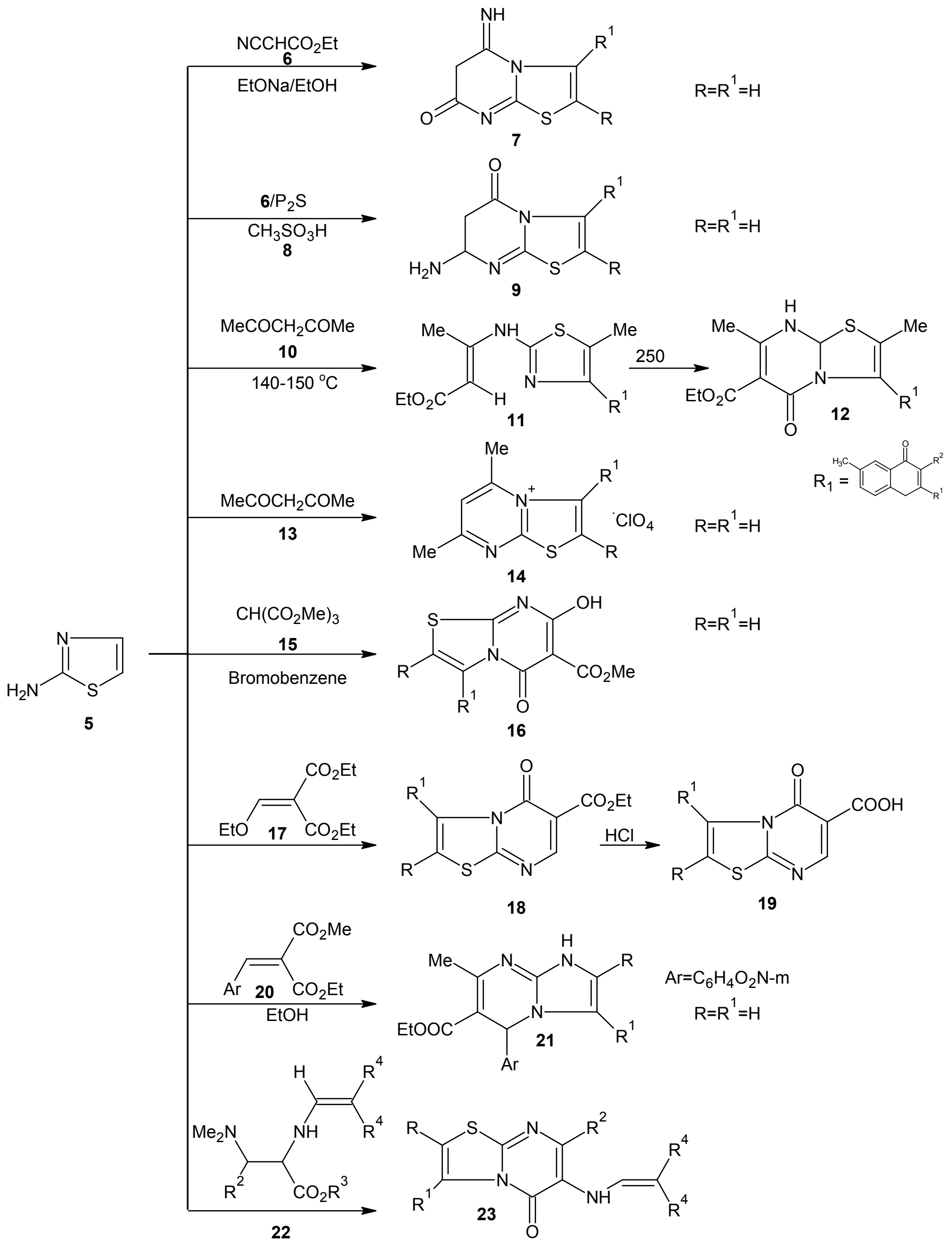


Chart-1

27. K. Takenaka, T. Tsuji; *J. Heterocycl. Chem.,* **33,** 1367 (1996).

28. A. Kutyrevand, T. Kappe; *J. Heterocycl. Chem.,* **36,** 237 (1999).

29. D. R. Shridhar, M. Jogibhukta, V. S. Krishnan; *Indian J. Chem. Sec. B,* **25B(3)**, 345 (1986).

30. T. Shioiri, K. Ninomija, S. Yamada; *J. Am. Chem. Soc.,* **94,** 6203 (1972).

31. T. Mishina, N. Tsuda, A. Inui, Y. Miura; Jpn. Kokai Tokkyo Koho JP 62, 169, 793; (1987); *Chem. Abstr.* **108,** 56120e (1988).

32. L. Selic, S.G. Grdadolnik, B. Stanovnik; *Heterocycles,* **45(12),** 2349 (1997).

33. G. Sorsak, S.G. Grdadolnik, B. Stanovnik; *J. Heterocycl. Chem.,* **35,** 1275 (1998).

34. L. Selic, B. Stanovnik; *J. Heterocycl. Chem.,* **34,** 813 (1997).

35. G. Sorsak, A. Sinur, L. Golic and B. Stanovnik; *J. Heterocycl. Chem.,* **32,** 921 (1995).

36. B. Stanovnik, H. Van de Bovenkamp, J. Svete, A. Hvala, I. Simonic, M. Tisher; *J. Heterocycl. Chem.,* **27,** 359 (1990).

- The reaction of 2-aminothiazole **(5)** with 2-hydropolyfluoroalk-2-enoate **(24)** in basic medium gave two isomers, 7-oxo **(25)** and its isomeric 5-oxo **(26)**. The structure of both **(25)** and **(26)** was established through ^{1}H NMR, ^{19}F NMR and mass spectra.[37]
- 2-Aminothiazole derivatives **(5),** (R1¼H, CO_2Et; R2¼Ph, aryl, Me), reacted with the acetylenic derivative **(27)** and ester derivative **(28)** in ethanol and polyphosphoric acid, respectively, to give the isomeric oxothiazolopyrimidine derivatives **(29)** and **(30)**, in 5–32% and 8–97% yield, respectively.[38]
- Condensation of 2-aminothiazole **(5)** in absolute ethanol with the sodium salt of ethyl oximinocyanoacetate **(31)** gave after acidification (pH 6) with diluted hydrochloric acid, the nitroso derivative **(32)** in 92% yield.[39]
- Treatment of the 2-aminothiazaole derivatives **(5)** with the hydrazone derivatives **(33)** gave the oxothiazolo [3,2-*a*] pyrimidine derivatives **34**.[40]
- Compound **(37)** was prepared in 70–95% yield by thermal (160 °C) condensation of arylaminobisthiazole **(35)** with two equivalents of bis(2,4,6-trichlorophenyl)methyl malonate **(36)**[41] **(Chart 2).**

37. Y.-S. Liu, W.-Y. Huang; *J. Chem. Soc. Perkin Trans.,* **1(6),** 981 (1997).
38. D. Janietz, B. Goldmann, W.-D. Rudorf ; *J. Prakt. Chem.,* **330(4),** 607 (1988).
39. M.R.D. Giudice, A. Borioni, C. Mustazza, F. Gatta, B. Stanovnik; *J. Heterocycl. Chem.,* **32,** 1725 (1995).
40. M. A. Metwally, A. M. Ismaiel, Y. M. Yousif, F. Eid; *J. Indian Chem. Soc.,* **66(3),** 179 (1989).
41. G.O. Mbagwa, R. Garland; *J. Heterocycl. Chem.,* **25,** 571 (1988).

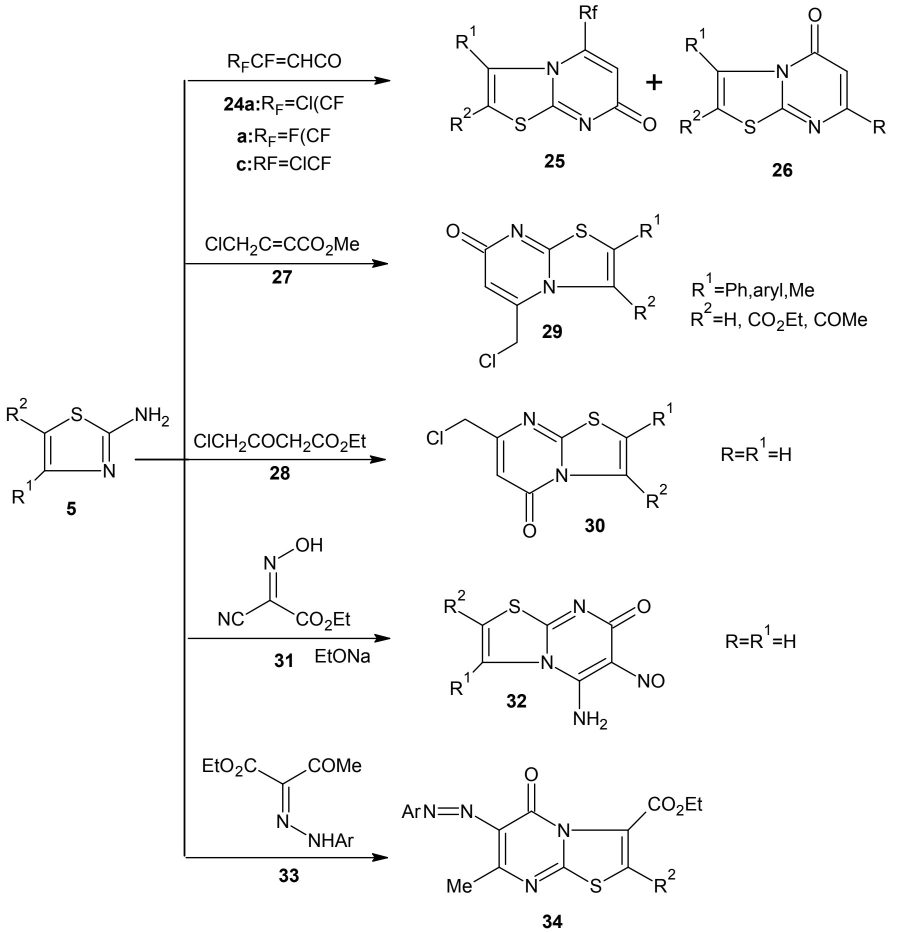
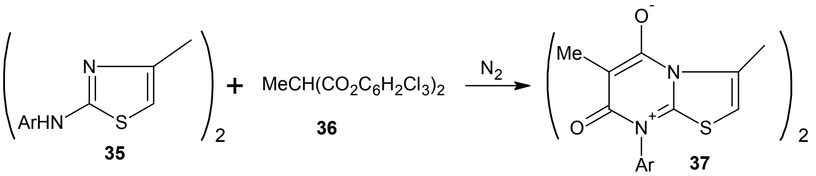


Chart-2



2.2 Azine Approaches

Starting from pyrimidine ring and subsequent construction of the thiazole in the terminal step.

Pyrmidinethione derivatives **(37-39)** were alkylated with monochloroacetic acid or chloroacetyl chloride and then cyclized to give thiazolopyrimidine derivatives.[42-56] Thus, pyrimidinethione **(37)** reacted with **(40)** or **(41)** in DMF[42] or in an acetic anhydride/pyridine mixture[44,46] to give thiazolo-pyrimidines **(42)** and **(43).** Alkylation with **(40)** in the presence of an aromatic aldehyde[42-44, 47, 49-52, 54] gave the ylidene derivatives **(44).** Similarly, pyrimidinethione derivatives **(38)** and **(39)** reacted with monochloroacetic acid in acetic acid/acetic anhydride/sodium acetate mixture[47,48] or with chloroacetyl chloride in dry dioxane to give the corresponding thiazolopyrimidines. Table I summarizes the pyrimidine-thione derivatives **(37-39)** used in the synthesis of various thiazolopyrimidines.

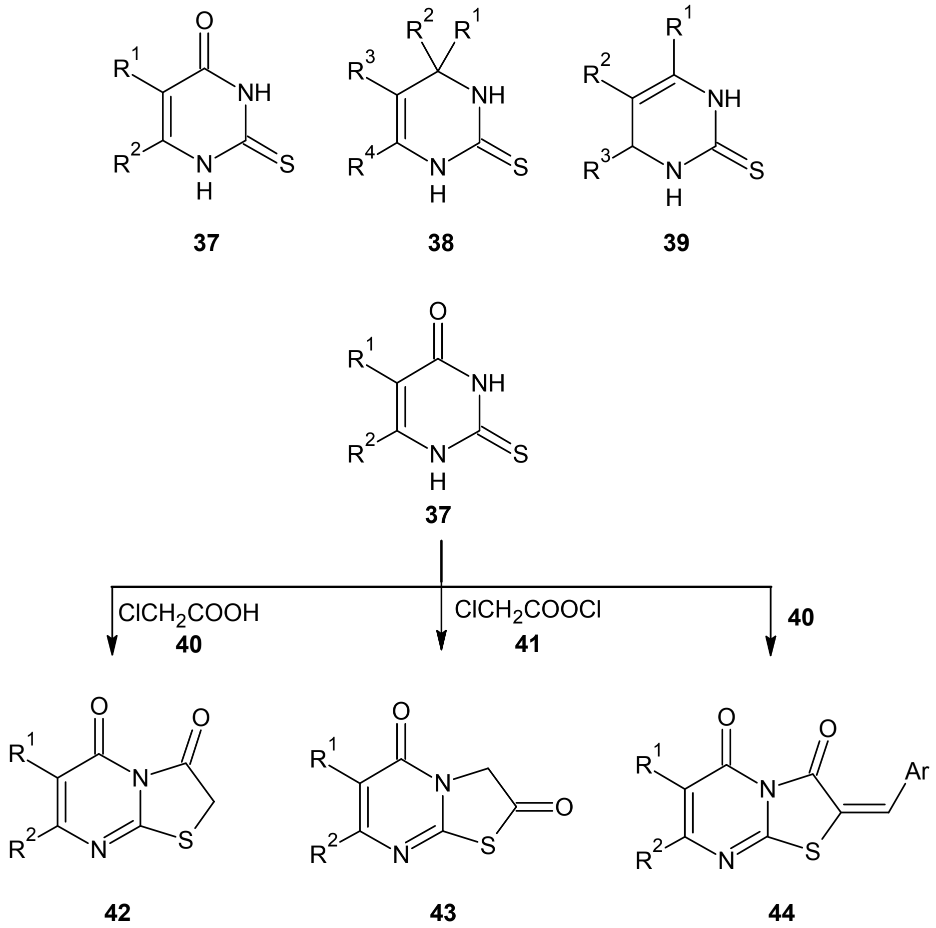
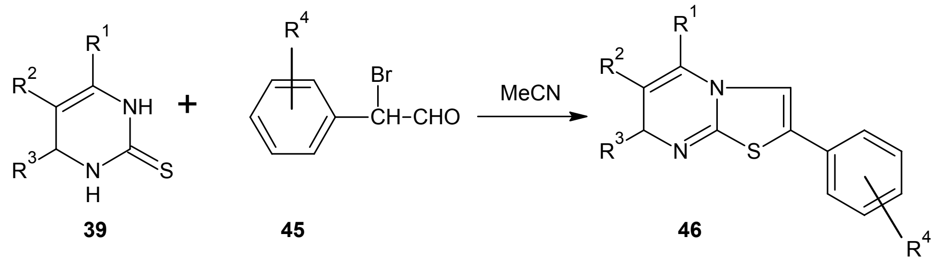


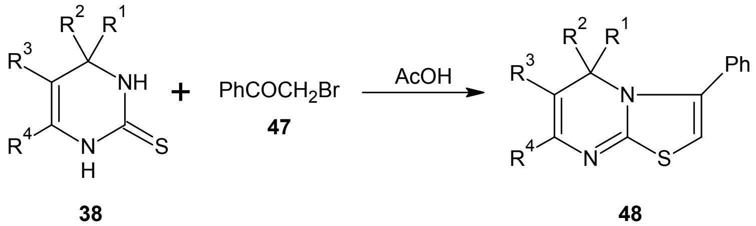
Chart-3

42. S.A. Abdel-Aziz; *Phosphorus, Sulfur Silicon Relat. Elem.,* **116,** 39 (1996).
43. F.M. Manhi, A.M. Abdel-Fattah *Egypt. J. Pharm.,* **33(5–6),** 825 (1992); *Chem. Abstr.,* **121,** 83254b (1994).
44. J.M. Parmar, A.R. Parikh; *Heterocycl. Commun.,* **4(5),** 463 (1998).
45. M. Ghorab, Y.A. Mohamed, S.A. Mohamed, Y.A. Ammar; *Phosphorus, Sulfur Silicon Relat. Elem.,* **108(1–4),** 249 (1996).
46. K.M. Ghoneim, M.Y.H. Essawi, M.S. Mohamed, A.M. Kamal; *Pol. J. Chem.,* **72(7),** 1173 (1998).
47. M.R. Mohamoud, A.Y. Soliman, H.M. Bakeer; *Indian J. Chem. Sec. B,* **29B(9),** 830 (1990).
48. H.R. Champaneri, S.R. Modi, H.B. Naik; *Asian J. Chem.,* **6(3),** 737 (1994); *Chem. Abstr.,* **121,** 300842y (1994).
49. H.Y. Moustafa; *Indian J. Heterocycl. Chem.,* **7(4),** 273 (1998).
50. M.A. Salama, H.H. Mousa, M. Al-Shaikh; *Orient. J. Chem.,* **7(4),** 185 (1991); *Chem. Abstr.,* **116,** 151713z (1992).
51. M.A.F. Sharaf, F.A. Abdel Aal, A.M. Abdel Fattah, A.M.R. Khalik; *J. Chem. Res. (S),* 354 (1996).
52. B. Tozkoparan, M. Ertan, P. Kelicen , R. Demirdamar; *Farmaco,* **54(9),** 588 (1999).
53. M.S. Akhtar, M. Seth, A.P. Bhaduri; *Indian J. Chem. Sec. B,* **26B(6),** 556 (1987).
54. M.A. Salama, H.H.A Mousa, S.A. El-Essa; *Orient. J. Chem.,* **7(3),** 128 (1991); *Chem. Abstr.,* **116,** 41405t (1992).
55. R.M. Abdel-Rahman, M. Fawzy, I. El-Baz; *Pharmazie,* **49,** 729 (1994).
56. C.D. Kappe, P. Roschger; *J. Heterocycl. Chem.,* **26,** 55 (1989).

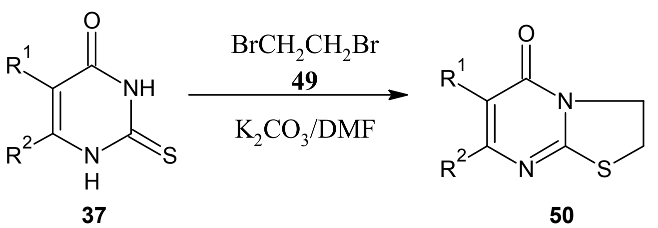
The Hantzsch-type condensation of dihydropyrimidines **(39)** with a substituted 2-bromophenylacetaldehyde **(45)** led to the thiazolopyrimidine derivatives **(46)**.[57,58]



Thiazolo[3,2-*a*]pyrimidine derivatives **(48)** were prepared by refluxing pyrimidinethione derivative **(38)** with phenacyl bromide **(47)** in glacial acetic acid.[59-61]



Reaction of pyrimidinethiones **(37)** with 1,2-dibromoethane **(49)** gave the oxothiazolopyrimidine derivatives **(50)** in the presence of DMF/K_2CO_3 at 70–80 °C.[62-65]



57. G. Adam, S. Kolczewski, V. Mutel, J. Wichmann; Eur. Pat. Appl. Ep 891, 978, (1999); *Chem. Abstr.,* **130,** 125087t (1999).
58. J. Wichmann, G. Adam, S. Kolczewski, V. Mutel, T. Woltering; *Bioorg. Medi. Chem. Lett.,* **9,** 1573 (1999).
59. A. Balkan, S. Uma, M. Ertan, W. Wiegrebe; *Pharmazie,* **47(9),** 687 (1992).
60. A. Balkan, M. Ertan, T. Burgemeister *Arch. Pharm. (Weinheim, Ger.),* **325(8),** 499 (1992).
61. S.A. Abdel-Aziz, H.A. Allimony, H.M. El-Shaaer, U.F. Ali, R.M. Abdel-Rahman; *Phosphorus, Sulfur Silicon Relat. Elem.,* **113,** 67 (1996).
62. V.J. Ran, A. Goel, M. Nath, P. Srivastava; *Bioorg. Med. Chem. Lett.,* **4(22),** 2653 (1994).
63. V.J. Ram; *Arch. Pharm. (Weinheim, Ger.),* **324(11),** 837 (1991).
64. A. Mobinikhaledi, N. Foroughifar, F. Goodarzi; *Phosphorus, Sulfur Silicon Relat. Elem.,* **179,** 507-512 (2004).
65. A. Mobinikhaledi, N. Foroughifar, B. Ahamadi; *Phosphorus, Sulfur Silicon Relat. Elem.,* **180,** 339-345 (2005).

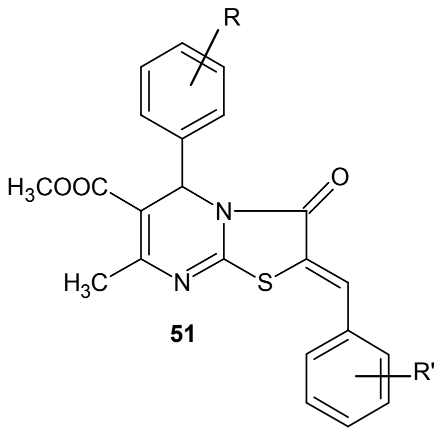
2.3 Retro Diels-Alder reaction

Both azole and azine approaches were considered as major methods for synthesis of the thiazolopyrimidines. Whereas, the retro Diels-Alder reaction was considered a minor method. Other reported methods include: (i) cyclization of propargylthiopyrimidines in the presence of Pd(II) salts, (ii) metabolism of a *N*-allylpyrimidine and (iii) reaction of thiazole derivative with isocyanates.

3 BIOLOGICAL PROFILE

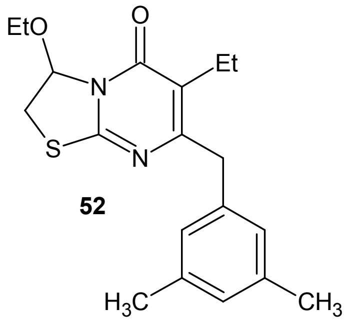
Thiazolo[3,2-*a*]pyrimidines have generated recently interest due to their interesting biological and pharmaceutical activities. Thus, these ring systems have following significant activities.

Various derivatives of thiazolo[3,2-*a*]pyrimidine **(51)** have been synthesized by B. Tozkoparan et al.[66] and tested for their anti-inflammatory activities at the 100 mg/kg dose level compared with indomethacin.



51

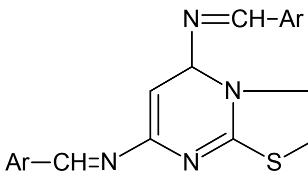
Krzysztof Danel and co-workers[67] have been synthesized substituted 2,3-dihydro-7H-thiazolo[3,2-*a*]pyrimidin-7-ones and considered as annulated analogues of HEPT. 2,3-Dihydro-5-[(3,5-dimethylphenyl)methyl]-3-ethoxy-6-ethyl-7*H*-thiazolo[3,2-*a*]pyrimidin-7-one **(52)** were found most active against *HIV-1*.



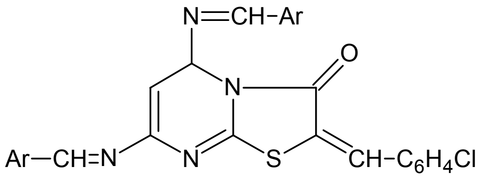
52

66. B. Tozkoparan, M. Ertan, P. Kelicen, R. Demirdamar; *Il Farmaco,* **54,** 588–593 (1999).
67. K. Danel, E. B. Pedersen, C. Nielsen; *J. Med. Chem.* **41,** 191-198 (1998).

Antimicrobial activity of thiazolo[3,2-*a*]pyrimidine **(53, 54)** have been screened by H. M. Sayed et al.[68] against various gram positive, gram negative pathogens and fungi.



53

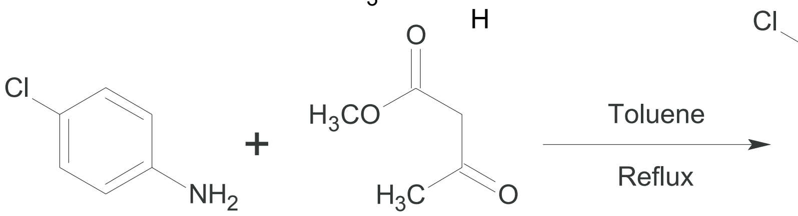


54

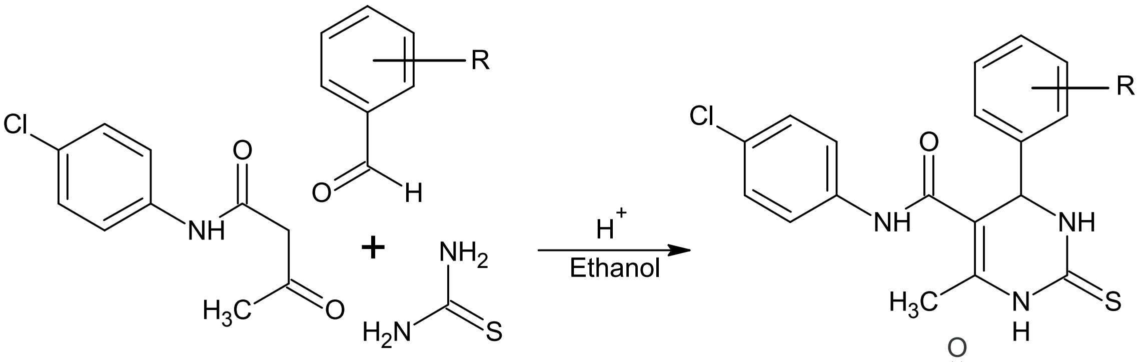
68. H. H. Sayed, A. H. Shamroukh, A. E. Rashad; *Acta Pharm.,* **56,** 231–244 (2006).

4 REACTION SCHEME

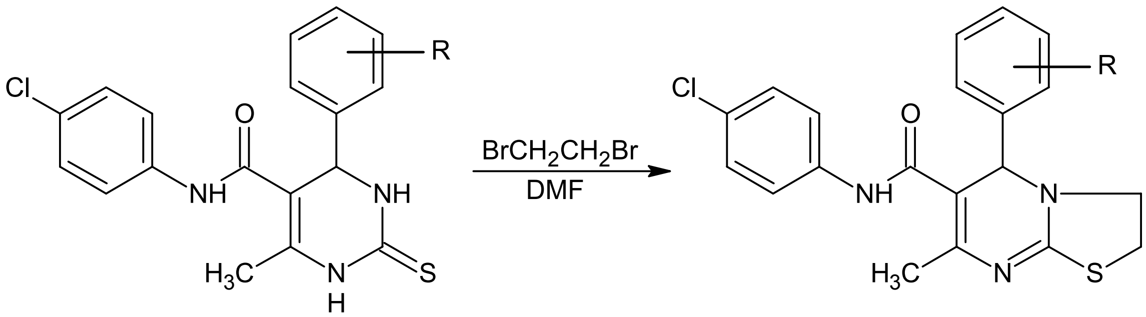
Step-1



Step-2



Step-3



5 EXPERIMENTAL

Melting points of all the synthesized compounds were taken in open capillary bath on controlled temperature heating mental. The crystallization of all the compounds was carried out in appropriate solvents. TLC was carried out on silicagel-G as stationary phase. Ethyl acetate: Hexane (5:5) was used as a mobile phase. The other solvent system like acetone: benzene was also employed.

Step-1 ***Synthesis of N-(4-chlorophenyl)-3-oxobutanamide.***

As per Part-B (Section-I), page No.224.

Step-2 ***Synthesis of N-(4-chlorophenyl)-6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide.***

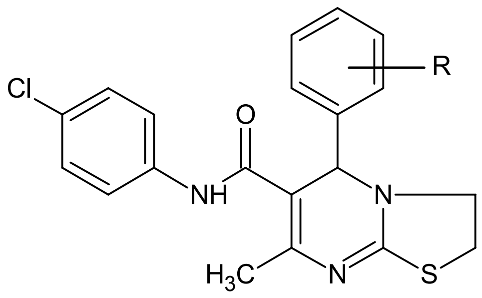
As per Part-B (Section-I), page No.224.

Step-3 ***Synthesis of N-(4-chlorophenyl)-7-methyl-5-phenyl-2,3-dihydro-5H-thiazolol[3,2-a]pyrimidine-6-carboxamide.***

1,2-Dibromoethane (0.011 mol, 1.88 gm) was added to a boiling solution of N-(4-chlorophenyl)-6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (0.01 mol, 3.57 gm) in dimethyl formamide (5 ml) and then refluxed for 50 minutes. After completion of the reaction, the reaction mass was poured into ice cold sodium bicarbonate solution and precipitated were isolated by filtered through a Buchner funnel and purified by column chromatography. Yield 82%. m.p. 205-206 ^{o}C, Anal. Calcd. for $C_{20}H_{18}ClN_{3}OS$ Calcd: C, 62.57; H, 4.73; N, 10.95%, Found: C, 62.66; H, 4.68; N, 10.87%.

Similarly, other *N*-(4-chlorophenyl)-7-methyl-5-aryl-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamide were prepared and crystallized from appropriate solvents, in some cases the products were purified by column chromatography. The reaction time and percentage yields of the respective reactions are depicted in the physical data **Table-2a**.

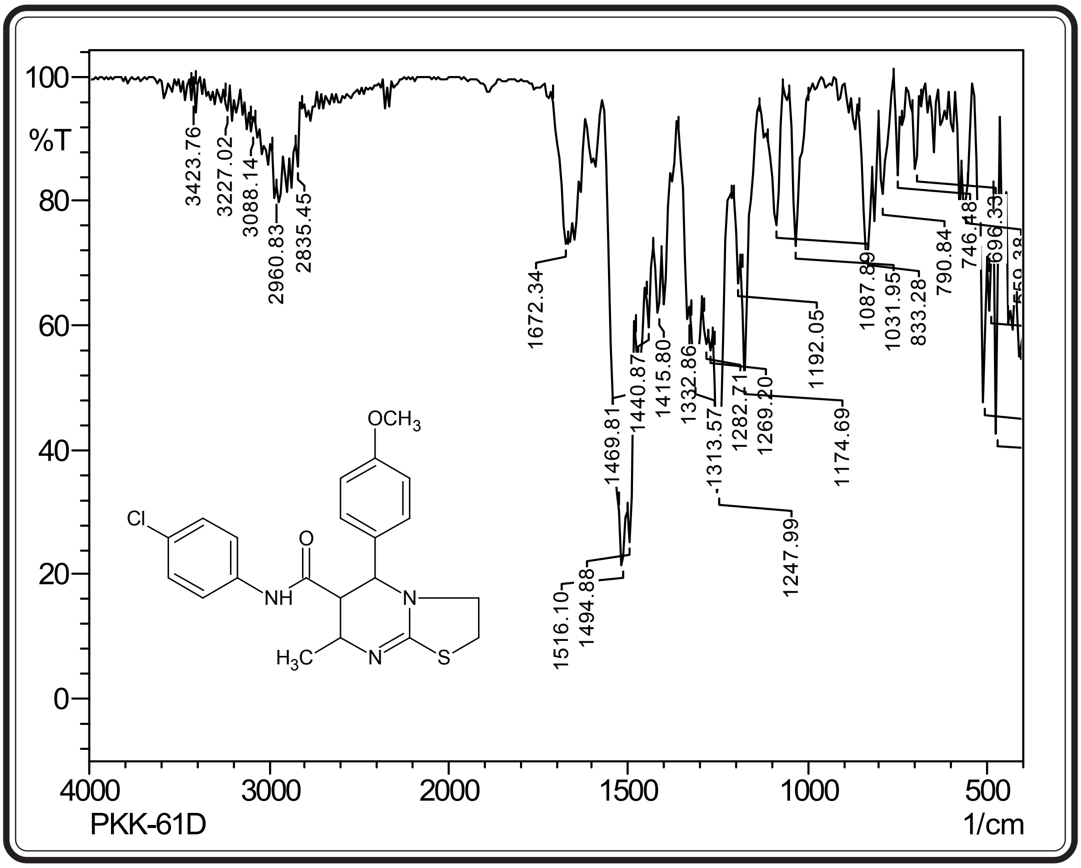
Table-2a: Physical constatnt of N-(4-chlorophenyl)-7-methyl-5-aryl-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamides.



Sr. No.	Substitution R	Molecular Formula/ Molecular weight	M.P. oC	Yield %	% Composition Calcd./Found		
					C	H	N
2a	H	$C_{20}H_{18}ClN_3OS$ 383.89	205-206	82	62.57 62.66	4.73 4.68	10.95 10.87
2b	4-OCH_3	$C_{21}H_{20}ClN_3O_2S$ 413.92	206-207	81	60.94 60.89	4.87 4.78	10.15 10.06
2c	3-NO_2	$C_{20}H_{17}ClN_4O_3S$ 456.94	201-202	69	56.01 55.91	4.00 4.11	13.06 13.01
2d	4-Cl	$C_{20}H_{17}Cl_2N_3OS$ 418.33	214-216	80	57.42 57.36	4.10 4.17	10.04 10.06
2e	3,4-$(OCH_3)_2$	$C_{22}H_{22}ClN_3O_3S$ 443.94	199-200	77	59.52 59.46	4.99 4.87	9.47 9.42
2f	2,5-$(OCH_3)_2$	$C_{22}H_{22}ClN_3O_3S$ 443.94	212-213	82	59.52 59.48	4.99 4.89	9.47 9.45
2g	2-OCH_3	$C_{21}H_{20}ClN_3O_2S$ 413.92	222-223	79	60.94 60.88	4.87 4.78	10.15 10.21
2h	2-OH	$C_{20}H_{18}ClN_3O_2S$ 399.89	219-220	76	60.07 60.01	4.54 4.47	10.51 10.43
2i	4-NO_2	$C_{20}H_{17}ClN_4O_3S$ 456.94	189-190	75	56.01 55.95	4.00 4.07	13.06 12.99
2j	3-OC_6H_5	$C_{26}H_{24}ClN_3O_2S$ 478.00	235-237	78	65.33 65.30	5.06 5.01	8.79 8.72
2k	4-F	$C_{20}H_{19}ClFN_3OS$ 403.90	215-215	72	59.47 59.44	4.74 4.70	10.40 10.36

6 SPECTRAL STUDIES

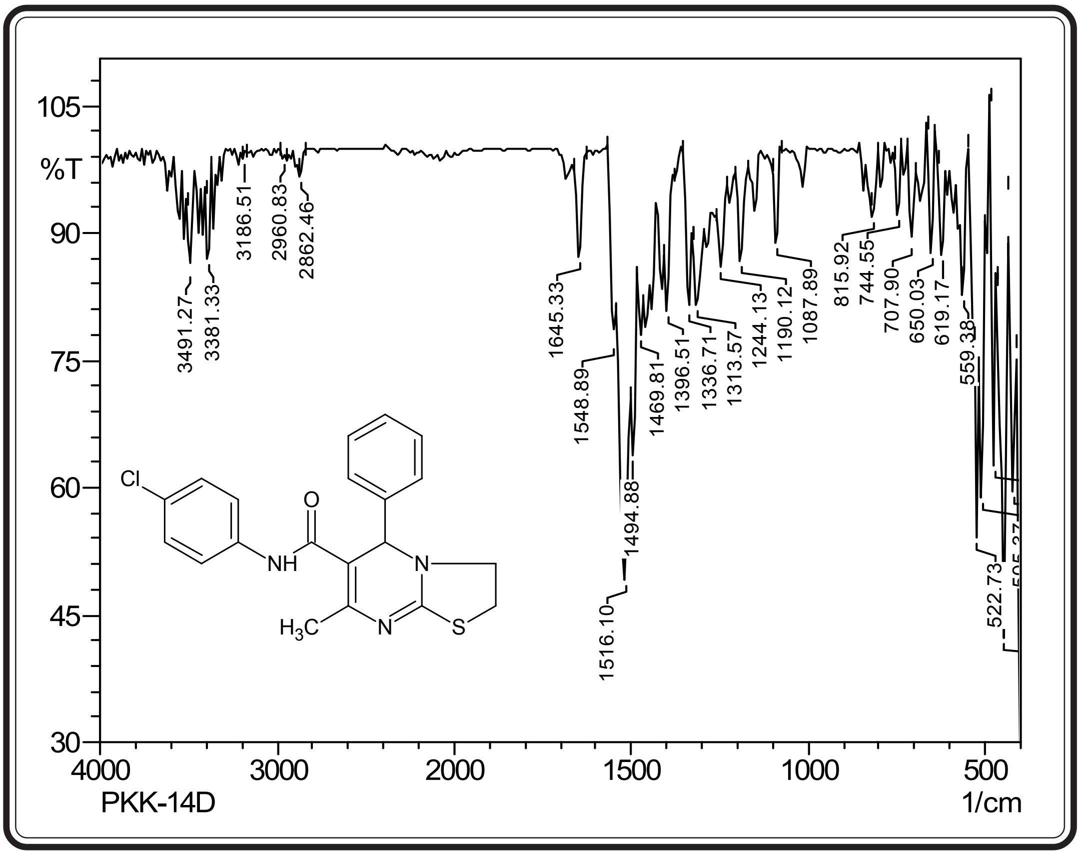
IR spectrum of *N*-(4-chlorophenyl)-7-methyl-5-(4-methoxyphenyl)-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamide.



Instrument: Shimadzu FTIR-8400 using KBR DRS techniques. The percentage transmittance is given in cm^{-1} and frequence range is between 400-4000cm^{-1}.

Type	Vibration Mode	Frequency cm^{-1}
Alkane $-CH_3$	C-H str. (asym.)	2960
	C-H str. (sym.)	2835
	C-H i.p.d (asym)	1440
	C-H o.o.d (sym)	1332
Aromatic	C-H str.	3088
	C=C (skeleton)	1469, 1494, 1516
	C-H i.p. bending	1031
	C-H o.p bending	833
Carbonyl	-C=O	1672
Amine	-NH str.	3423
	-NH def.	1546
Halide	-C-Cl	746

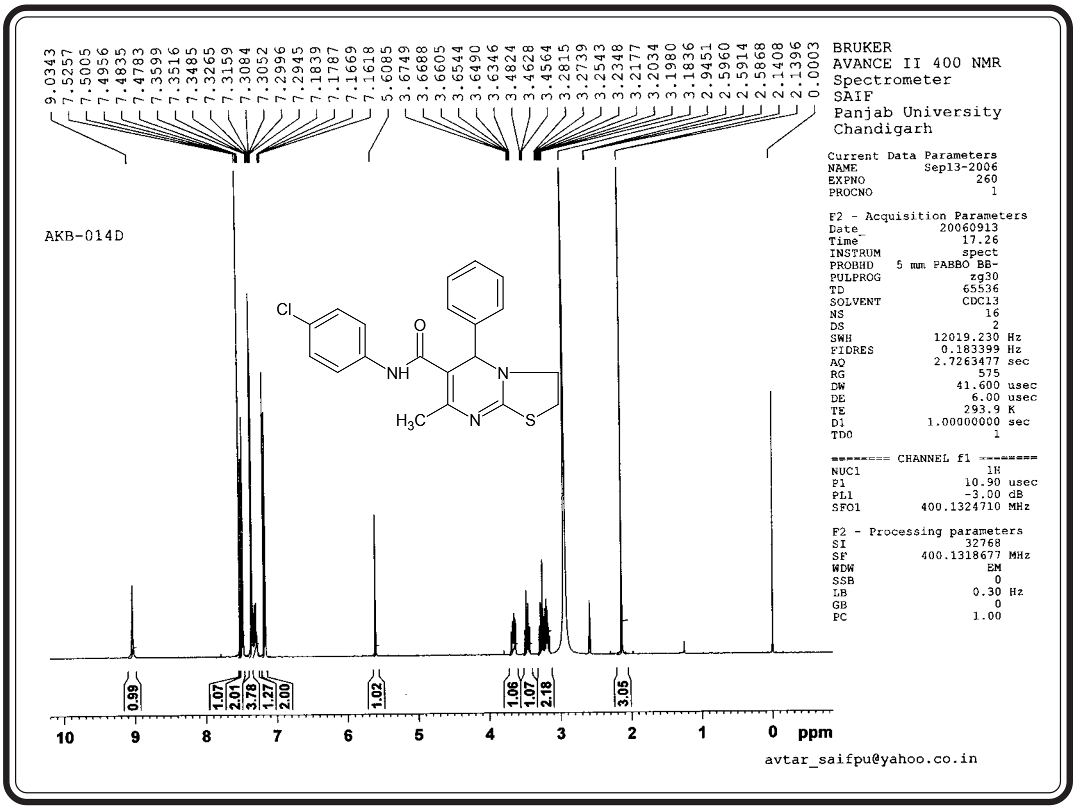
IR spectrum of *N*-(4-chlorophenyl)-7-methyl-5-phenyl-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamide.

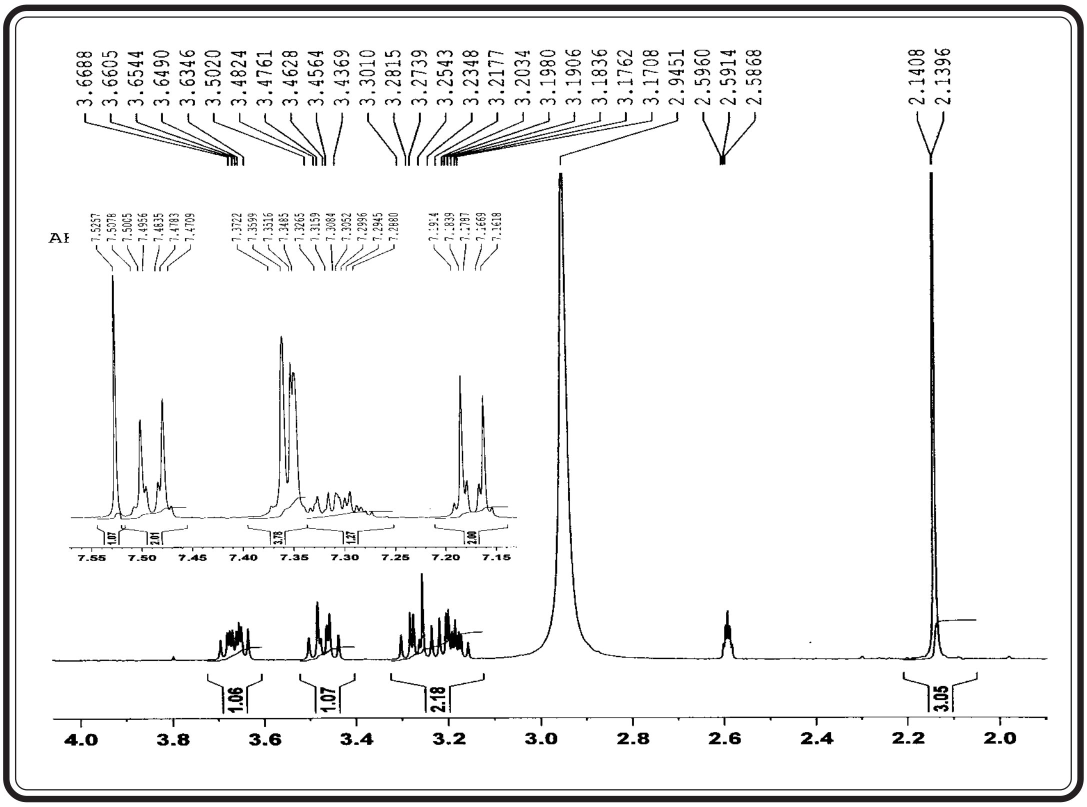


Instrument: Shimadzu FTIR-8400 using KBR DRS techniques. The percentage transmittance is given in cm^{-1} and frequence range is between 400-4000cm^{-1}.

Type	Vibration Mode	Frequency cm^{-1}
Alkane $-CH_3$	C-H str. (asym.)	2960
	C-H str. (sym.)	2862
	C-H i.p.d (asym)	1469
	C-H o.o.d (sym)	1396
Aromatic	C-H str.	3186
	C=C (skeleton)	1494, 1516
	C-H i.p. bending	1087
	C-H o.p bending	815
Carbonyl	-C=O	1645
Amine	-NH str.	3381
	-NH def.	1548
Halide	-C-Cl	707

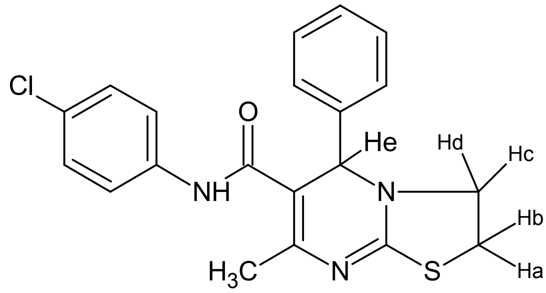
[1]H-NMR spectrum of *N*-(4-chlorophenyl)-7-methyl-5-phenyl-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamide.





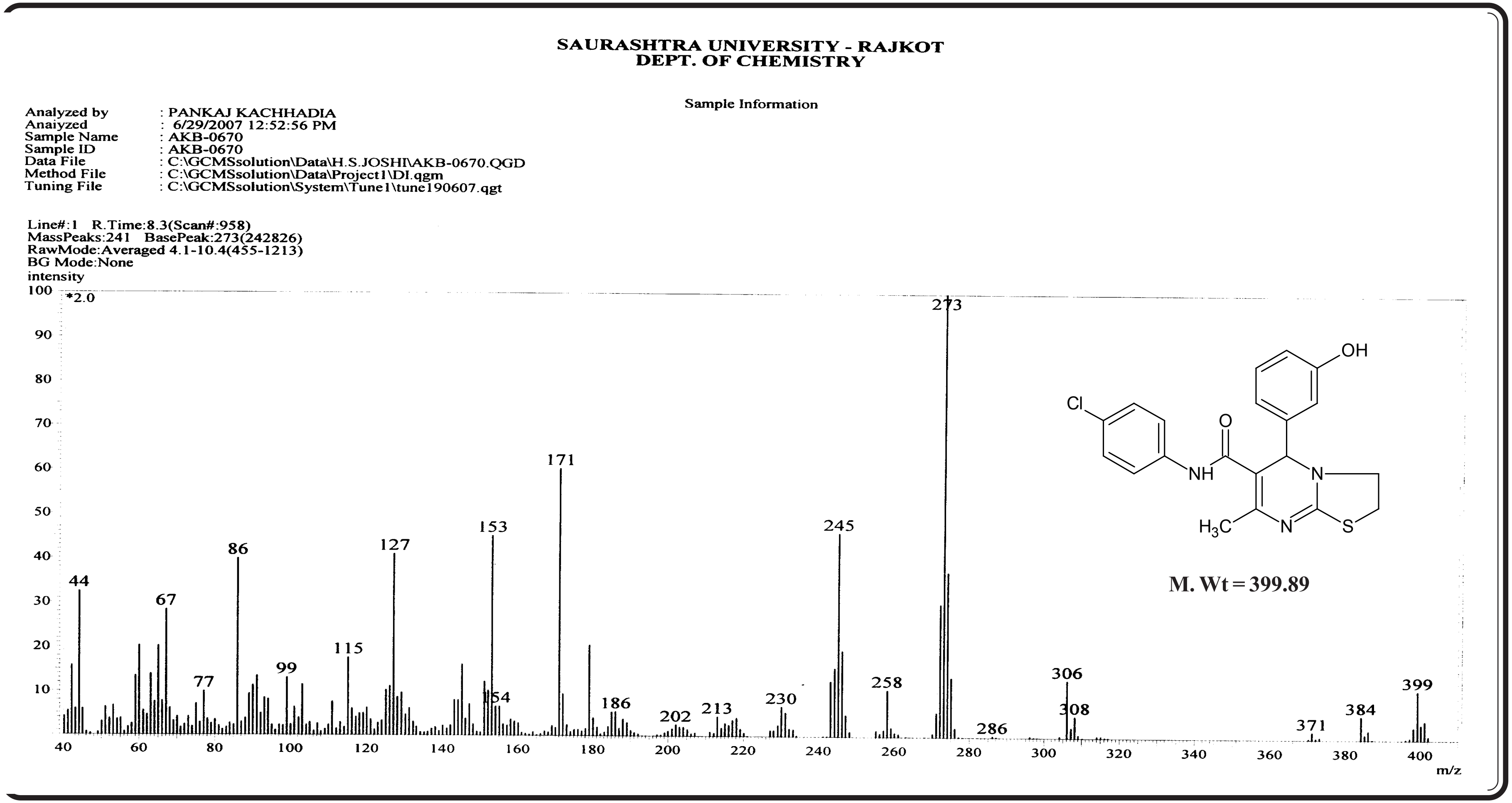
Instrument: BRUKER 400 MHz (Avance - II), **Internal reference:** TMS,
Solvent: DMSO [d_6].

Assignment of proton vlues of *N*-(4-chlorophenyl)-7-methyl-5-phenyl-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamide.



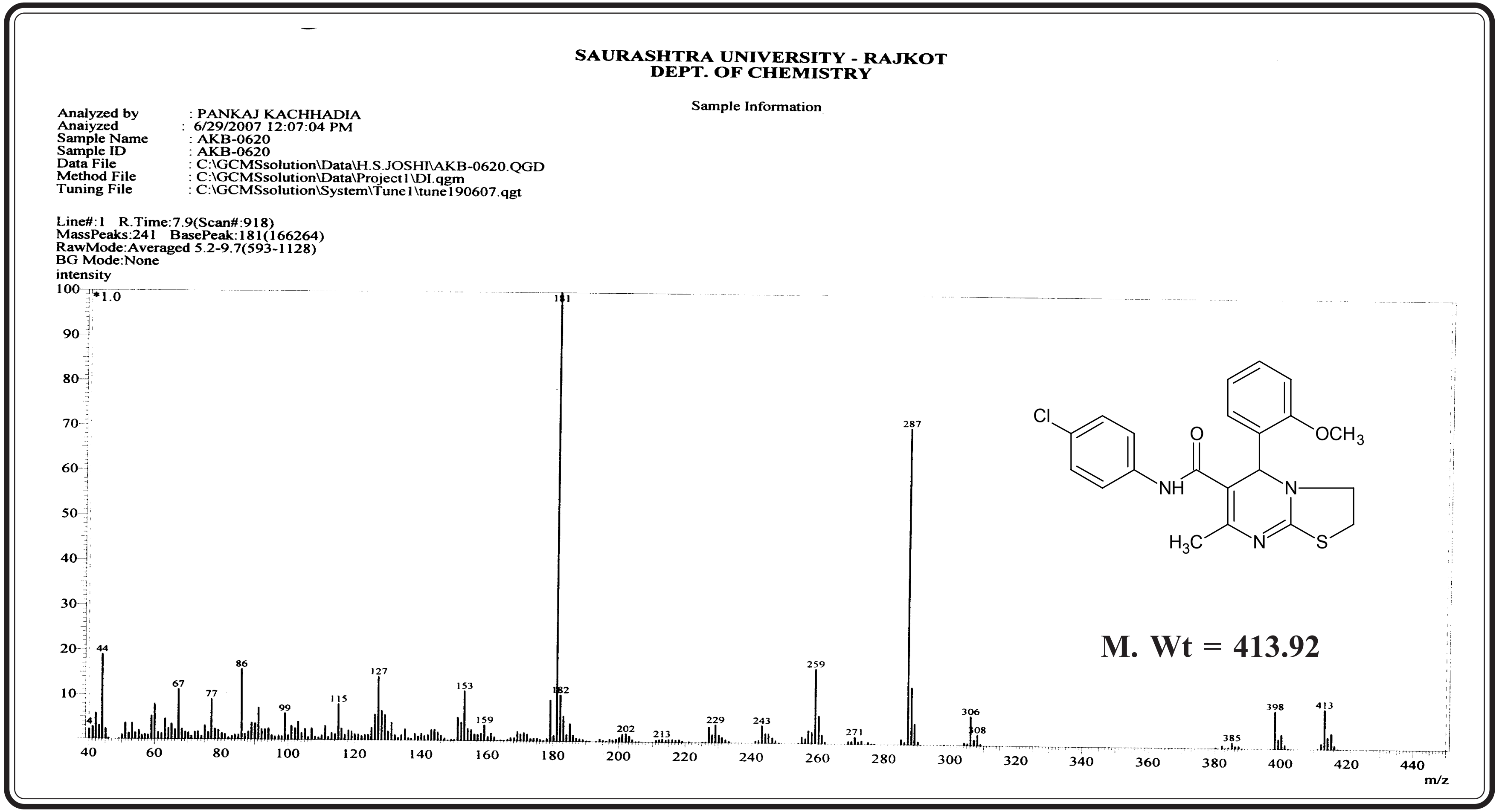
Sr. No.	Chemical shift in ppm	Relative No. of Protons	Multiplicity	Inference	J value in Hz
1	2.14	3H	singlet	$-CH_3$	-
2	3.18-3.28	2H	multiplet	-Ha & -Hb	-
3	3.45-3.48	1H	multiplet	-Hc	-
4	3.63-3.67	1H	multiplet	-Hd	-
5	5.60	1H	singlet	-He	-
6	7.16-7.52	9H	multiplet	Ar-H	-
7	9.03	1H	singlet	-NH (Amide)	-

EI-MS spectrum of *N*-(4-chlorophenyl)-7-methyl-5-(3-hydroxyphenyl)-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamide.



Instrument: Shimadzu GC-MS QP-2010, DI-probe, EI-method.

EI-MS spectrum of *N*-(4-chlorophenyl)-7-methyl-5-(2-methoxyphenyl)-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamide.



Instrument: Shimadzu GC-MS QP-2010, DI-probe, EI-method.

Table-2b: **Antimicrobial activities of *N*-(4-chlorophenyl)-7-methyl-5-aryl-2,3-dihydro-5*H*-thiazolol[3,2-*a*]pyrimidine-6-carboxamides.**

Sr. No.	Antibacterial Activity				Antifungal Activity
	S. aureus	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*A. niger*
2a	**18** (0.85)C_1, (0.72)C_2 (0.72)C_3, (1.20)C_4	**16** (0.66)C_1, (0.66)C_2 (0.94)C_3, (0.88)C_4	**18** (0.85)C_1, (0.72)C_2 (0.72)C_3, (1.20)C_4	**14** (0.70)C_1, (0.66)C_2 (0.72)C_3, (0.70)C_4	**16** (0.66)C_5
2b	**15** (0.66)C_1, (0.60)C_2 (0.62)C_3, (0.83)C_4	**12** (0.50)C_1, (0.50)C_2 (0.70)C_3, (0.66)C_4	**15** (0.66)C_1, (0.60)C_2 (0.62)C_3, (0.83)C_4	**16** (0.76)C_1, (0.64)C_2 (0.64)C_3, (1.06)C_4	**21** (0.87)C_5
2c	**14** (0.70)C_1, (0.61)C_2 (0.77)C_3, (0.70)C_4	**14** (0.58)C_1, (0.58)C_2 (0.82)C_3, (0.77)C_4	**14** (0.63)C_1, (0.56)C_2 (0.58)C_3, (0.77)C_4	**12** (0.57)C_1, (0.48)C_2 (0.48)C_3, (0.80)C_4	**15** (0.62)C_5
2d	**12** (0.60)C_1, (057)C_2 (0.66)C_3, (0.60)C_4	**21** (0.87)C_1, (0.87)C_2 (1.23)C_3, (1.16)C_4	**12** (0.54)C_1, (0.48)C_2 (0.50)C_3, (0.66)C_4	**11** (0.52)C_1, (0.44)C_2 (0.44)C_3, (0.73)C_4	**17** (0.70)C_5
2e	**21** (1.05)C_1, (1.00)C_2 (1.16)C_3, (1.05)C_4	**10** (0.40)C_1, (0.41)C_2 (0.58)C_3, (0.55)C_4	**14** (0.63)C_1, (0.56)C_2 (0.58)C_3, (0.77)C_4	**13** (0.60)C_1, (0.52)C_2 (0.52)C_3, (0.86)C_4	**12** (0.50)C_{54}
2f	**14** (0.70)C_1, (0.66)C_2 (0.77)C_3, (0.70)C_4	**12** (0.50)C_1, (0.50C_2 (0.70)C_3, (0.66)C_4	**11** (0.50)C_1, (0.44)C_2 (0.45)C_3, (0.61)C_4	**16** (0.76)C_1, (0.64)C_2 (0.64)C_3, (1.06)C_4	**17** (0.70)C_{54}

2g	**11** (0.55)C_1, (0.52)C_2 (0.61)C_3, (0.55)C_4	**19** (0.79)C_1, (0.79)C_2 (1.11)C_3, (1.55)C_4	**15** (0.68)C_1, (0.60)C_2 (0.62)C_3, (0.83)C_4	**17** (0.80)C_1, (0.68)C_2 (0.68)C_3, (1.13)C_4	**19** (0.79)C_5
2h	**19** (0.95)C_1, (0.90)C_2 (1.05)C_3, (0.95)C_4	**10** (0.41)C_1, (0.41)C_2 (0.58)C_3, (0.55)C_4	**14** (0.63)C_1, (0.56)C_2 (0.58)C_3, (0.77)C_4	**11** (0.52)C_1, (0.44)C_2 (0.44)C_3, (0.73)C_4	**15** (0.62)C_5
2i	**15** (0.75)C_1, (0.71)C_2 (0.83)C_3, (0.75)C_4	**11** (0.45)C_1, (0.45)C_2 (0.64)C_3, (0.61)C_4	**11** (0.50)C_1, (0.44)C_2 (0.45)C_3, (0.61)C_4	**18** (0.85)C_1, (0.72)C_2 (0.72)C_3, (1.2)C_4	**14** (0.58)C_5
2j	**14** (0.70)C_1, (0.66)C_2 (0.77)C_3, (0.70)C_4	**17** (0.70)C_1, (0.70)C_2 (1.00)C_3, (0.94)C_4	**19** (0.86)C_1, (0.76)C_2 (0.79)C_3, (1.05)C_4	**15** (0.71)C_1, (0.60)C_2 (0.60)C_3, (1.00)C_4	**13** (0.54)C_5
2k	**12** (0.60)C_1, (0.57)C_2 (0.66)C_3, (0.60)C_4	**16** (0.66)C_1, (0.66)C_2 (0.88)C_3, (0.83)C_4	**17** (0.77)C_1, (0.68)C_2 (0.70)C_3, (0.94)C_4	**10** (0.47)C_1, (0.40)C_2 (0.40)C_3, (0.66)C_4	**18** (0.75)C_5
C_1	**20**	**24**	**22**	**21**	**00**
C_2	**21**	**24**	**25**	**25**	**00**
C_3	**18**	**17**	**24**	**25**	**00**
C_4	**20**	**18**	**18**	**15**	**00**
C_5	**00**	**00**	**00**	**00**	**24**

Activity index = Inhibition zone of the sample / Inhibition zone of the standard

For antibacterial activity : C_1 = Amoxicillin, C_2 = Ciprofloxacin, C_3 = Cephalexin, C_4 = Erythromycin.

For antifungal activity : C_5 = Greseofulvin.

List of publications

1. Development and validation of HPLC method for assay and content uniformity determination of ezetimibe in tablet formulation, Ashish S. Doshi, Pankaj K. Kachhadia, H. S. Joshi*, ***Journal of Chromatographia.***67(2008)137-142.
2. Synthesis and selective antitubercular and antimicrobial inhibitory activity of 1-acetyl-3,5-diphenyl-4,5-dihydro-(1*H*)-pyrazole derivatives P. T. Chovatia, J. D. Akbari, P. K. Kachhadia, P. D. Zalavadia and H. S. Joshi* ***J. Serb. Chem. Soc****. 71* (7) 713-720 (2007).
3. Development and validation of a stability indicating HPLC assay method for determination of nebivolol in tablet formulation, Pankaj K. Kachhadia, Ashish S. Doshi, H. S. Joshi*, ***Journal of AOAC International*** (accepted)
4. Validated HPLC method for determination of Aspirin and Clopidogrel in combined dosage form in presence of degradation product formed under ICH recommended stress condition. Pankaj K. Kachhadia, Ashish S. Doshi, H. S. Joshi*, ***Journal of AOAC International*** (accepted).
5. Synthesis Of some new 1,2,3,4-tetrahydropyrimidine-2-thiones and their thiazolo[3,2-*a*] pyrimidine derivatives as a potential biological agents. J. D. Akbari, P. K. Kachhadia, S. D. Tala, A. H. Bapodra. M. F. Dhaduk and H. S. Joshi* ***Phosphorous, Sulfur, Silicon and the related elements*** (accepted).
6. New validated stability-indicating high-performance liquid chromatographic assay method for the simultaneous determination of tramadol hydrochloride and aceclofenac in commercial tablet. Pankaj K. Kachhadia, Ashish S. Doshi, Vijay R. Ram H. S. Joshi* (Under communication).